

Searching for Novel Diagnostic- Prognostic Biomarkers in Gastric Cancer

Pesquisa de Novos Biomarcadores em Cancro do Estômago

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Porto, 2012

Tese de Doutoramento

Programa Doutoral em Medicina e Oncologia Molecular

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Dissertação de candidatura ao grau de Doutor na área científica de Medicina, programa doutoral “Medicina e Oncologia Molecular” apresentada à Faculdade de Medicina da Universidade do Porto.

Artigo 48º, § 3º - A Faculdade não responde pelas doutrinas expendidas na dissertação. (Regulamento da Faculdade de Medicina da Universidade do Porto – Decreto-Lei Nº 19 337, de 29 de Janeiro de 1931).

Nota explicativa

A presente tese está escrita em inglês na sua quase totalidade pelo facto de o Professor Franco Roviello ter sido co-orientador dos trabalhos nela apresentados.

Lista de Publicações

Ao abrigo do Artigo 8º do Decreto-Lei nº 388/70, fa zem parte desta dissertação os seguintes trabalhos já publicados:

Corso G, Marrelli D, Pedrazzani C, Machado JC, Mancini S, Seruca R, Roviello F. Gastric cardia carcinoma is associated with the promoter -77T>C gene polymorphism of X-ray cross-complementing group 1 (*XRCC1*). *Journal of Gastrointestinal Surgery* 2009;13:2233-8.

Corso G, Pedrazzani C, Pinheiro H, Fernandes E, Marrelli D, Rinnovati A, Pascale V, Seruca R, Oliveira C, Roviello F. E-cadherin genetic screening and clinico-pathologic characteristics of early onset gastric cancer. *European Journal of Cancer* 2011;47:631-9.

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Velho S, **Corso G**, Oliveira C, Seruca R. KRAS signaling pathway alterations in microsatellite unstable gastrointestinal cancers. *Advances in Cancer Research* 2010;109:123-43; 2010.

Os próximo artigos não fazem parte integrante desta dissertação mas foram usados na Introdução:

Marrelli D, Pedrazzani C, Berardi A, **Corso G**, Neri A, Garosi L, Vindigni C, Santucci A, Figura N, Roviello F. Negative *Helicobacter pylori* status is associated with poor prognosis in patients with gastric cancer. *Cancer* 2009;115:2071-80.

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Roviello, F., **Corso, G.**, Marrelli, D. Long-term results after R0 resection in the surgical treatment of gastric cancer. *Surgery in Multimodal Management of Gastric Cancer* 2011, cap. 17, (eds) Springer-Verlag Italia 2011, in press.

Roviello F, Pedrazzani C, Marrelli D, Di Leo A, Caruso S, Giacomuzzi S, **Corso G**, de Manzoni G. Super-extended (D3) lymphadenectomy in advanced gastric cancer. *European Journal of Surgical Oncology* 2010;36:439-46.

Roviello, F., **Corso, G.**, Marrelli, D. Standard and extended lymphadenectomy: technical notes. Surgery in Multimodal Management of Gastric Cancer 2011, cap. 33, (eds) Springer-Verlag Italia 2011, in press.

MacConaill, L.E., Campbell, C.D., Kehoe, S.M., Bass, A.J., Hatton, C., Niu, L., Davis, M., Yao, K., Hanna, M., Mondal, C., Luongo, L., Emery, C.M., Baker, A.C., Philips, J., Goff, D.J., Fiorentino, M., Rubin, M.A., Polyak, K., Chan, J., Wang, Y., Fletcher, J.A., Santagata, S., **Corso, G.**, Roviello, F., Shivdasani, R., Kieran, M.W., Ligon, K.L., Stiles, C.D., Hahn, W.C., Meyerson, M.L., Garraway, L.A. Profiling critical cancer gene mutations in clinical tumor samples. PLoS One 2009;4:e7887.

Em cumprimento do disposto no referido Decreto-Lei, declaro que participei activamente na recolha e análise de dados e que escrevi os artigos em colaboração com os co-autores.

Para os Portugueses, amigos e exemplo de vida

Agradecimentos/Acknowledgments/Ringraziamenti

Prof. Raquel Seruca (IPATIMUP, Instituto de Patologia e Imunologia Molecular da Universidade do Porto, Portugal)

Prof. Franco Roviello (Dipartimento di Patologia Umana ed Oncologia, Sezione di Chirurgia Generale ed Oncologica, Università degli Studi di Siena, Italia)

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ABBREVIATIONS

AJCC	American joint committee cancer
AKT	v-AKT murine thymoma viral oncogene
AO	Aorta
ASCO	American society of clinical oncology
BRAF	v-RAF murine sarcoma viral oncogene homolog B
CEA	Common hepatic artery
c-MET	met proto-oncogene (hepatocyte growth factor receptor)
CT	Celiac trunk
DGC	Diffuse gastric cancer
EGJ	Esophagogastric junction
EGFR	Epidermal growth factor receptor
EODGC	Early onset diffuse gastric cancer
ERBB	v-erb-b2 erythroblastic leukemia viral oncogene homolog
ERBB1	v-erb-b2 erythroblastic leukemia viral oncogene homolog 1
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2
ERBB3	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3
ERBB4	v-erb-b2 erythroblastic leukemia viral oncogene homolog 4
FGC	Familial gastric cancer
FOLFIRI	Leucovorin/fluorouracil/irinotecan
FOLFOX	Leucovorin/fluorouracil/oxaliplatin
FUFIRI	Irinotecan/fluorouracil/leucovorin
FUFOX	Oxaliplatin/fluorouracil/leucovorin
XELOX	Capecitabine/oxaliplatin
GC	Gastric cancer
HDGC	Hereditary diffuse gastric cancer
HER2	Hairy-related 2
HNPCC	Hereditary non-polyposis colon cancer
<i>H. Pylori</i>	<i>Helicobacter pylori</i>
IGCLC	International gastric cancer linkage consortium
IRGGC	Italian research group for gastric cancer

IVC	Inferior vein cava
JCGC	Japanese classification of gastric carcinoma
JGCA	Japanese gastric cancer association
KRAS	Kirsten rat sarcoma virus oncogene
LN	Lymph-node
LNR	Lymph-node ratio
LOH	Loss of heterozygosity
LRV	Left renal vein
M	Mucosa
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MLH1	MutL homologue 1
MLK3	Mixed lineage kinase 3
MMR	Mismatch repair
MP	Muscularis propria
MSH2	MutS homologue 2
MSH3	MutS homologue 3
MSH6	MutS homologue 6
MSI	Microsatellite instability
mTOR	Mammalian target of rapamycin
NOC	N-nitroso compound
OOR	Overall response rate
PAI	Pathogenicity island
PAN	Para-aortic node
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3-kinase
PIK3CA	Phosphoinositide-3-kinase, catalytic alpha polypeptide
PMS1	Postmeiotic segregation increased 1
PMS2	Postmeiotic segregation increased 2
PolyA	Poly-adenylation
R	Residual tumor
RAS	Rat sarcoma virus oncogene

RRA	Right renal artery
RSV	Right spermatic vein
RTK	Receptor tyrosine kinase
SA	Splenic artery
SM	Submucosal
SMM	Small mucosa
SRCs	Signet ring cells
SS	Subserosa
TKI	Tyrosine kinase inhibitor
TNM	Tumor node metastasis
TRM	Tumor ratio metastasis
3'-UTR	3'-untranslated region
UICC	International union against cancer
XRCC1	X-ray-cross-complement 1

1. INTRODUCTION

1.1 Incidence and mortality

Until recently, GC was the second most common cancer worldwide, but now, with an estimated 934,000 new cases per year in 2002 (8.6% of new cases), it is in fourth place behind cancers of lung, breast and colon and rectum. Moreover, it is the second most common cause of death from cancer (700,000 deaths annually) [1].

Almost two-thirds of cases occur in developing countries and 42% in China alone. The geographical distribution of GC is characterized by wide international variations; high-risk areas include East Asia (China and Japan), Eastern Europe, and parts of Central and South America. Incidence rates are low (<10 per 100,000 in men in Southern Asia, North and East Africa, North America, and Australia and New Zealand) (figure 1). Patterns in woman are broadly similar to those in men [1]. Although it has been suggested that GC is rare in Africa [2], the incidence in Central (Middle) Africa is not low [3], with an estimated age standardized rate in women (12.6 per 100,000) similar to that in Easter Europe [1].

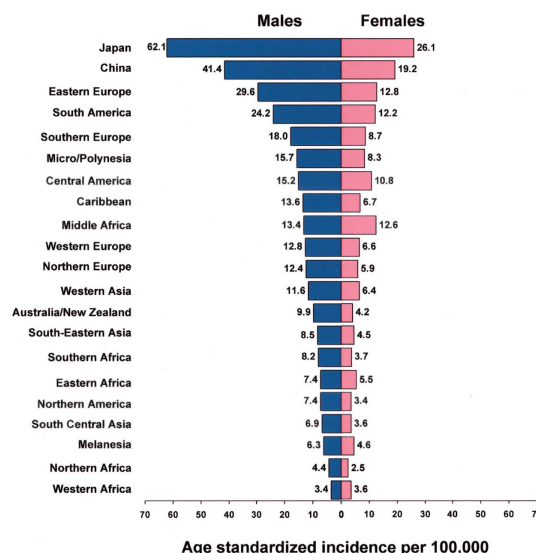


Figure 1. Worldwide distribution and incidence of gastric cancer; Japan, China and Eastern Europe are the higher incidence areas for gastric carcinoma. Data shown per 100,000 by gender. (Adopted from “Global Cancer Statistics, 2002”; CA A Cancer J Clin 2005, 55,74-108).

In Europe GC represents the fifth cancer most common, after colon and rectum, breast, lung and prostate, with an incidence of 149,000 per year; respectively 89,000 for male and 59,000 for female genders. Mortality rate for GC is 116,000, in men 69,000 and in woman 47,000 [4] (figure 2). The highest European risk area for GC is the Eastern zone with an incidence of 49,000 for male and 21,000 for female (Belarus area) [4]. Portugal and Italy represent one of the most high risk areas for stomach cancer, in particular respectively with incidence in 2008 of 28,300 for male and 12,800 for female and in Italy with incidence of 22,100 for male and 11,300 for female. Mortality rate for stomach cancer in Portugal is 22,000 for male and 10,000 for female and in Italy 14,000 for male and 7,000 for female. Moreover, the estimated number of new cancer cases in 2008 was in Portugal about 170 for male and 100 for female, and in Italy about 910 for male and 630 for female; its mortality was in Portugal of 140 for male and 90 for female, and in Italy 600 for male and 430 for female [4]. In Italy, Tuscany and the central regions are considered the highest risk areas for stomach cancer with a mortality rate of 4,2/10,000 inhabitants (2002; The Italian National Institute of Statistics-ISTAT) (figure 3).

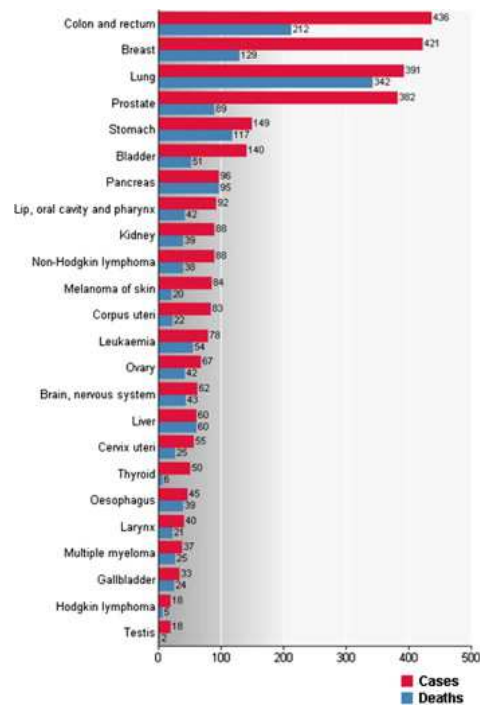


Figure 2. Estimated numbers of cancer cases and cancer deaths in European countries (in thousands) (Adopted from “Estimated cancer incidence and mortality in Europe in 2008”; Eur J Cancer 2010, 46:765-781).

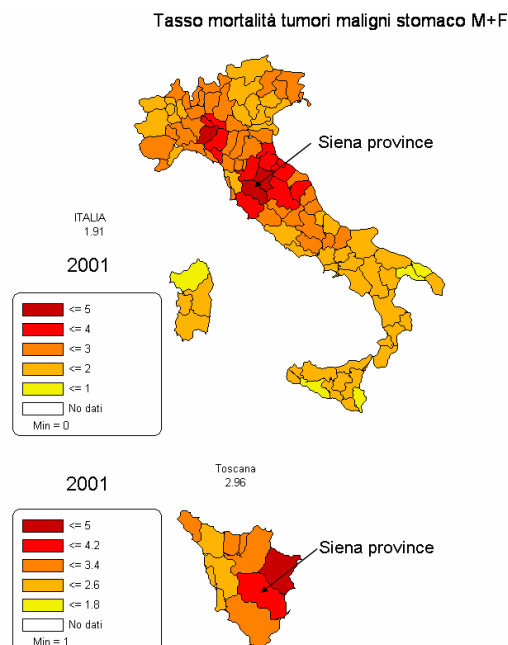


Figure 3. Mortality rate for gastric carcinoma in Italy. Tuscany region, in particular the province of Siena, are the higher risk-area for GC (adopted from www.istat.it).

1.2 Pathologic classification

a) Early GC

a.1) *Macroscopic aspects.* Early GC is a malignant epithelial neoplasm limited to the mucosa and submucosa. It can be divided into 2 groups: intramucosal and submucosal carcinoma, both likely to produce lymph node metastases. In eastern countries, where people undergo screening for the early diagnosis of GC, early gastric carcinoma reaches a percentage of 30-50%. In western countries where screening is not performed, the figure decreases to 16-24%. The follow up of dysplastic lesions does not appear to influence the prevalence of early GC. Most early carcinomas are located in the distal section of the stomach and these tumors are divided into three main types according to their endoscopic appearance: a) type I (protruding), b) type II (superficial), c) type III (excavated) (figure 4) [5].

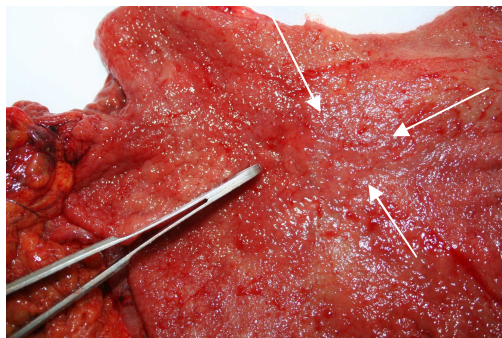


Figure 4. Early GC, type IIb. Arrows indicated the tumoral area.

a.2) *Histologic classification.* Early carcinomas of type I (protruding) are often well differentiated, intestinal-type carcinomas with tubular or papillary formations. Type II carcinomas histologically correspond to intestinal carcinomas, ranging from well to poorly differentiated ones. Early carcinomas of type III can be intestinal or diffuse. *Kodama* has divided intramucosal carcinomas in small mucosal carcinomas (<4cm SM) and superficial carcinomas (>4cm= SUPER) [6]. Both these types of intramucosal carcinoma can be strictly confined to the mucosa (small mucosa M and superficial M) or focally infiltrate the submucosa. In the penetrating variant (which includes two subcategories, Pen A e Pen B) the

invasion of the submucosa is more extensive than in the two variants mentioned before. Pen A is characterised by infiltration of the submucosa along the whole margin of the neoplasia. Pen B, on the other hand, is the more frequent of the two and penetrates muscularis mucosa discontinuously at multiple sites. The prognosis is worse in Pen carcinomas than in small mucosal and superficial ones and Pen A is the subcategory presenting the least favourable evolution of all. Kodama's classification agrees with the theory according to which the frequency of lymph node metastases depends on the depth of infiltration of the mucosa and submucosa. The incidence of metastases in intramucosal carcinomas is 4% while it increases to 17% in early carcinomas infiltrating the submucosa. In spite of these probabilities of developing metastases, early carcinomas have a more favourable prognosis than advanced carcinomas. Five years after gastrectomy patients with early carcinomas have a survival rate of 80% which decreases to 20% in patients with advanced gastric carcinoma.

Other histologic types of gastric carcinoma are a) mucinous carcinoma (muroid, gelatinous), b) adenosquamous carcinoma and squamous carcinoma c) hepatoid carcinoma, d) parietal cell carcinoma, e) lymphoepithelioma-like carcinoma (undifferentiated carcinoma with plentiful lymphoid stroma), and f) carcinosarcoma.

b) Advanced GC.

b.1) Macroscopic aspects. These carcinomas infiltrate the gastric wall beyond the submucosa. They are mainly located in the distal stomach, in the antro-pyloric region or along the lesser curvature while they are less frequent in the body of the stomach. Carcinomas originating in the cardia or at the junction of the cardia and the oesophagus are increasingly frequent and are generally smaller in size than tumours of the distal stomach. Advanced carcinomas are divided into 4 macroscopic types according to the criteria proposed by *Borrmann*: a) polypoid, b) fungating, c) ulcerated; d) infiltrative (figure 5, 6).

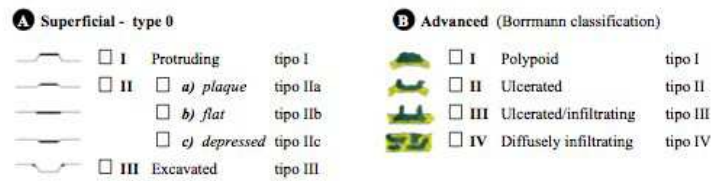


Figure 5. Macroscopic classification of early and advanced GC.



Figure 6. Ulcerated and advanced gastric adenocarcinoma; Borrmann IV. Arrows indicated the tumoral area.

According to the Japanese classification of gastric carcinoma [5], GC can be macroscopically categorised into five types: a) type 0 (superficial), b) type 1 (polypoid), c) type 2 (ulcerated), d) type 3 (ulcerated/infiltrating), e) type 4 (diffusely infiltrating).

b.2) Histologic classifications. The most widely used histological classification is the *Lauren* classification, which classifies advanced carcinomas according to 3 different types [6]: a) intestinal, b) diffuse (signet-ring cell carcinoma), c) mixed (presence of both intestinal and diffuse aspects).

The intestinal type is the most frequent in countries with a high incidence of GC and correlates with the action of exogenous factors (as *H. Pylori* infection or a diet rich in salt). The most frequent site for this type of carcinoma is the antro-pyloric region and its predominant macroscopic appearance is fungating. The frequency of intestinal carcinoma is declining in Western countries and this is due to a reduction of environment-related risk factors. In contrast, the frequency of diffuse carcinoma tends to remain steady. This type of carcinoma is responsible for rare cases of gastric carcinoma affecting patients with early onset of age and for hereditary carcinomas, as carriers of *CDH1* germline mutations. Intestinal type carcinomas have a glandular structure similar to that of carcinomas arising in the small or large intestine. Multifocal chronic gastritis with glandular atrophy and intestinal metaplasia is the gastric pathology, which more frequently gives rise to intestinal carcinoma. The degree of differentiation of intestinal carcinoma varies considerably from case to case but generally, in inverse proportion to the size of the tumour. Sometimes, the carcinoma is so well differentiated that it looks like an intestinal metaplasia of complete type. On the contrary, poorly differentiated variants may appear solid.

Diffuse carcinoma consists of poorly cohesive cells which infiltrate the gastric wall and do not form glands. The cells are generally small and round, either arranged as single, scattered cells, or clustered in ill-defined, lacy, microglandular, reticular formations. The cells tend to accumulate intracytoplasmic mucin which makes the nucleus move to either pole of the cell (signet-ring image). Diffuse carcinoma is often associated with stromal desmoplasia (stromal production of a great many collagenous fibres) and inflammatory reaction. However, the most frequent macroscopic appearance of diffuse carcinoma is ulcerated (type 3) or infiltrative (type 4).

Carneiro has proposed a new classification of advanced gastric carcinoma along the following lines [7]: a. glandular type and b. isolated cell type which generally corresponds to Lauren's intestinal and diffuse carcinomas, c. solid type formed by clusters, trabeculae and solid nests of undifferentiated cells without the formation of glands d. mixed type which is a cross between the glandular type and the isolated cell type. In a sample of Portuguese patients the glandular type was found to be the most frequent, followed by the mixed, solid and isolated cell types (figure 7).

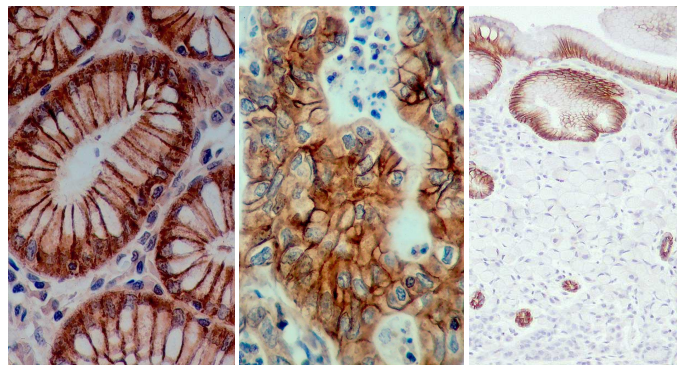


Figure 7. The Carneiro classification; respectively normal mucosa, glandular and isolated cell types stained for E-cadherin.

1.3 Staging system

a) *The AJCC-UICC TNM staging system.* According to the 7th edition of the TNM classification [9], all tumors with an epicenter in the stomach greater than 5 cm from the EGJ or those within 5 cm of the EGJ without extension into the esophagus are staged using the gastric carcinoma scheme. Tumors arising at the EGJ, or arising in the stomach within 5 cm from EGJ and crossing the EGJ are classified and staged using the TNM system for esophageal adenocarcinoma.

a.1) *Primary tumor (T).* A tumor may penetrate the muscularis propria with extension into the gastrocolic or gastrohepatic ligaments, or into the greater or lesser omentum, without perforation of the visceral peritoneum covering these structures. In this case, the tumor is classified T3. If there is perforation of visceral peritoneum covering the gastric ligaments or the omentum, the tumor should be classified T4. The adjacent structures of the stomach include the spleen,

transverse colon, liver, diaphragm, pancreas, abdominal wall, adrenal gland, kidney, small intestine and retroperitoneum. Intramural extension to the duodenum or esophagus is classified by the depth of the greatest invasion in any of these sites, including the stomach. This classification does not mention the mesocolon as an adjacent structures involved in GC. A recent study has shown as the clinicopathologic characteristics of tumor with invasion of this anatomical structure are more similar to those of the tumor with infiltration of adjacent structures (T4b) than the tumor with infiltration of serosa (T4a). Furthermore, the survival rate of patient affected by tumor with mesocolon invasion is lower than that of the patients with T4a tumor but similar to that with T4b tumor [10].

The 7th edition of TNM staging system [5] has some changes with respect to the previous 6th edition [11], providing a more detailed classification of prognosis, particularly between T2 and T3 carcinomas [12]. In the most recent edition, tumor involving muscularis propria and tumor involving SS are respectively the categories T2 and T3, which are now considered in the stage grouping. In the previous edition, although cancer invading muscularis propria was the subcategory T2a and cancer invading SS was the subcategory T2b, these subcategories were not maintained in stage grouping. These changes were justified because patients with GC invading MP have less nodal involvement, lower recurrence rates, more favorable prognosis and longer disease free survival than patients with cancers invading SS [13-17].

Despite the new staging system [5] better represents the prognosis for patients with MP or SS involvement, the prognostic impact of different subclassifications has been investigated in these tumors. It was reported that the prognosis of T2 tumor invading the outer longitudinal muscle bands of MP is similar with subserosal cancer (T3), but significantly poorer than T2 cancer invading the inner circular muscle bands of MP [18]. In subserosal tumor (T3), a recent study has shown that the infiltrating growth pattern of invasion of subserosal layer with an indistinct border, according to the JCGC [5], is an independent negative prognostic factor and it is closely related to peritoneal recurrence [19]. Moreover, it was reported that the horizontal width of spread of T3 tumor in the subserosal layer has a prognostic impact. The prognosis of cancer

with SS invasion of less than 20 mm in width (SS-narrow cancer) is similar to that of T2 cancer, while cancer with SS invasion of more than 20 mm in width (SS-wide cancer) behaves very similar to T4a cancer [20]. With respect to the previous edition, the 7th edition reports modifications in the categorization of tumor with serosal or deeper involvement. The subcategory T4a (cancer perforates serosa) was the category T3 and the subcategory T4b (cancer invades adjacent structures) was the category T4, but these two subcategories are still maintained in the stage grouping.

a.2) Regional LNs (N). Regional LNs categories are defined on basis of the number of metastatic lymph nodes. Involvement of the nodes into the hepatoduodenal ligament, retropancreatic, mesenteric and paraaortic areas is classified as distant metastasis (M1). According to the 6th edition [11], N stage was categorized as N0 (no metastatic LNs), N1 (1 to 6 metastatic LNs), N2 (7-15 metastatic LNs), N3 (>15 metastatic LNs). The recent 7th edition [9] introduced a new grouping of N categories: former N1 was subdivided in current N1 (1 or 2 metastatic LNs) and N2 (3 to 6 metastatic LNs); former N2 and N3 categories are now named N3a and N3b (figure 8).

Location	LMU/MUL MLU/UML	LD/L	LM/M/ML	MU/UM	U	E+
Lymph node station						
No. 1 rt paracardial	1	2	1	1	1	
No. 2 lt paracardial	1	M	3	1	1	
No. 3 lesser curvature	1	1	1	1	1	
No. 4sa short gastric	1	M	3	1	1	
No. 4sb lt gastroepiploic	1	3	1	1	1	
No. 4d rt gastroepiploic	1	1	1	1	2	
No. 5 suprapyloric	1	1	1	1	3	
No. 6 infrapyloric	1	1	1	1	3	
No. 7 lt gastric artery	2	2	2	2	2	
No. 8a ant comm hepatic	2	2	2	2	2	
No. 8p post comm hepatic	3	3	3	3	3	
No. 9 celiac artery	2	2	2	2	2	
No. 10 splenic hilum	2	M	3	2	2	
No. 11p proximal splenic	2	2	2	2	2	
No. 11d distal splenic	2	M	3	2	2	
No. 12a lt hepatoduodenal	2	2	2	2	3	
No. 12b.p post hepatoduod	3	3	3	3	3	
No. 13 retropancreatic	3	3	3	M	M	
No. 14v sup mesenteric v.	2	2	3	3	M	
No. 14a sup mesenteric a.	M	M	M	M	M	
No. 15 middle colic	M	M	M	M	M	
No. 16a1 aortic hiatus	M	M	M	M	M	
No. 16a2,b1 paraaortic, middle	3	3	3	3	3	
No. 16b2 paraaortic, caudal	M	M	M	M	M	
No. 17 ant pancreatic	M	M	M	M	M	
No. 18 inf pancreatic	M	M	M	M	M	
No. 19 infradiaphragmatic	3	M	M	3	3	2
No. 20 esophageal hiatus	3	M	M	3	3	1
No. 110 lower paraesophag	M	M	M	M	M	3
No. 111 supradiaphragmatic	M	M	M	M	M	3
No. 112 post mediastinal	M	M	M	M	M	3

M : lymph nodes regarded as distant metastasis

E+ : lymph node stations re-classified in cases of esophageal invasion

Figure 8. The LN classification in accord to the Japanese guidelines (JGCC) [The new GC classification was recently revised in Gastric Cancer 2011;14:101-112].

Adequate dissection of regional nodal areas is important to warrant appropriate determination of the pN status. Although it is suggested that at least 16 regional nodes should be assessed pathologically, a pN0 determination may be assigned on basis of the actual number of nodes evaluated microscopically. The extent of LN dissection performed by the surgeon strongly influences the number of LNs examined by the pathologist. Anatomical studies [21, 22] identified a median number of 25 LNs per patient after extended LN dissection (D2 according to the JGCA [5] and 43 to 52 LNs per patient after super-extended LN dissection (D3 according to JGCA). A recent analysis of the IRGGC on the quality assessment of LN dissection [23] identified a median of 14 examined LNs after D1 LN dissection, 29 after D2 and 46.5 after D3. Another study reported a median of 18 examined LNs per patient after D1 α - β lymphadenectomy and 31 after D2 [24]. The incidence of inadequate LN examination (<15 examined LNs per patient) was 54.5% after D1 [19], 34.1% after D1 α - β [24], 2.6 to 6.2% after D2 and 1.4% after D3 lymphadenectomy (figure 9 and 10). These figures are far away from those reported by Western population-based studies [25-27], with a median of 10

examined LNs per patient and incidence of inadequate lymph node examination exceeding 60% of cases.



Figure 9. Classification of locoregional LNs (adopted from “Japanese classification of gastric carcinoma, 2nd English edition”. Gastric Cancer 1998,1:10-24).

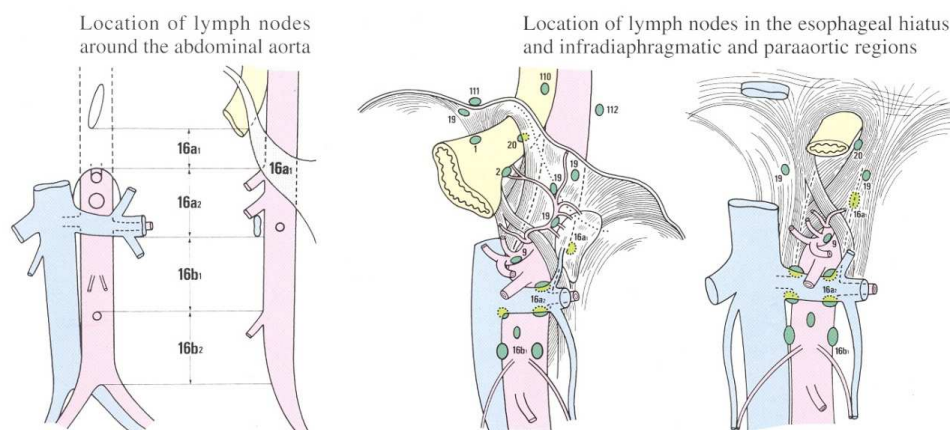


Figure 10. Classification of LNs around the abdominal aorta (n° 16) the oesophageal hiatus, infradiaphragmatic and para-aortic regions (adopted from “Japanese classification of gastric carcinoma, 2nd English edition”. Gastric Cancer 1998,1:10-24)..

Although the TNM staging system, the most widely used, is simple and reproducible, the appropriate classification of nodal status is still debated and different staging systems have been proposed and investigated. Its limitations are that it demands the examination of at least 15 LNs and inadequate staging

(understaging) is obtained in case of limited node dissection [28]. In the JCGC, the status of regional LN metastasis is defined according to their anatomical locations in relation with the site of the primary tumor (upper, middle and lower third of the stomach) [5]. When limited lymphadenectomy (D1 dissection) is performed, no information is obtained about extra-perigastric nodes and a complete nodal staging cannot be assessed.

In order to overcome the problem of stage migration induced by extended LN dissection [29, 30], during the last years a new independent prognostic factor was largely investigated and validated: the LNR, defined as the absolute ratio between metastatic and examined LNs, appeared to be a strong independent indicator of prognosis [31-34], even in case of inadequate nodal staging (<15 examined lymph nodes) [35]. The IRGGC defined four prognostic intervals for LNR: 0; 0.01-0.09; 0.10-0.25; >0.25. These intervals corresponded to a definite different prognosis even within the same pN status, independently from the extent of nodal dissection [31]. It was already demonstrated that LNR is independently influenced by the number of metastatic LNs but not by the number of examined LNs [24, 31, 33], being therefore suitable as a prognostic factor independent from the stage-migration phenomenon. Moreover, LNR reflects both characteristics of the tumor (number of metastatic nodes) and of the surgical treatment (extent of LN dissection). As a consequence, a TRM instead of a TNM staging system was suggested [36].

In the recent new 7th Edition of the TNM staging system [9], the UICC elected to retain the pN classification based on the number of metastatic nodes. It seems that this new pN stage-grouping is more accurate as a prognostic predictor than its precursor [37]. It will be very interesting, in the future, to investigate the prognostic yield of this new TNM stage-grouping faced to LNR. Recently, another classification based on size of LN metastasis has been proposed. In any TNM stage of patients with LN metastasis, this classification yields different prognostic groups. Different cut-off have been proposed, 8 mm [38] and 2 cm [39], to define two N subcategories: n1, LN metastasis with largest size less than the cutoff and n2, LN metastasis with largest size equal or larger than the cutoff.

a.3) *Distant metastasis (M)*. Absence and presence of distant metastasis are respectively defined M0 and M1. Liver, peritoneal surface and distant LNs are the most common metastatic sites in GC. Metastases to central nervous system and lung occur but are less frequent.

The TNM staging system classifies positive peritoneal cytology as metastatic disease (M1). Although the cytological detection of free cancer cells in peritoneal washing is a marker of poor prognosis and peritoneal recurrence [40], in Japanese [41] and Western series [42, 43], it was reported that in peritoneal lavage cytology does not increase the prognostic information already provided by the pathologic T and N after curative resection [41]. Moreover, it should be considered that peritoneal lavage cytology is not widely practiced in Western centers [44] and that it is characterized by a high specificity in predicting peritoneal recurrence but it has a low sensitivity [40].

1.4 Risk factors

a) *Tobacco consumption and dietary habits*. Smoking is the major risk factor for esophageal and gastric cardia adenocarcinomas, accounting for approximately 40% of cases. It was shown that most but not all studies observed a higher risk in the cardia than in the noncardia site in the specific cigarette smoking comparing with nonsmokers; conversely alcohol consumption may be associated with an increased risk of distal and total GC [45, 46]. Risk of gastric cardia adenocarcinomas is increased among current cigarette smokers with a reduction observed until 30 years after smoking cessation; the risk rose with increasing intensity and duration of smoking. Because of the long lack time before risk of these tumors is reduced among ex-smokers, smoking may affect early stage carcinogenesis.

Recently, we verified that the risk of cardia gastric carcinoma is associated with cigarette smoking and with the -77CC single nucleotide polymorphism located on the promoter region of *XRCC1* gene, that is involved in the DNA excision repair system (paper I) [47].

In gastric carcinogenesis, diet is determinant. Rather than only one kind of agent, factors like dietary habits, consumption frequency, amount of the etiologically important agents, traditional food-preparation techniques, and storage methods, all play major roles in the etiology of GC. Excessive salt consumption is also a confirmed factor. Excessive consumption of fish and other seafood plays the lead in the GC etiology in Japan and other Far-East countries. Lower consumption of fresh fruits and vegetables are other important etiologic factors. Dietary factors and improvement in food storage caused a significant decline in the worldwide prevalence of GCs. Interestingly, Palli and Colleagues showed in another study performed in the same geographical region an association between GC and high consumption of red meat [48]. Etiopatogenesis of gastric cardia carcinoma is related with nitrosamines, some of which are present in tobacco and tobacco smoke that are a class of compounds whose carcinogenic activities have been well established. Similarly, cured fish and meat could contain NOC and probably the mechanism of carcinogenesis of tobacco consumption and some diet habits is due to the common presence of NOC. The role of NOC in GC etiology is related with the alteration of the pH gastric juice; in the specific at the presence of NOC pH juice is low (1.0-2.5), leaving to a phenomenon called chemical gastric nitrosation that proceeds as an acid-catalyzed reaction in the normal acidic stomach before premalignant lesions develop. High concentration of NOC has been identified *in vivo* also in bacterial inflammatory of gastric mucosa with a high pH level (6.0-8.5); at the same way, high pH level step induced to the bacterial gastric nitrosation. This chemical modification induces an increased tolerance to DNA damage with reduced activity of MMR genes activity leading to an accumulation of errors during the DNA replication.

b) *H. pylori* infection. Several studies reported a strong association between chronic *H. pylori* infection and GC risk; as such, the World Health Organization's International Agency for Research on Cancer recognized *H. pylori* as a Group 1 carcinogen for humans [49]. Chronic gastric inflammation and the interaction between *H. pylori* and gastric epithelial cells have been suggested as potential mechanisms in gastric carcinogenesis. However, only a few individuals infected by

H. pylori eventually develop GC, probably this is due to other environmental factors, host-inflammatory genetic susceptibility and variation of the bacterial strains [50-52]. *H. Pylori* is a Gram-negative bacterium that colonizes the human gastric epithelium, the severity of *H. pylori* related disease is correlated with the presence of *cag* PAI associated with production of the *cagA* antigen. The *cagA* gene, the strain-specific *H. pylori* gene, is located in the right half of the PAI, and encodes the protein *cagA*, which is secreted via a type IV secretion system and translocated into gastric epithelial cells affecting host cell physiology. Infection with *cag* PAI-positive *H. pylori* strains has been recognized as a marker for strains that confer increased risk for peptic ulcer disease, gastric mucosal atrophy and GC. The *cagA* gene is one of several genes of a PAI called the *cag* PAI and the presence of *cagA* is considered to be a marker of the presence of the *cag* PAI. The *vacA* gene is present in all *H. pylori* strains and encodes the vacuolating cytotoxin *vacA*, which induces epithelial cell injury. *H. pylori* colonizes the atrophic stomach poorly, and intestinal metaplasia hardly at all, suggesting that the bacteria may create the environment for intestinal-type gastric carcinogenesis (atrophy and hypochlorhydria) rather than causing the cancer directly. The risk of developing GC for infected persons is estimated to be between two and three times higher compared to none infected ones. More recently, the role of individual susceptibility has been stressed and pro-inflammatory cytokine gene polymorphism has been demonstrated to interact in the compound process of gastric carcinogenesis in several studies [53] In particular, some Authors investigated the relationship between interleukin-1 gene polymorphism and GC risk. The conclusions supported the theory in which host genetic factors affecting the inflammatory and immune response to *H. pylori* infection may determine a higher risk of GC development, in particular in high risk area [54]. In addition, a variety of studies have indicated that salted, smoked, pickled and preserved foods (rich in salt, nitrite, and preformed N-nitroso compounds) as well as meat intake are associated with an increased risk of non-cardia GC [55]. In GC, the absence of *H. Pylori* infection is related with specific clinicopathologic factors impacting GC long term survival. Negative *H. pylori* status is associated with cardia tumor location, advanced pT classification, noncurative surgery, and a lower 5-year survival rate after R0 resection. Marrelli

and Colleagues demonstrate that *H. pylori* status is a significant prognostic factor in which negative *H. pylori* status appears to be an indicator of poor prognosis in patients with GC (paper 10.1) [56].

1.5 Sporadic and hereditary forms of GC and CDH1 gene

About 90% of GC cases appear in a sporadic setting, whereas familial clustering is observed in the remaining 10%. Of these, only 1-3% is hereditary. Among the cases with familial aggregation of GC, the most common disease is the hereditary diffuse gastric carcinoma syndrome (HDGC) related with the *CDH1* germline mutations, identified firstly in 1998 by Guilford and Colleagues in Maori kindred from New Zealand.

CDH1 gene maps to chromosome 16q22.1 and consists of 16 exons that encodes for a 120 kDa protein called E-cadherin that is a member of transmembrane glycoproteins family expressed on epithelial tissue and responsible for calcium-dependent cell-to-cell adhesion. Human E-cad is considered an invasion suppressor and has been demonstrated to be critical for establishing and maintaining polarized and differentiated epithelia through intercellular adhesion complexes. The structure of E-cad is presented in figure 11. In GC important epigenetic and structural mechanisms of *CDH1* inactivation/deregulation are somatic mutations, LOH and promoter hypermethylation. *CDH1* deregulation has been identified in early tumors of patients with *CDH1* germline mutations demonstrating to be an initial event in HDGC [57, 58].

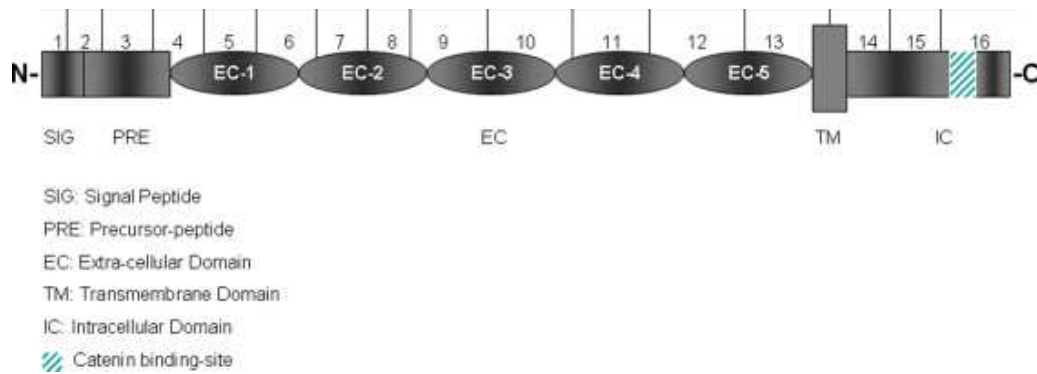


Figure 11. Structure of E-cadherin protein (adopted from “E-cadherin and hereditary diffuse gastric cancer”. Surgery 2007,142:645-657).

To date, many studies reported different type of *CDH1* alterations in HDGC families and in EODGC; it has been identified 122 germline mutations, among these 71.9% are non-missense (truncating, splice site, non-sense, large deletion) and 28.1% are missense mutations (personal and unpublished data) (table 1). For E-cad germline missense mutations functional assessment with *in vitro* and *in vivo* analysis is required, since it could be appear as pathogenic or neutral variant [59-62].

Table 1. Overall *CDH1* germline mutations identified to date in hereditary and early onset diffuse gastric carcinoma

Reference	<i>CDH1</i> _mut	Mut_syte	Mut_type	Ancestry
Bacani (2006)	5'UTR(-117)G>A	Promotor	5'UTR sub.	USA (1)
Bacani (2006)	5'UTR(-71)C>G	Promotor	5'UTR sub.	USA (1)
Corso (2010)	-63C>A	Promotor	5'UTR sub.	Italy (1)
Oliveira (2009)	Del 5'UTR-ex 1	Promotor	Deletion	Spain (1)
				Ireland (1)
Oliveira (2009)	del ex 1-2	Exon 1-2	Deletion	Canada (1)
				Lithuania (1)
Guilford (2010)	2T>C	Exon 1	Missense	Unknow (1)
Suriano (2005)	3G>C	Exon 1	Nonsense	Canada (1)
Bacani (2006)	41delT	Exon 1	Deletion	USA (1)
Oliveira (2002)	45insT	Exon 1	Insertion	UK (1)
Guilford (2010)	46insTGC	Exon 1	Insertion	Unknow (1)
Bacani (2006)	48+5G>C	Intron 1	Splice Site	USA (1)
Bacani (2006)	48+15C>G	Intron 1	Splice Site	USA (1)
More (2007)	49-2A>C	Intron 1	Splice site	Europe (1)
Richards (1999)				UK (1)
Moran (2005)	49-2A>G	Intron 1	Splice Site	Ireland (1)
Humar (2002)	53delC	Exon 2	Deletion	Europe (1)
Richards (1999)	59G>A	Exon 2	Non sense	UK (1)
Guilford (2002)	70G>T	Exon 2	Non sense	USA (1)
Shinmura (1999)	185G>T	Exon 3	Missense	Japan (1)
Gayther (1998)	187C>T	Exon 3	Non sense	USA (1)
Guilford (1999)	190C>T	Exon 3	Non sense	New Zealand (1)
Dussaulux-G. (2001)	283C>T	Exon 3	Non sense	France (1)
More (2007)	353C>G	Exon 3	Missense	Europe (1)
Keller (1999)	372delC	Exon 3	Deletion	Germany (1)
Kaurah (2007)	377delC	Exon 3	Deletion	Unknow (1)
Brooks-W. (2004)	382delC	Exon 3	Deletion	Europe (1)
Bacani (2006)	388+26C>T	Intron 3	Splice Site	USA (1)
Kaurah (2007)	515C>G	Exon 4	Missense	Unknown (1)
Avizienyte (2001)	525 C>G	Exon 4	Missense	Finland (1)
Kaurah (2007)	532-18C>T	Intron 4	Splice site	UK (1)
Oliveira (2004)	532-18C>T	Intron 4	Splice site	Portugal (1)
Guilford (1999)	586G>T	Exon 5	Non sense	Europe (1)
Brooks-W. (2004)	531+1G>A	Intron 5	Splice site	Europe (1)
Frebourg (2006)	531+2T>A	Intron 5	Splice site	France (1)
Mateus (2009)	641T>C	Exon 5	Missense	Europe (1)
Corso (2010)	670C>T	Exon 5	Missense	Italy (1)
More (2007)	715G>A	Exon 6	Missense	Europe (1)
Yoon (1999)	731A>G	Exon 6	Missense	Korea (1)
Guilford (2010)	753insG	Exon 6	Insertion	Unknown (1)
Kaurah (2007)	808T>G	Exon 6	Missense	Unknown (1)
Oliveira (2002)	832G>A	Exon 6	Splice site	Pakistan (1)
Rogers (2008)	833-2A>G	Intron 6	Splice site	USA (1)
Brooks-W. (2004)	892G>A	Exon 7	Missense	Europe (1)
Jonsson (2002)				Sweden (1)
Suriano (2005)	1003C>T	Exon 7	Non sense	Canada (2)
Guilford (1998)	1008G>T	Exon 7	Splice site	New Zealand (1)
Oliveira(02)Zhang(06)	1018A>G	Exon 8	Missense	EU (1) China (1)
Guilford (2010)	1023T>G	Exon 8	Nonsense	Unknown (1)
Suriano (2005)	1063delT	Exon 8	Deletion	Canada (1)
Brooks-W. (2004)	1064insT	Exon 8	Insertion	UK (1)
More (2007)	1107delC	Exon 8	Deletion	Spain (1)
Roviello (2007)	1118C>T	Exon 8	Missense	Italy (1)
Brooks-W. (2004)	1134del8ins5	Exon 8	Deletion/Ins	Netherlands (1)
More (2006)	1137G>A	Exon 8	Splice site	Europe (2)
Guilford (1999)	1137+1G>A	Intron 8	Splice site	USA (1)
Brooks-W. (2004)	1212delC	Exon 9	Deletion	Europe (1)
Brooks-W. (2004)	1226T>C	Exon 9	Missense	Europe (1)
Wang (2003)	1243A>C	Exon 9	Missense	Japan (1)
Suriano (2005)	1285C>T	Exon 9	Missense	Canada (1)
Brooks-W. (2004)	1212delC	Exon 9	Deletion	Europe (1)
Brooks-W. (2004)	1226T>C	Exon 9	Missense	Europe (1)
Suriano (2005)	1285C>T	Exon 9	Missense	America (1)
Mayrbaeurl (2010)	1306_1303insA	Exon 9	Del/ Ins	Austria (1)
More (2007)	1306_1307delTT			
	1391-1392delTTC	Exon 10	Deletion	Europe (1)

Yoon (1999)	1460T>C	Exon 10	Missense	Korea (1)
Barber (2008)	1466insC	Exon 10	Insertion	UK (1)
Oliveira (2002)	1472insA	Exon 10	Insertion	UK (1)
Brooks-W. (2004)	1476delAG	Exon 10	Deletion	Europe (1)
Guilford (1999)	1487del7	Exon 10	Deletion	New Zealand (1)
Jiang (2004)	1507C>T	Exon 10	Non sense	China (1)
Humar (2002)	1565+1G>T	Intron 10	Splice site	New Zealand (1)
Rogers (2008)	1565+2insT	Intron 10	Insertion	USA (1)
Guilford (1999)	1588insC	Exon 11	Insertion	USA (1)
Rodriguez-S. (2006)	1610delC	Exon 11	Deletion	Spain (1)
Keller (2004)	1619insG	Exon 11	Insertion	Germany (1)
Kaurah P (2007)	1682insA	Exon 11	Insertion	Ireland (1)
Humar B (2002)	1710delT	Exon 11	Deletion	USA (1)
Gayther (1998)	1711insG	Exon 11	Insertion	USA (1)
Brooks-Wilson (2004)	1711+5G>A	Intron 11	Splice site	Europe (1)
Salahshor (2001)	1774 G>A	Exon 12	Missense	Sweden (1)
Brooks-W. (2004)	1779insC	Exon 12	Insertion	Europe (1)
Suriano (2005)	1792C>T	Exon 12	Non sense	UK (1)
Kaurah (2007)	1795A>T	Exon 12	Missense	Unknown (1)
Brooks-W. (2004)	1779insC	Exon 12	Insertion	Europe (1)
Suriano (2003)	1849G>A	Exon 12	Missense	USA (2)
Kaurah (2007)	1876T>A	Exon 12	Missense	Unknown (1)
Kaurah, More (2007)	1901C>T	Exon 12	Miss/splice site	UK (1) New Zeal (1) Portugal (1)
Suriano (2003)	1913G>A	Exon 12	Nonsense	Spain (1)
Bacani (2006)	1937-13T>C	Intron 12	Splice Site	USA (1)
Brooks-W. (2004)	2061delTG	Exon 13	Deletion	Europe (1)
Rogers (2008)	2064delTG	Exon 13	Deletion	USA (1)
Guilford (98) More (07)	2095C>T	Exon 13	Non sense	New Zeal (1) China (1)
Suriano G (2005)	2161C>G	Exon 13	Splice site	Canada (1)
Kaurah (2007)	2164+5G>A	Exon 14	Splice site	Unknwon (1)
Oliveira (2009)	del 14-16	Ex 14-16	Deletion	Europe (1)
Brooks-Wilson (2004)	2195G>A	Exon 14	Missense	Europe (1)
Kaurah (2007)	2245C>T	Exon 14	Missense	Colombia (1)
Simoës-Correia (08)	2269G>A	Exon 14	Missense	Portugal (1)
Ghaffari (2009)	2275G>T	Exon 14	Nonsense	Iran (1)
Suriano (2005)	2276delG	Exon 14	Deletion	Canada (1)
Guilford (2010)	2287G>T	Exon 14	Nonsense	Unknown (1)
Humar (2002)	2295+5G>A	Intron 14	Splice site	Europe (1)
Brooks-Wilson (2004)	2310delC	Exon 15	Deletion	Europe (1)
Kaurah (2007)	2343A>T	Exon 15	Missense	UK (1)
Guilford (1998)	2381insC	Exon 15	Insertion	New Zealand (1)
Kaurah (2007)	2392G>A	Exon 15	Missense	Unknown (1)
Rogers (2008)	2395delC	Exon 15	Deletion	USA (1)
Keller G (2004)	2396C>G	Exon 15	Missense	Germany (1)
Kaurah (2007)	2398delC	Exon 15	Deletion	Ireland (2)
Bacani (2006)	2439+31G>A	Intron 15	Splice site	USA (1)
Caron (2008)	2399delG	Exon15	Deletion	USA (1)
More (2007)	2440-6C>G	Intron 15	Splice site	Europe (1)
Yabuta (2002)	2494G>A	Exon 16	Missense	Japan (1)
Oliveira (2009)	Del ex 16	Exon 16	Deletion	Europe (1)

Recently, Oliveira and Colleagues identified a novel germline mechanism of *CDH1* inactivation found in family with aggregation for DGC (paper 10.2) [63].

Analyzing all identified *CDH1* germline mutations, it has been calculated that approximately 30% of the HDGC families harbouring *CDH1* germline mutations [64]. In 1999, the IGCLC defined the following criteria for identification of HDGC families: 1) two or more documented cases of DGC in first or second degree relatives, with at least one diagnosed before the age of 50; 2) three or more

documented cases of DGC in first or second degree relatives, independently of age of onset. After demonstrating the presence of *CDH1* germline mutations in patients not belonging to the IGCLC criteria, Brooks-Wilson and colleagues recommended their modification considering: 1) two or more documented cases of DGC in first degree relatives, with at least one diagnosed before age 50; 1A) two or more cases of GC, with at least one DGC diagnosed before age 50; 2) three or more documented cases of DGC in first degree relatives, diagnoses at any age; 2A) three or more cases of GC, diagnosed at any age, with at least one documented case of DGC; 3) isolated individual diagnosed with DGC at less than 45 years of age; 4) isolated individual diagnosed with both DGC and lobular breast cancer (no other criteria met); 5) one family member diagnosed with DGC and another with lobular breast cancer (no other criteria met); 6) one family member diagnosed with DGC and another with signet ring carcinoma of the colon (no other criteria met) [65]. These novel criteria introduced a new clinical entity called EODGC, in which E-cadherin mutations occur with a frequency of about 3%, 35 years old at diagnosis is the cut off age for the *CDH1* genetic screening (paper II) [66]. In the last consensus conference of Cambridge in 2008, the IGCLC modified the abovementioned criteria for *CDH1* genetic screening as following: a) two GC cases in family, one confirmed DGC <50; b) three confirmed DGC cases in first or second degree relatives independent of age; c) age <40; d) personal or family history of DGC and lobular breast cancer, one diagnosed at <50 [64]. DGC with SRCs is the predominant histological type in carriers of *CDH1* germline mutations. Early stage HDGC is characterized by the presence of multiple foci of diffuse type SRCs carcinoma confined to the superficial gastric mucosa [67]. The majority of neoplastic foci observed in the resected specimen after prophylactic gastrectomy are less than 1 mm in diameter, and all lay under normal appearing surface epithelium [68]. Multifocal microscopic disease is very common with a preferentially localization at the corpo/fundus of the epithelial wall. Carneiro and Colleagues proposed a histological model for GC development in E-cad mutation carriers: at the beginning, histopathological analysis shows a pattern of *in situ* SRCs carcinoma with early pagetoid spread with pagetoid proliferation of SRCs and, at last invasive SRCs carcinoma results evident. *H. pylori* infection is not

detected in gastric mucosa obtained from prophylactic gastrectomies [69]. E-cad truncating germline mutation carriers have a fairly high lifetime risk of developing GC; the estimated cumulative risk by age 80 years is 67% for men (95% CI, 39–99) and 83% for women (95% CI, 58–99) with a mean age at diagnosis of 40 years (range, 14-85) [64, 70]. Prophylactic total gastrectomy has been recommended for truncating *CDH1* mutation carriers and it remain to clarify the role of prophylactic gastrectomy in missense mutation carriers, because to date no prophylactic surgery has been performed in missense mutation cases [68]. Strategy for clinical management includes review of family history, genetic testing, chromoendoscopic surveillance and prophylactic surgery (paper III) [71]. Prophylactic total gastrectomy remains the only method to abolish the inherited risk for GC in *CDH1* truncating mutation carriers.

In HDGC the *CDH1* second hit of inactivation can be either due to structural alteration as LOH or somatic mutations or to an epigenetic event as promoter hypermethylation. In HDGC primary tumors promoter hypermethylation occur with the frequency of 50% and LOH is documented in 13% of patients. These events can appear concomitantly in about 19% of cases [72]. In sporadic GC, tumors can present promoter hypermethylation in about 55% of cases and somatic mutations or LOH respectively in 55% and 4% [73].

Recently, we analyzed 246 primary GC, aiming to assess *CDH1* somatic alterations and studying the associated clinicopathologic features. We verified that promoter hypermethylation occurred in 20.7% of sporadic GC and structural alterations (LOH and somatic mutations) in 11% of the cases. In particular, we found that *CDH1* structural alterations were associated to poor prognosis in GC patients (abstract I) [74].

1.6 MSI and oncogenic mutations.

MSI phenotype or mutator phenotype is caused by defect in the MMR system responsible for the correction of mismatches that occur during DNA replication. In gastric carcinoma this phenotype is found in about 25% of the

cases [75]. The MMR system is composed of at least seven proteins, *h-MLH1*, *h-MLH3*, *h-MSH2*, *h-MSH3*, *h-MSH6*, *h-PMS1*, and *h-PMS2* [76]. Genetic and epigenetics alterations occurring at these MMR system effectors, namely in *h-MLH1* and *h-MSH2*, and less frequently in *h-MSH6* and *h-PMS2*, are the main mechanisms by which MMR system failure occurs in MSI gastrointestinal cancer [77]. In Lynch syndrome, germline mutations at MMR genes are frequently detected [78]. Conversely, in MSI gastric carcinoma, no germline mutations have been described in MMR genes in familial GC and, somatic mutations in MMR genes are rare in gastric carcinoma (paper IV) [79, 80]. MSI GC from patients with familial history of GC were recently found to display similar clinico-pathologic features to those of MSI sporadic cases (papers IV, V) [80, 81].

Gastric tumors with MSI phenotype shows distinct clinicopathologic features; most MSI tumors are of intestinal histotype located in the distal part of the stomach, with limited LN involvement, more frequently in older women [82]. Survival analysis of patients with MSI status demonstrated a better long term prognosis in subjects with advanced tumors and marker instability (paper VI) [83]. MSI gastrointestinal cancers display particular epigenetic and genetic background associated with specific pathway, as the activation and deregulation of the *MAPK* cascade and the *PI3K* pathways (figure 12).

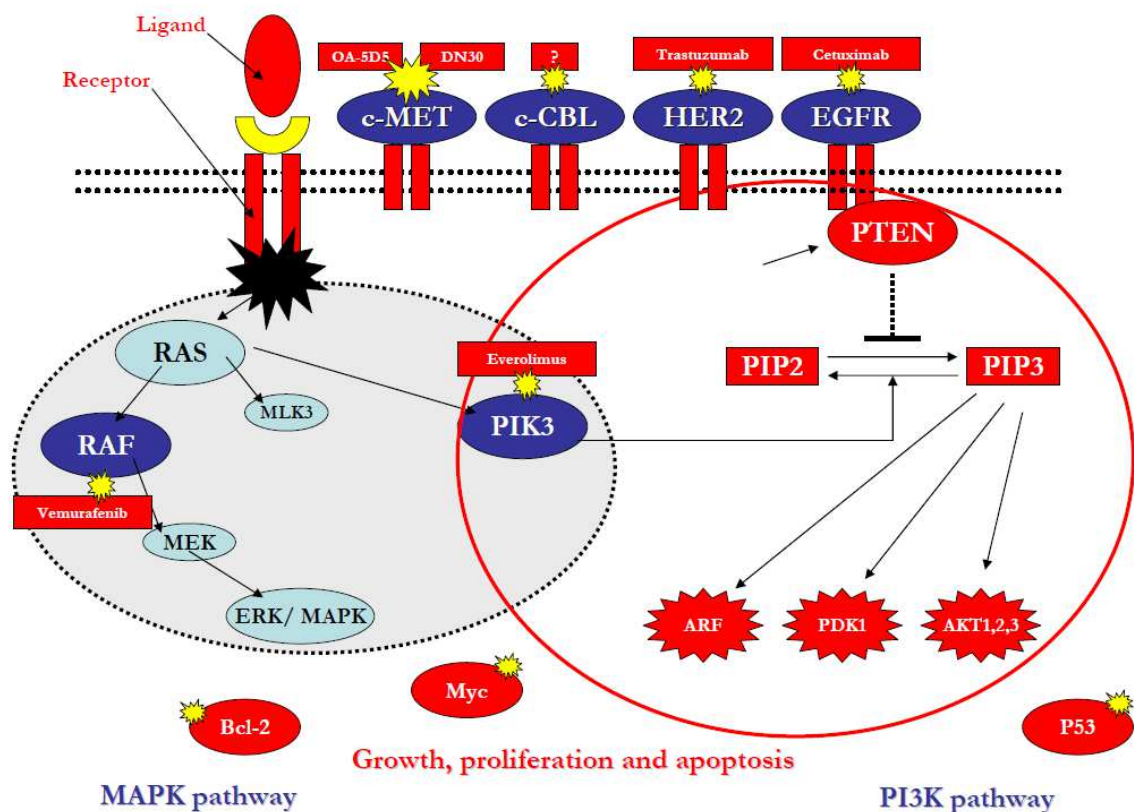


Figure 12. The MAPK/PI3K cascade and pathway. The future of tumor treatment will include the molecular-blockage of targeted oncogene/receptors. In this figure we propose a model of different receptors and oncogenes associating with its respective chemotherapies.

Several oncogene mutations belonging to *EGFR*- *MAP* and PI3K pathway are associated with MSI GC. In GC overall, *KRAS* gene mutations occur in 3-8% of the cases and whenever present, cluster in the MSI subset (~17% of MSI cases). In contrast, *BRAF* mutations are rare or absent in GC. *PIK3CA* oncogenic mutations occur with a frequency of about 14%. *EGFR* is frequently involved in the signaling pathway in MSI GC; in gastric carcinoma, although the frequency of *EGFR* overexpression is high, the mechanisms responsible of this deregulation remain undiscovered in most cases [84].

EGFR increased copy number was reported in about 13% of GCs, conversely, hotspot somatic mutations were found very rarely [85]; recently a novel mechanism underlying *EGFR* overexpression was described in MSI colon cancer [86]. High levels of *EGFR* expression were detected in cell lines due to the

presence of novel deletion in a polyA (13) repeat element within the 3'-UTR of the *EGFR* gene. This discovery defined a novel mechanism of *EGFR* activation and overexpression. Other oncogenes are rarely involved (3%) like *MLK3* as example. Detection of oncogenic mutations in *MAPK/PI3K* downstream can open new avenues regarding the stratification of MSI GC patients for novel target therapies, in particular with the *EGFR* inhibitors.

1.7 Prognosis and long-term survival

Long-term survival in patients with GC after curative (R0) resection is strongly influenced by pTNM stage classification, in which lymphatic dissemination is the main predictor of tumor recurrence. Furthermore, it has been demonstrated that extended lymphadenectomy may decrease the risk of tumor relapse after gastric resection, with several other prognostic factors indicative of long-term outcome. Indeed, overall 5-year survival for GC after curative (R0) resection is extremely variable, and significant differences in survival have been reported from institutions throughout the world.

In R1 and R2 cases, prognosis is determined primarily by the absence or presence of distant metastases while the pTNM classification is of minor significance; by contrast, in R0 patients, pTNM is a good predictor of prognosis. In this chapter, we review the overall survival of GC patients who underwent radical (R0) surgery, as reported by several institutions located in different geographic areas.

The R classification system was defined by Hermanek and Colleagues in 1994 [87, 88]. Surgery is defined as “potentially curative” (R0) when the surgical procedure is concluded without any evidence of macroscopic residual tumor in the tumor bed, lymph nodes, and/or at distant sites. In addition, macroscopic and microscopic examination of resection margins is negative. R1 and R2 indicate, respectively, microscopic and macroscopic residual tumor. According to this definition, the R classification is considered as a predictor of prognosis, in which good long-term survival can be expected only in R0 patients. However, this

classification system is currently discussed controversially by different authors because the definitions do not consider the presence of tumor dissemination through the blood stream, peritoneal dissemination, and/or lymphatic involvement. Consequently, the presence of microscopic and distant residual disease could be missed [89]. In this regard, clinical pre-operative staging is fundamental, including the selection of the appropriate modality to determine the extent of tumor spreading: endoscopic ultrasound, computed tomography, positron emission tomography, magnetic resonance imaging, or laparoscopic exploration [90]. More recently, the UICC/AJCC system defined as R0 resection a complete resection of the primary cancer without macroscopic or microscopic residual disease. Patients with circulating intraperitoneal cancer cells, identified by cytology examination after lavage, are classified as having R1 disease. According to the new UICC/AJCC TNM, the number of positive lymph nodes is considered a strong prognostic determinant, and cases with positive peritoneal cytology are considered as M1 and included in stage IV [91, 92].

Long-Term Results after R0 Resection in High-Incidence Areas. In this and the following sections, we analyze R0 GC cases and the long-term results, as reported from areas around the world where there is either a high or a low incidence of GC. Among the areas with a high GC incidence are Japan, Korea, China, and Latvia. In Korea and Japan, extended lymph node dissection (D2 or D3) is routinely performed.

Three studies of interest were carried out in Japan. Maruyama and Colleagues [93] analyzed the data of 8851 patients with primary gastric carcinoma (JGCA 2006). The authors identified 4959 GC patients as having R0 disease (namely curability A); the cumulative 5-year survival was 88.5%. A Japanese study based on tumor histology grade was performed on 1119 GC patients, including a subset of R0 cases. The tumors were graded as follows: grade 1, well differentiated; grade 2, moderately differentiated; and grade 3, poorly differentiated [94]. Five-year survival was 71% for patients with grade 1, 65.7% for those with grade 2, and 66.7% for those with grade 3 disease. In the third Japanese study [94], the outcome of 587 GC patients who underwent R0 gastrectomy with D2

lymphadenectomy was analyzed. Five-year survival according to the AJCC/UICC staging system was 94.6% for Ia, 88.4% for Ib, 70.6% for II, 54.1% for IIIa, 35.5% for IIIb, and 25.6% for IV. A population of 9998 GC patients was analyzed by Ahn and Colleagues [95] from Korea, which has the world's highest incidence of GC. Overall 5-year survival in their R0 resection patients was 66.4%. According to the TNM stage, the 5-year disease-free survival of patients with stages Ia, Ib, IIa, IIb, IIIa, IIIb, and IIIc was 95.1, 88.4, 84, 71.7, 58.4, 41.3, and 26.1%, respectively. Among Asian countries, a paper described 2159 patients in China who underwent both en bloc resection of primary GC and D2/D3 lymphadenectomy, without microscopic or macroscopic residual disease (R0). The 5-year disease-free survival of patients with stages I, II, III, and IV was 84, 50.8, 29.1, and 12.4%, respectively [96]. The advantage of these Asian studies was that in each one screening programs led to the diagnosis of GC at an early stage of the disease. Furthermore, most patients achieved radical (R0) resection with D2 or D3 lymphadenectomy. Good results were also reported in a Latvian study of 444 patients with GC who underwent R0 surgery [97]. Five-year survival was 52.5% but considering pTNM stage was 89.7% for stage Ia, 72.6% for stage Ib, 52.9% for stage II, 34.8% for stage IIIa, 27.4% for stage IIIb, and 18.7% for stage IV. Considered as a whole, the overall 5-year survival of patients from high-incidence areas treated by R0 surgery is approximately 44-88%. The lowest overall survival was registered in China (44%) and the highest in Japan (88.5%).

Long-Term Results after R0 Resection in Low-Incidence Areas. Among the countries with a low GC incidence, we consider studies from the USA, Germany, and Norway.

In the USA, the 711 patients analyzed in the study of Strong and Colleagues [98] were treated in a specialized center by R0 resection, with extended lymphadenectomy performed in most cases. The authors reported an overall 5-year survival rate of 58%. The study of Cunningham and Colleagues [99] consisted of 436 patients; overall 5-year survival after R0 resection in this group was 33%. In a trial comparing chemoradiotherapy after surgery vs. surgery alone, Macdonald and Colleagues [100] reported an overall 5-year survival of 44% in 281

patients treated by gastrectomy and chemoradiotherapy vs. 28% in 275 patients treated by surgery alone. Notably, in that trial only 10% of patients were treated by D2 dissection. A study from Germany analyzed 124 GC patients who underwent curative surgery (R0) with D2 lymphadenectomy [101]; the overall survival rate over a period of 94 months was 51%. A multicentric study was conducted in Germany by Siewert and Colleagues [102], in which 1182 consecutive patients underwent radical surgery with D1 or D2 lymphadenectomy. The 5-year survival rate with respect to stage was: Ia 82.8%, Ib 68.3%, II 42.9%, IIIa 28.2%, IIIb 16.7%, and IV 17.3%. Long-term survival was better in patients receiving R0 surgery and D2 lymphadenectomy at stage II (29% vs. 56.7%). In another Western study, from Norway [103], 97 patients underwent R0 surgery for gastric carcinoma with D1 lymphadenectomy; 5-year survival was 39%.

In low-incidence areas (USA, Norway, Germany), there are more cases of advanced stage disease and thus a lower overall 5-year survival (28-58%) than in high-incidence areas (Korea, Japan, China, Latvia), where survival is 44-67%. Specifically, the American data showed lower overall 5-year survival in GC patients with R0 resection (28-58%), with similar results in the population from Norway (39%). In Germany, the overall survival rate (42-51%) is within the range of that in high-incidence areas (44-88.5%). Generally, however, patients with R0 GC in countries with a higher incidence of the disease have a better prognosis than analogous patients in low-incidence countries.

The Experience of the IRGCC. The IRGCC assessed a prognostic score predicting tumor recurrence in GC patients with curative resection (R0) [104] based on 536 patients who underwent UICC R0 resection at three surgical department in Italy (University of Siena, University of Verona, and Morgagni Hospital of Forlì). During the follow-up period, tumor relapse was identified in 50.7% of these patients. The results of this study demonstrated that the predicted and observed risk of recurrence overlapped, with a sensitivity of 83.5% and specificity of 81.1%. The statistical model identified nodal status, pT stage, tumor location, extent of lymphadenectomy, and advanced age as independent predictors of recurrence.

Extended lymphadenectomy (D2) is considered the standard approach for the treatment of advanced GC, with benefits in long-term survival. The IRGGC demonstrated that the 5-year overall survival was 54% in patients with R0 surgery and D2 lymphadenectomy, and with a real survival benefit for patients with locoregional lymph node involvement [105]. Another study [106], conducted on 286 R0 GC patients treated with super-extended (D3) lymphadenectomy, demonstrated a potential benefit of this procedure, especially in patients with pT2 disease and positive nodes (5-year overall survival 60%).

The overall geographic Italian area is considered as low-risk for GC, but in some regions of the country the incidence is relatively high [107, 108]. In Tuscany, for example, the estimated incidence of GC is three-fold higher than in Southern Italy. GC patients shows a 5-year survival probability of 63.2% in Tuscany patients, compared to 48% in Southern patients.

The following table 2 reviews the overall survival rate reported in the literature for GC patients undergoing potentially curative surgery (R0).

Table 2

Country	Risk area	Number of patients	UICC	Lymphadenectomy	Follow-up end-time (months)	Overall survival (%)
Korea	High	9.998	R0	D2-D3	60	55.6
Japan	High	4.959	R0	D2	60	88.5
Japan	High	1.119	R0	Unknown	60	67.8
Japan	High	587	R0	D2	60	61.5
China	High	2.159	R0	D2-D3	60	44.0
Latvia	High	444	R0	D1	60	52.5
USA	Low	711	R0	D2	60	58.0
USA	Low	436	R0	Unknown	60	33.0
USA	Low	556	R0	D1	60	28-44
Germany	Low	124	R0	D2	94	51.0
Germany	Low	1.182	R0	D1-D2	60	42.7
Norway	Low	97	R0	D1	60	39.0
Italy-Tuscany	High	545	R0	D2-D3	60	63.2
Italy-Southern	Low	89	R0	D2-D3	60	48.0

Radical resection (R0) for GC offers the possibility of cure although it requires complete surgical removal of the tumor. The UICC classification for GC considers curative resection (R0) as a good predictive prognostic factor; nevertheless, the limitation of this classification (R0 vs. R1) is the risk of overlooking microscopic tumor diffusion, especially in cases with hematogenous or

peritoneal metastasis. Therefore, the R classification may represent a more valid prognostic indicator when associated with pTNM stage.

The overall survival of R0 patients who undergo GC resection is 30-70%, and varies notably in different institutions worldwide. The survival rate of R0 GC patients is better in high-incidence than in low-incidence areas. This difference has been explained by: (a) differences in the disease itself; (b) stage migration; and (c) differences in treatment. These explanations are probably not mutually exclusive. Moreover, environmental factors can interact with and influence molecular pathways, as can single-nucleotide polymorphisms and dietary and life-style habits.

The widespread introduction of screening programs in high-incidence countries has resulted in the detection of early stage GC, which in turn would explain differences in prognosis; in addition, those countries generally have a better diagnostic and therapeutic approach, which is also likely play a prognostic role. This points to the need for a standard surgical approach to the treatment of GC; for example, it has been demonstrated that extended (D2) and superextended (D3) lymphadenectomy are associated with better R0 outcome in advanced cases of GC, also in low-risk areas. Figure 13 shows the clinical and surgical management of GC in accord to the R classification.

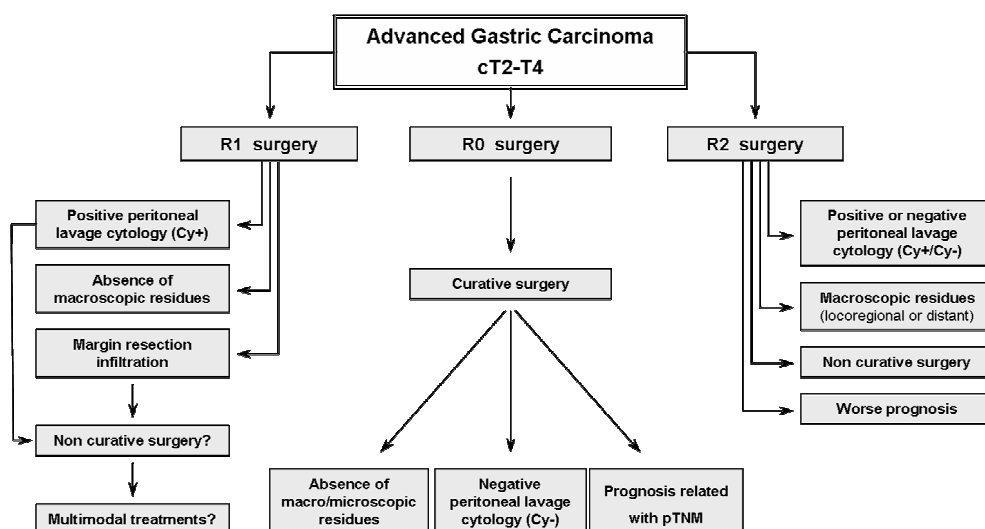


Figure 13. R classification and management of advanced GC

[Note. This paragraph was extracted from the chapter n° 17 of the book “*Surgery in Multimodal Management of Gastric Cancer*”; Roviello F, Corso G, and Marrelli D. (eds) Springer-Verlag Italia 2011 (paper 10.3)]

1.8 Extended (D2) and super-extended (D3) lymphadenectomy

The JGCA guidelines define and recommend D2 LN dissection for most gastric cancers. Limited lymphadenectomy, i.e., D1+ α or D1+ β LN dissection, is indicated for selected patients with stage T1 cancers. D1+ α dissection refers to the removal of level 1 (cardia perigastric group) stations plus station 7 (left gastric artery), with the addition of station 8a (common hepatic artery), and D1+ β dissection to the removal of level 1 (right paracardial group) stations plus stations 7 (left gastric artery), 8a (common hepatic artery), and 9 (celiac artery) [109, 110]. Gastrectomy with D1+ α is indicated for T1N0 tumors limited to the M and for differentiated SM tumors < 1.5 cm in diameter. Submucosal tumors that do not meet this condition and T1N1 (M, SM) tumors < 2.0 cm should be treated by gastrectomy with D1+ β [111].

Following publication of the 13th edition of the JCGC, in June 1999, the standard for advanced carcinomas has been the D2 dissection [112]. Indeed, GC who patients underwent R0 surgery with standard D2 lymphadenectomy were found to have better long-term survival [105]. The more recent, extended lymphadenectomy, D3 dissection, is indicated also in advanced gastric cancer, as shown in a selected sub-group of these patients who had longer survival after this procedure [106]. Clearly, adequate knowledge of LN classification is essential in gastric cancer in order to correctly perform a D2 standard or D3 extended lymphadenectomy, as defined by the JCGC.

In the late 1980s, Keiichi Maruyama and colleagues at the National Cancer Center Hospital in Tokyo created a computer program, known as the “Maruyama program,” that analyzed a large series of gastric cancer patients treated by extended lymphadenectomy (i.e., D2 or more). The program estimates the rate of metastasis in each of the 16 main LN stations. The Maruyama program has been used to assess Japanese, German, and Italian populations and found to be highly accurate [113-115]. It provides a highly valuable tool for surgeons in pre-operative

or intra-operative planning, as a convenient means of rationally determining an adequate lymphadenectomy for the treatment of gastric cancer.

D2 lymphadenectomy represents the standard surgical procedure for advanced gastric carcinoma. The technique has proven to be safe when conducted in specialized centers and is associated with a low risk of post-operative complications and mortality. D3 lymphadenectomy requires removal of the PANs. This procedure is an emerging technical approach especially indicated for patients with upper-third gastric cancers. It has an acceptable morbidity rate and no increase in mortality compared to earlier approaches (paper 10.4).

D2 Standard Lymphadenectomy and LN Stations. In patients with advanced gastric carcinomas, D2 dissection depends on the tumor's location.

Upper third. D2 classification of these tumors includes the following LN stations: right (station 1), and left (station 2) paracardial, lesser curvature (station 3), short gastric, right and left gastroepiploic vessels (stations 4sa, 4sb, 4d), supra- and infra-pyloric (stations 5, 6), left gastric artery (station 7), common hepatic artery (anterosuperior group station 8a), celiac artery (station 9), proximal (station 11p), and left hepatoduodenal (station 12a). Lymphadenectomy along the splenic hilum (station 10) and distal splenic artery (station 11) should be performed in cases of T3–T4 tumors with serosal infiltration.

Middle and middle/lower third. For gastric tumors located at this site, the D2 LN stations are: right (station 1), and left (station 2) paracardial, lesser curvature (station 3), short gastric, right and left gastroepiploic vessels (stations 4sa, 4sb, 4d), supra- and infra-pyloric (stations 5, 6), left gastric artery (station 7), common hepatic artery (anterosuperior group, station 8a), celiac artery (station 9), proximal splenic artery (station 11p), and hepatoduodenal ligament (along the hepatic artery, station 12a) (figure 14). Lymphadenectomy along the splenic hilum (station 10) and distal splenic artery (station 11) should be performed in cases of T3–T4 tumor with macroscopic lymphatic metastases.

Lower third. The D2 classification for antral/pyloric gastric tumors comprises the following: right paracardial group (station 1), lesser curvature (station 3), right and left gastroepiploic vessels (stations 4sb, 4d), supra- and infra-pyloric (stations

5, 6), left gastric artery (station 7), common hepatic artery (anterosuperior group, station 8a), celiac artery (station 9), proximal splenic artery (station 11p), hepatoduodenal ligament (along the hepatic artery, station 12a), and proximal mesenteric vein (station 14v).

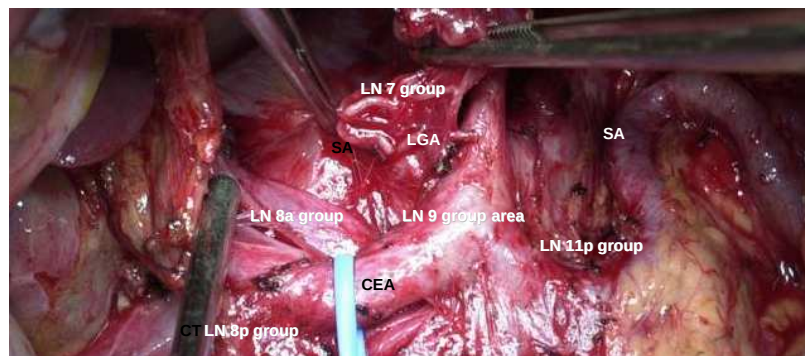


Figure 14. Dissection of LN stations no. 7, 8a and 8p, 9 and 11p. Isolation and section of LGA and removal of lymph node 7; exposition of the CT with lymphadenectomy of station 9, this procedure expose the CT ramifications as the CEA and the SA with removal respectively of lymph nodes no. 8a (anterior), 8p (posterior) and 11p (splenic proximal).

Lymph Node Stations in D3 Extended Lymphadenectomy. Similar to the D2 classification, LNs of the D3 groups are ranked according to tumor location.

Upper third. Suprapyloric (station 5) and infrapyloric stations (station 6), common hepatic artery (posterior group, station 8p), hepatoduodenal ligament (anterior and posterior hepatic artery, stations 12a, 12p), middle para-aortic (station 16a2,b1), infradiaphragmatic (station 19), and esophageal hiatus (station 20).

Middle and middle/lower third. Left paracardial (station 2), short gastric (station 4sa), common hepatic artery (LN posterior group; station 8p), splenic hilum (station 10), hepatoduodenal ligament (posterior hepatic artery, station 12p), retropancreatic (station 13), superior mesenteric vein (station 14v), and middle para-aortic (station 16a2,b1).

Lower third. Left gastroepiploic vessels (station 4sb), common hepatic artery (posterior group, station 8p), hepatoduodenal ligament (posterior hepatic artery, station 12p), retropancreatic (station 13), and middle para-aortic (station 16a2,b1) (figure 15).

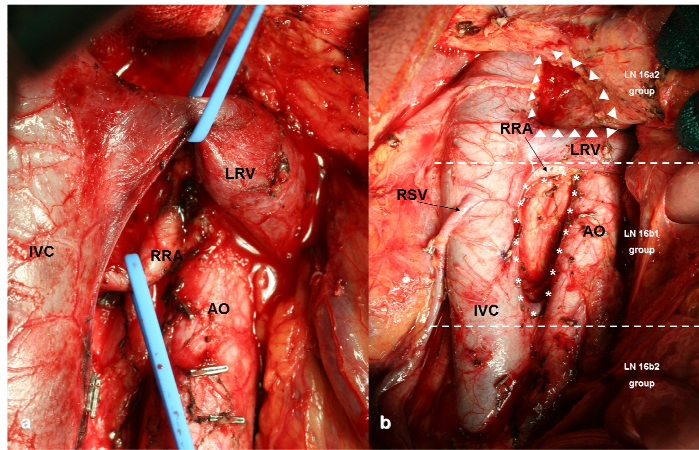


Figure 15. Dissection of LN station no. 16. a) Exposition of AO, IVC, LRV and RRA. b) Arrows indicate the lymph node station no. 16a2, and asterisks the lymph node station no. 16b1; identification of RSV.

[Note. This paragraph was extracted from the chapter n° 33 of the book “*Surgery in Multimodal Management of Gastric Cancer*”; Roviello F, Corso G, and Marrelli D. (eds) Springer-Verlag Italia 2011 (paper 10.5)]

1.9 Novel adjuvant treatments, the EGFR inhibitors

Despite the recent improvements in surgical approach and conventional chemotherapy treatments, advanced GCs are still associated with poor clinical outcomes. The increased knowledge of novel molecular mechanisms as the *MAPK/PI3K* cascade (figure 12) and its potential usefulness of targeted therapies open new strategies in gastric carcinoma treatment.

The *EGFR* belongs to the *ERBB* family, a family of four related cell-surface receptors: *ERBB1* (*EGFR*), *ERBB2* (*Her-2/neu*), *ERBB3* and *ERBB4*. Ligand binding to the extracellular domain leads to *EGFR* activation, which homodimerizes, resulting in the phosphorylation of the intracellular tyrosine kinases and initiating a series of intracellular signals including the central *RAS/RAF/MAPK* or the *PI3K/AKT/mTOR* pathway. These downstream signaling cascades are potent regulators of intracellular/intercellular processes, such as cell cycle progression, apoptosis and cell survival, proliferation, angiogenesis and metastasis. This is the rationale for the development of agents able to block the activity of *EGFR*.

The *EGFR* inhibitors can be classified in mAbs directed against cell-surface antigens and in TKIs that inhibit the *EGFR* autophosphorylation and signal transduction (figure 16) [116].

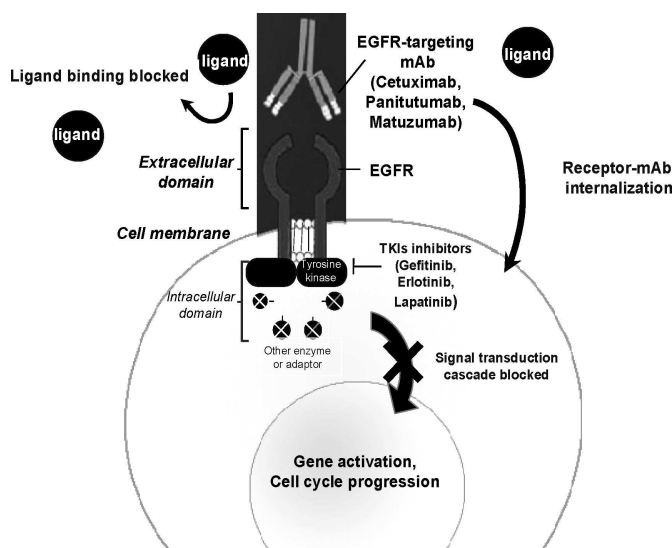


Figure 16. Anti-*EGFR* pathway therapies. (Adopted from “Advanced gastric cancer and cancer of the gastro-oesophageal junction: focus on targeted therapies”. Crit Rev Oncol Hematol 2011, in press)

The mAbs comprise Cetuximab (Erbix[®]), Matuzumab (EMD72000, Merck), and Panitumumab (Vectibix[®], Amgen) that inhibit competitively the binding of epidermal growth factor; in particular in gastric carcinoma several phase II-III trials have been studied the benefit of cetuximab. The higher OOR was reported by Yeh and Colleagues [117] with a percentage of 69%; the Authors in a phase II trial evaluated the activity and safety of cetuximab combined with 5-fluorouracil/leucovorin/irinotecan in metastatic GCs. In general, it has been observed a satisfactory OOR (41-69%) when cetuximab was associated with conventional chemotherapies, as cetuximab + FOLFIRI (OOR 44%) [118], or + cisplatin/docetaxel (OOR 41%) [119], or + FOLFOX (OOR 50%) [120], or + FUFOX (OOR 65%) [121], or + XELOX (OOR 52%) [122], or + FUFIRI (OOR 42%) [123], or + 5-FOLFIRI (OOR 69%) [117], or + capecitabine/cisplatin (OOR 48%) [124], or + oxaliplatin/irinotecan (OOR 63%) [125].

Matuzumab has been evaluated in combination with epirubicin/cisplatin/capecitabine in patients with cardia gastric adenocarcinoma; in EGFR positive cases the disease control was achieved in 57% [126].

Efficacy of Panitumumab was verified in combination with epirubicin/oxaliplatin/capecitabine in patients with advanced gastric and oesophageal cancer [127].

The ToGA trial reported an overall survival benefit for Trastuzumab (Herceptin[®], Genentech), a mAbs against the ERBB2 receptor (Her-2/neu), if adding cisplatin/5-fluorouracil/capecitabine, in patients with cardia gastric adenocarcinoma [128].

Among the *EGFR* TKIs class are described two small oral molecules namely Erlotinib (Tarceva[®], Roche-Genetech) and Gefitinib (Iressa[®], AstraZeneca Pharmaceuticals); evidences of its efficacy in gastric carcinoma or in cardia GC was modest or scarce [129, 130].

Considering these data-results, it is evident that a real benefit of mAbs in OOR was verified whenever conventional chemotherapies are added to Cetuximab, or Panitumubab, or Matuzumab treatments.

2. RATIONALE AND AIMS

2.1 Background

GC remains one of the leading causes of cancer-related deaths worldwide, even though a decline has been observed in its incidence and the mortality rate in recent decades. The exact mechanisms underlying gastric carcinogenesis are not yet fully understood. In Tuscany, Italy and Portugal are considered the highest risk areas in Europe. Despite advances in treatment, the overall outcome for patients remains poor. The overall 5 year survival is around 25%. This is largely due to the advanced stage of disease at presentation so that most patients only receive symptomatic and palliative treatment. The standard correct surgical treatment still plays the main role. Various multimodal therapy regimens are used to improve the patient prognosis, but the majority of patients do not respond to treatment. In GC, detection of invasive disease and prediction of response to treatment and clinical prognosis are all very limited. The general goal of this project is to contribute to tackling GC by identifying molecular parameters that can predict prognosis, response to treatment and thus improving the management of the disease. In order to achieve our long term goal we studied a large series of GC in which materials from GC patients operated using standard Japanese surgical approach to minimize, as much as possible, surgical bias.

We focused in sporadic GC cases but we included in our study a series of GC with familial aggregation. Within the latter setting we analysed different epigenetic risk factors, considering the high incidence rate of familial clustering in Tuscany area.

2.2 General aim

The general goal of this working plan is to identify molecular parameters that can predict prognosis, response to treatment and thus improving the management of GC. Specifically we aim in this PhD proposal to study the role of

EGFR signalling cascade in GC and to identify novel diagnostic-prognostic biomarkers.

We expect to achieve our goal by tackling the problem on one side by following a bottom-up approach that will address the role of alterations of selected genes (namely E-cadherin) in GC progression.

2.3 Specific aims

1. *Address the role of MSI as prognostic biomarkers.* In this task we will correlate the MSI phenotype with clinicopathologic characteristics of GC patients and tumours; moreover we assess the frequency of MSI in GC with familial aggregation.
2. *Analysis of the promoter XRCC1 -77T/C gene polymorphism in GC risk.* *XRCC1* is one of the major DNA repair proteins involved in the base-excision repair pathway. Several single nucleotide polymorphisms in the *XRCC1* gene are identified and related with increased cancer risk development. In particular, the -77T/C polymorphism located on the promoter region relates with increased risk in lung cancer patients. The aim of this task is to analyse the -77T/C allelic frequencies in a population composed by 456 primary GC and 507 blood donor controls.
3. *Address the role of E-cadherin deregulation in GC progression.* To this end we will search for the presence of somatic E-cadherin mutations as well as E-cadherin promoter hypermethylation in a large series of GC patients. In addition we will characterize the E-cadherin protein expression in tumour material. Results from this study will be used to define the basis for the use of demethylating agents as possible novel anticancer therapy. Furthermore we will select families which fulfil the HDGC criteria as well as juvenile GC cases and search for the presence of E-cadherin germline mutations. In positive families, the possibility of total prophylactic gastrectomy will be considered.
4. *Address the role of EGFR activation in GC progression.* To this end we will search for the presence of *EGFR* somatic mutations within the kinase

domain. We will also characterize in the same series of cancers the *EGFR* protein expression. Results from this study are expected to bring new insight into the use of *EGFR* pharmacological inhibitors as anticancer therapy in GC. On the other side they will also be useful to characterize the possibility of using *EGFR* activation as biomarker for GC, in particular to predict recurrence.

3. MATERIALS AND METHODS

3.1 Material

Data information on 500 GC patients were collected in a database Access, observed with 90 variables for each patient including clinical, diagnostic, surgical, pathological, and follow up data. Moreover, information is available for most of patients on disease free time and mode of recurrence and date of last exam. The mean period of follow-up is 49 months (74 months, if considering surviving patients). All GC patients were submitted to a standard surgery in accord to the guidelines of the JGCA.

The biological material of 500 GCs includes tumoral and normal gastric tissue flash frozen, directly collected in the operating room, and tumoral or normal genomic DNA. All materials were obtained from the Department of Human Pathology and Oncology, section of General Surgery and Surgery Oncology, University of Siena, Italy.

3.2 Methods

The methods used in the different studies are briefly described in this section. A more detailed description can be found in the section papers.

Pentaplex Polymerase Chain Reaction

Used for the amplification of five quasimonomorphic mononucleotide repeats named BAT-26, BAT-25, NR-24, NR-21 and NR27, and for the analysis of loss of heterozygosity at three *CDH1* intragenic markers D16S3025, D16S496 and D16S3067.

GeneScan run and analysis

Applied for the determination of MSI status, evaluating the pick area of the five abovementioned markers, and for the *CDH1* loss of heterozygosity score.

Polymerase Chain Reaction

Used for the amplification of promoter *XRCC1*, for the screening of *CDH1* (promoter and exons 1 to 16), *KRAS* (exon 1), *BRAF* (exon 15), *PI3KCA* (exons 9 and 20), *MLK3* (exons 1 to 10), *EGFR* (exons 19 to 21 and 3'-UTR polyA).

Bisulphite modification

Tumoral genomic DNA was submitted to bisulphite treatment in order to perform methylation analysis in *CDH1* and *MLH1* promoter genes.

Sequencing

Applied for the screening of *CDH1* (promoter and exons 1 to 16), *KRAS* (exon 1), *BRAF* (exon 15), *PI3KCA* (exons 9 and 20), *MLK3* (exons 1 to 10), *EGFR* (exons 19 to 21 and 3'-UTR polyA).

Restriction fragment length polymorphism assay

Used for the genotyping characterization of the -77TC promoter polymorphism of *XRCC1* gene.

Characterization of the effect of CDH1 germline missense mutation

In silico and in vivo analysis were used for the evaluation of *CDH1* mutation pathogenicity. Matrigel assay was applied to determine the invasive potential of cells expressing mutant form of *CDH1*.

Immunohistochemistry

Used for evaluation of the *EGFR* expression.

Statistical analysis

Student's t-test, log-rank test, Kaplan-Meier method, Cox proportional hazards regression model.

4. RESULTS AND DISCUSSION

This section focuses on the results and discussion from data obtained concerning gastric carcinoma.

4.1 MSI as predictive biomarker of good prognosis

MSI phenotype was identified in 63 (25.2%) of 250 cases. Further, in this study we confirmed that MSI phenotype is associated to a specific clinicopathologic profile. Significant differences in clinicopathologic characteristics and long-term prognosis were verified between MSS tumors, tumors with instability at 2-4 markers (MSI/5) and tumors with instability at all 5 markers (MSI/5) were described. A more favourable prognosis was confirmed for MSI phenotype at univariate ($P < 0.001$) and multivariate analysis ($P = 0.051$).

When microsatellite instability was verified in 5 markers (MSI/5 phenotype) was strongly related to older age ($P < 0.001$), female gender ($P = 0.001$), antral location ($P < 0.036$), intestinal histotype ($P < 0.003$), less infiltration of the serosa ($P = 0.006$). Lymph node involvement was rare ($P < 0.001$) and, if present, limited to an extremely low number of metastatic nodes (median 3; $P = 0.001$). Long-term survival of MSI/5 phenotype was favorable and its positive effect confirmed at multivariate analysis: RR of MSI/5 with respect to MSS, 0.32 (95% CI, 0.16 to 0.63) ($P = 0.002$). MSI tumors showed peculiar clinicopathologic features and a better prognosis compared to stable tumors. Tumors with instability at all 5 loci had a very good prognosis.

Some of the MSI patients with a very good prognosis although having advanced tumors. This fact led us to study in detail this subset of patients. Within the series there was a case of a 31-year-old woman who had undergone palliative subtotal gastrectomy with macroscopic residual (R2) tumor. Interestingly 63 months previously this patient had a non-intestinal pT4N2 tumorm but was still alive at the time of MSI detection. The favorable survival in patients with the MSI/5 phenotype was also evident among patients who underwent palliative resection (median survival, 58.4 months; 95% confidence interval, 0.0-132.5 months).

Older age, female sex, antral tumor location, intestinal histotype, less invasion of the serosa, and involvement of fewer lymph nodes significantly correlated with MSI/5 phenotype. The number of involved lymph nodes was particularly low in patients having tumors with instability at all 5 markers. Among 22 patients with MSI/5 pN+ disease, 21 had fewer than 6 positive lymph nodes, while the 31-year-old patient already mentioned had 8 positive lymph nodes.

Significantly lower rates of lymphovascular invasion were consistently observed for MSI/5 and MSI/<5 tumors compared with MSS tumors. These data should correlate with less tumor aggression, lower disease stage, and older age in patients with MSI/5 tumors. The MSI/5 phenotype delineates a subset of tumors with a similar growth pattern but with fewer propensities to invade serosal layers and spread systemically or via lymph nodes. Considering the locoregional growth of this class of tumors, one could argue for an aggressive surgical approach even in very advanced cases.

Survival analysis confirms high survival rates for patients with MSI/5 tumors. Survival of patients with the MSI/5 phenotype was significantly better than that of patients with the MSI/<5 phenotype, and this difference was confirmed in multivariate analysis. These novel findings identify a particular subset of MSI tumors with less biologic aggression and with a favourable prognosis (paper VI). In conclusion, our data confirmed previous evidences that tumors with MSI demonstrate distinct clinicopathologic features and better prognosis compared with patients having stable tumors. These results evidence the role of MSI phenotype as predictive biomarkers of good prognosis in advanced GC cases.

Data concerning MSI and familial GC, revealed that MSI status was significantly more frequent in patients with familial aggregation of GC (38.7% vs. 19.4%; $p=0.001$). A similar frequency of MSI was observed in patients with a positive familial history of GC and in patients with other family members affected by colon cancer ($p=0.96$). Conversely, in families with other members affected by lung cancer the frequency of MSI was significantly lower (5.6%) ($p=0.007$). Similar MSI rates have been observed in GC patients with other family members affected by GC or colon cancer. The same does not occur in families with other members

affected by lung cancer. Our data seems to suggest that the first two types of families share common etiological factors in contrast to families with gastric and lung cancers (paper V).

In one other study conducted on 410 GC patients, we identified that 23.2% showed a MSI phenotype; considering family history for GC, 4.6% fulfilled the HGC and 2.4% GC patients belong to families with incomplete criteria, namely as FGC. The overall frequency of MSI in the familial setting was 24.1% and was not different from that of the sporadic setting, where MSI was found in 23.1% of the tumors. Specifically within the familial setting, 26.3% of HGC patients and 20% of FGC displayed tumor with MSI phenotype.

The different results reported in these two papers can be due to the different GC population setting in which environmental factors could play a significant role in MSI generation or more probably, this divergence is related to clinical criteria used to select family history of GC.

If in colon cancer, MSI phenotype is the hallmark of the HNPCC syndrome, we concluded that in gastric carcinoma MSI does not represent a standard biomarker of hereditary or familial setting (paper IV).

4.2 The promoter XRCC1 -77T/C gene polymorphism is associated with cardia GC risk

A total of 963 subjects were enrolled in this study; 456 cases were GC patients who underwent surgical treatment for resectable GC. The control group included 507 unselected healthy blood donors.

The frequencies of the TT, TC, and CC genotypes were 34%, 48%, and 18% in the cancer group versus 37%, 47%, and 16% in normal healthy individuals, respectively. We found that the -77C/C homozygous genotype is associated significantly with increased risk of gastric cardia carcinoma ($p=0.023$) with an odds ratio (OR) of 1.65 [95% confidence interval (CI) 1.14 to 2.4]. In the family history stratification, we report a significant association ($p=0.043$) between the -77T/C polymorphism and GC cases with familial GC and other family members with lung cancer.

Our results suggest that the *XRCC1* -77T/C polymorphism is a relevant host susceptibility factor for gastric cardia cancer development and is associated with familial GC cases also involving an excess of lung cancer (paper I).

We are convinced that the -77T/C polymorphism has clinical potential. In fact we plan to offer to carriers with -77T/C and smoking habits and with a family history for lung cancer, a strictly follow-up with annual upper endoscopy using the chromoendoscopy method. In positive cases a surgical approach or neo-adjuvant treatment will be proposed.

4.3 Structural CDH1 alteration as predictive biomarker of worse prognosis in GC

Within the 246 cases analysed for E-cadherin alterations promoter hypermethylation was the most frequent event occurring in 20.7% (51 of 246) of the cases. We found 26 (10.6%) cases affected by structural alterations (three with somatic mutations and 23 with LOH). The identified somatic mutations were 1105insACCAAC, 1119A>G and c1320+5 G>A (figure 17).

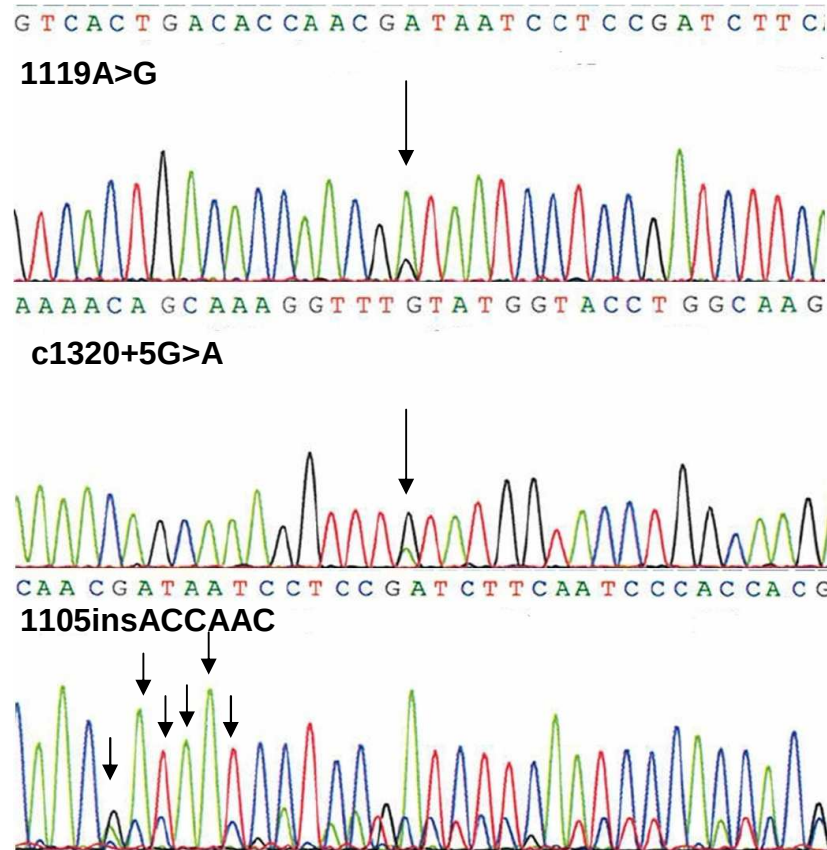


Figure 17. Somatic *CDH1* mutations identified in 86 diffuse GC.

Promoter *CDH1* methylation was associated with the diffuse type of GC ($p=0.017$) and the presence of distant lymph node metastasis ($p=0.043$). We identified association between male gender and structural *CDH1* alterations ($p<0.001$), R1/2 radicality of resection ($p=0.015$). We verified a correlation between advanced stages ($>pT2$ stage) and *CDH1* promoter methylation ($p=0.033$). Kaplan-Meier survival analysis estimates that *CDH1* structural alterations participate as poor prognostic factor in GC patients ($p = 0.017$) (figure 18).

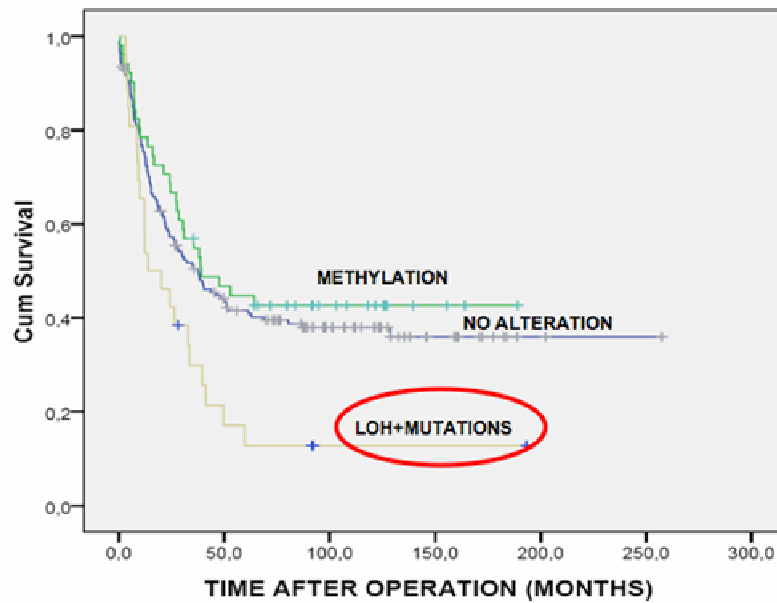


Figure 18. Patients carrier LOH and/or *CDH1* somatic mutations showed a worse prognosis at multivariate analysis.

A significant association ($p=0.023$) between *CDH1* promoter hypermethylation and a positive familial history was found for the first time (Table 3). We determined the survival rate of patients and verified that cases with LOH and E-cadherin mutations showed a worse survival rate, in contrast to cases with hypermethylation or without E-cadherin alterations (abstract I).

Table 3. Molecular mechanisms at *CDH1* in 86 primary diffuse gastric cancers.

Cancer setting	Lesions analyzed	Positive	Negative
2 nd hit mechanism	n=86	n=44	n=42
Sporadic	67	21* (31.3%)	46* (68.7%)
Methylation	67	17 [#] (25.4%)	50 [#] (74.6%)
LOH	67	3 (4.5%)	64 (95.5%)
Mutations	67	3 (4.5%)	64 (95.5%)
Familial	19	10* (52.6%)	9* (47.4%)
Methylation	19	10 [#] (52.6%)	9 [#] (47.4%)
LOH	19	0 (0%)	19 (100.0%)
Mutation	19	0 (0.0%)	19 (100.0%)

* $P= 0.0207$; [#] $P= 0.023$

With these results we support the evidence that E-cadherin alterations are a frequent event in sporadic diffuse gastric carcinoma. Further, we show that epigenetic changes of E-cadherin leading to its decrease protein expression induce an invasive behaviour to neoplastic cells. Moreover, we found a new molecular biomarker of prognosis in gastric cancer that can be used to predict the clinical prognosis of the patients.

Table 4 Resumes all *CDH1* alterations reported in our series of 246 primary gastric carcinomas.

Cancer setting	Total	CDH1 alteration in sporadic	CDH1 alteration in familial	% CDH1 alteration within positive cases
N° of cases	246	174	72	77
Methylation	51 (20.7%)	32 (18.4%)	19 (26.0%)	66.2%
LOH	18 (7.3%)	13 (7.7%)	5 (6.8%)	23.4%
Mutations	3 (1.2%)	3 (1.7%)	0 (0.0%)	3.9%
Methylation+LOH	5 (2%)	3 (1.7%)	2 (2.7%)	6.5%
Total alterations	77 (31.3%)	51 (29.3%)	29 (39.7%)	100%
Intestinal	160	107 (66.9%)	53 (33.1%)	49 (30.6%)
Methylation	24 (15%)	15 (14%)	9 (16.9%)	49.0%
LOH	20 (12.5%)	10 (9.3%)	5 (9.4%)	40.8%
Mutations	0 (0.0%)	0 (0.0%)	0 (0.0%)	0.0%
Methylation+LOH	5 (3.1%)	3 (2.8%)	2 (3.7%)	10.2%
Total alterations	49 (30.6%)	28 (26.2%)	16 (30.2%)	100%
Diffuse	86	67 (77.9%)	19 (22.1%)	33 (38.4%)
Methylation	27 (31.4%)	17 (25.4%)	10 (52.6%)	81.8%
LOH	3 (3.5%)	3 (4.5%)	0 (0.0%)	9.1%
Mutation	3 (3.5%)	3 (4.5%)	0 (0.0%)	9.1%
Methylation+LOH	0 (0.0%)	0 (0.0%)	0 (0.0%)	0.0%
Total alterations	33 (38.4%)	23 (34.3%)	10 (52.6%)	100%

We performed *CDH1* germline mutation screening in 21 EOGC patients and found two novel constitutional missense sequence variants. The first patient presented a missense 670C>T mutation that affects codon 224 of exon 5 leading to a substitution of the arginine to a cysteine (R224C). In the second patient the *CDH1* mutation was identified at the position -63 located at the 5'UTR region (-63C>A transversion). The R224C mutation was also indentified in a blood donor

subject (1/244) suggesting a population frequency of 0.4%. The -63C>A alteration was not found into the control population (0/244).

The R224C *CDH1* sequence variant was found in constitutional DNA, the tumor DNA was also analyzed in order to identify somatic mutations or methylation of *CDH1* promoter region. No somatic alterations were found in the tumor material of the patient harbouring the R224C constitutional sequence variant. Heterozygosity at the SNPs in the promoter region [-161 C/A (*rs16260*)], exon 13 [2076 T/C (*rs1801552*)] and 3'-UTR SNP (*rs1801026*) as well as the mutations is retained in the tumour.

Unfortunately, we were not able to characterize the second sequence variant (-63C>A mutation) due to lack of tumor material.

Slow aggregation on soft agar assays and Matrigel invasion assays represent valuable and reliable tools to assess *in vitro* the functional impact of E-cadherin mutations on the aforementioned functions. CHO E-cadherin negative cells engineered to stably express either the R224C mutant or the positive (wild-type *CDH1* cDNA) and negative controls (empty vector) were used as cell model. Cells expressing the R224C variant maintain the ability to form compact aggregates as seen for the wild-type *CDH1*, differently to what is observed for mock cells. When seeded on the Matrigel chambers the same association is evident: R224C expressing cells behave like the cells transduced with the wild-type form, retaining the capacity to prevent invasion in clear contrast to what can be seen for mock cells.

In our series we report *CDH1* germline sequence variants in two out of 21 EOGC cases (9.5%). Despite to the hypothetical deleterious nature of the R224C mutation by *in silico* prediction, E-cadherin function is preserved *in vitro* without impairing the ability to mediate cell-cell adhesion and suppress cell invasion. Considering the low frequency of 670C>T alteration also identified in control population (0.4%), we concluded that this alteration is a new rare polymorphic variant, never described so far. Our data suggest that functional assessment is mandatory to understand the role of *CDH1* missense mutations in diffuse GC screening and that only *in silico* analysis evaluation could fail.

Moreover, we revised all *CDH1* germline mutations in sporadic EODGC reported in literature. To date, 264 apparently sporadic diffuse or mixed EOGC patients aged 51 years old or less have been screened for the presence of *CDH1* germline sequence variants. From these 264 EOGC cases, 19 (7.2%) carried *CDH1* constitutional germline sequence variants but only 2.3% (6/264) of them did in fact represent variants with a proven potentially deleterious effect. This predicted pathogenicity was based on the type of mutation (frameshift) or on the results obtained from in vitro functional analysis.

The most interesting finding from the previous analysis is the fact that the 2.3% EOGC cases displaying deleterious *CDH1* germline alterations were always younger than 35 years old. These results are important as they impact the clinical management, counselling and molecular diagnosis to be offered to EOGC patients in the near future. Based on these strong evidences, *CDH1* mutation screening is now recommended for young patients with DGC and the cut-off for the age of diagnosis of EOGC is now accepted to be decreased from 50 to 35 years (paper II).

4.4 Oncogenic mutations as predictive therapeutic biomarkers in MSI GC

Thirty-five of 63 (55.6%) MSI GC showed oncogenic mutations in at least one of the screened genes (*EGFR*, *KRAS*, *BRAF*, *PIK3CA* and *MLK3*).

EGFR mutations

We did not find pathogenic mutations in the hotspot regions of *EGFR* (exons 18, 19, 20 and 21). However, deletions at the A13 repeat localized into the 3'-UTR of *EGFR* were found in 30/63 (47.6%) of MSI GC. Within the 30 mutated carcinomas, 15 (50%) showed a mononucleotide A deletion, 12 (40%) had a dinucleotide A deletion and, 3 (10%) a trinucleotide A deletion. All 63 normal gastric samples showed a wild-type A13 or A14 repeat of *EGFR* in a homozygous or heterozygous state. According with the findings from the *EGFR* 3-UTR mutation screening, we verified, by *EGFR* immunohistochemistry, that while two *EGFR* mutant GC (deletion [delAA(A)11]) showed an increased expression of *EGFR* in tumour cells in comparison to the surrounding normal tissue, a wild-type *EGFR*

GC case did not display *EGFR* expression in the tumour area. Due to the lack of good quality paraffin tissue, we can only consider this data as preliminary. This result needs to be validated in a larger series of MSI GC in order to confirm the correlation between the *EGFR* expression and the mutations in A13 deletions in the 30-UTR polyA repeat of *EGFR*.

KRAS and BRAF mutations

KRAS mutations were observed in 17.5% (11/63) of MSI GC cases. From these 11 cases harbouring somatic mutations, we found that codon 12 was mutated in 63.6% (7/11) and codon 13 in 36.4% (4/11). All but one *KRAS* mutation localized in codon 12 were G12D. All mutations in codon 13 were G13D. None of the MSI GC cases under study showed *BRAF* somatic mutations in the hotspot codon.

PIK3CA mutations

PIK3CA mutations were found in 14.3% (9/63) of the MSI GC studied. Among the mutated cases, eight somatic mutations were located in codons previously described as *PIK3CA* hotspots (codons 542, 545 and 1047). Five *PIK3CA* alterations occurred at the helical domain (codon 515, 542 and 545) and four mutations affected the kinase domain (codon 1047). A novel missense mutation (1544A>G) was identified within the helical domain at codon 515 (N515S). None of these mutations were present in the normal counterpart of these cases.

MLK3 mutations

MLK3 mutations were found in two carcinomas corresponding to a frequency of 3.2%: one was a missense mutation (2052G>A) localized in proline-serine-threonine rich domain (A684T) and the other was a frameshift mutation (c.69insG). Besides these two somatic mutations, that were tumour specific, we identified one splice site alteration (c.1069+10C>T) and one missense mutation (2190G>A) (R730H) that were present in both tumour and constitutional DNA, both with unknown pathogenic function. Moreover, we observed two silent *MLK3*

sequence variants (225A>G and 2259A>T) that did not change the aminoacid residues of the *MLK3* protein (A75A and P753P, respectively) and were previously described in the normal population.

Concomitant oncogenic mutations

We verified that within the 35 MSI GC harbouring oncogenic mutations, 14 (40.0%) of the mutant cases showed concomitant oncogenic alterations, always involving *EGFR* polyA mutations. From those cases with more than one mutation, seven had *EGFR* A13 repeat deletions and *KRAS* mutations (7/14 – 50%), three showed *EGFR* polyA deletion and a *PIK3CA* mutation (3/14 – 21.4%) and two had *EGFR* deletions and both *KRAS* and *PIK3CA* (2/14 – 14.3%) mutations. In one case we found an *EGFR* deletion and concomitant missense mutations in *MLK3* and *PIK3CA* and in another case an *EGFR* deletion and a *MLK3* mutation (paper VII-VIII-IX).

Overall, we identified a novel mechanism of *EGFR* activation in GC, located at the polyadenylation site in the 3'-UTR region. The presence of *EGFR* A-13 repeat mutations and the acknowledgment of the mutation status into the *MAPK/PI3K* pathway could be a novel target therapy for *EGFR* inhibitors in advanced GC patients, in particular with cetuximab-plus protocol. In particular, in this study we classified GC as potentially electable for anti-*EGFR* therapies when patients are carrier of mutation at poly-A *EGFR* site, and when the *MAPK/PI3K* cascade is wilde-type without oncogenic mutations; in fact the presence of alteration in the down-stream relates with resistance to anti-*EGFR* therapy, and this excludes the patients for anti-*EGFR* treatment.

Results from this study are expected to bring new insight into the use of *EGFR* pharmacological inhibitors as anticancer therapy in GC.

4.5 Published articles and abstracts

The following papers are the core of this Doctoral thesis. They are here presented in their original formatting.

PAPER I

Corso, G., *et al.*, **Gastric Cardia Carcinoma is Associated with the Promoter -77T>C Gene Polymorphism of X-Ray Cross-Complementing Group 1 (*XRCC1*)**. J Gastrointest Surg 13:2233–2238; 2009.

Gastric Cardia Carcinoma is Associated with the Promoter -77T>C Gene Polymorphism of X-Ray Cross-Complementing Group 1 (*XRCC1*)

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Received: 15 June 2009 / Accepted: 21 July 2009 / Published online: 7 August 2009
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Abstract

Purpose X-ray repair cross complementing group 1 (*XRCC1*) is one of the major DNA repair proteins involved in the base excision repair pathway. Several single-nucleotide polymorphisms in the *XRCC1* gene are identified and related with increased cancer risk development. In particular, the -77T>C polymorphism located on the promoter region relates with lung cancer risk development. The aim of this study is to analyze the -77T>C allelic frequencies in a population composed of 456 primary gastric cancer patients (GC) and 507 blood donor controls.

Methods GC patients were observed at the University of Siena, Italy; clinicopathological data and family history were available for the cancer group. The control group is composed of blood donors. Constitutional genomic DNA was PCR amplified, and *XRCC1* -77T>C was detected using restriction enzyme BsrB I and analyzed in a 3% agarose gel.

Results The -77T>C homozygous genotype was significantly associated with increased risk of gastric cardia carcinoma ($p=0.023$) with an odds ratio of 1.65 (95% confidence interval 1.14 to 2.4). In the family history stratification, we report a significant association ($p=0.043$) between the -77T>C polymorphism and GC cases with familial lung cancer aggregation.

Conclusions Our results suggest that the *XRCC1* -77T>C polymorphism is a relevant host susceptibility factor for gastric cardia cancer development and specific subsets of familial clustering of GC.

Keywords Gastric cancer · Single nucleotide genetic polymorphism · Risk factor

Introduction

Gastric cancer (GC) remains the second leading cause of cancer-related death and the fourth most common epithelial

neoplasia worldwide.^{1,2} Recently, a gradual decrease in incidence and mortality rate in intestinal GC has been observed;³ conversely, the diffuse histotype is showing a constant trend of increasing incidence in other geographic area.

Several genetic, epigenetic, and environmental factors interact causing a cumulative effect in the early steps of gastric carcinogenesis; among these, human genetic polymorphisms

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in some inflammation-related genes are related with an increased risk of *Helicobacter pylori* related GC.^{4–8} Continuous exposure to etiological risk factors, like *H. pylori*, causes an alteration of the DNA repair system leading to an increase mutation rate in the epithelial cells of the host.⁹

Functional polymorphisms in DNA repair genes have been demonstrated to alter the DNA repair activity and have been associated with different types of neoplasia, namely lung cancer.¹⁰ One of such genes is the X-Ray Cross-Complementing Group 1 (*XRCC1*; OMIM *194360) that is one of the major DNA repair proteins involved in the base-excision repair pathway.^{11,12}

In *XRCC1*, the functional polymorphism -77T>C (rs3213245), located in the promoter region, decreases the DNA repair efficiency leading to increased cancer susceptibility.¹² Recently, Hao and colleagues demonstrated that the -77T>C polymorphism in the *XRCC1* gene 5' untranslated region contributes to its diminished function since it shows high affinity to Sp1 leading to *XRCC1* reduced transcriptional activity. Further, C allele of *XRCC1* gene has been associated with increased risk of lung cancer.¹³

In the present study, we aimed at determining the association between T>C polymorphism of the *XRCC1* gene and risk of GC development using a series of 507 controls and 456 patients with primary GC from two Italian geographic areas (with high and low incidence of GC). Moreover, we studied the clinicopathologic features of patients and tumors, including the family history of these GC patients, namely, history related to members affected with lung cancer within the family.

Methods

Study Population

A total of 963 subjects were enrolled in this study; 456 cases were GC patients who underwent surgical treatment for resectable GC between 1989 and 2008 at the Department of Human Pathology and Oncology, Hospital of the University of Siena, Italy. The control group included 507 unselected healthy blood donors, recruited after obtaining informed consent in the period 2001–2003 at the same Hospital Center; all blood donors were case-unrelated.

We studied 176 women in the cases and 129 women in controls. The recruitment of the patients and controls was concentrated in two geographic areas: Central Italy (748 cases, 77.7%) and Southern Italy (215 cases, 22.3%). Central Italy represents a high-risk area and Southern Italy a low-risk area for GC.

In the GC group, 303 cases (66.4%) were of intestinal histotype and 153 (33.6%) of diffuse type; tumor location was subdivided into cardia (70 cases, 15.3%) and noncardia

(386 cases, 84.7%) carcinoma. In the cancer group, we evaluated the correlation between GC, the genotype polymorphism of *XRCC1* gene, and the smoking habits. Smoking history was available in 381 GC patients; overall, we stratified all individuals as smoker or no smoker and correlated the cigarette tobacco consumption with the -77T>C genotype. In the control population, no report of smoking habits was available.

Family History for Lung Cancer

This study was approved by the Local Ethics Committee, and informed consent was obtained from all recruited subjects. Oncological anamnesis and family history were investigated, adopting the detailed method described previously.^{14,15} Family history was available for 443 GC probands; familial data were not obtained from 13 individuals. A detailed family history of GC was carefully collected including the presence of patients with lung cancer in first-degree relatives within the family. Forty-three (9.4%) GC probands showed a family history for lung cancer at first-degree relatives in the patient's family.

DNA Extraction, Promoter Amplification, and Genotyping

For GC patients constitutional DNA was extracted from 50 mg of normal gastric tissue after histopathological examination, in accordance with the Puregene DNA Purification Kit Protocol (Gentra Systems, Minneapolis, USA); for healthy blood donors, constitutional DNA was extracted from whole blood using the same protocol. The *XRCC1* -77T>C was detected by using the polymerase chain reaction (PCR) restriction fragment length polymorphism assay. PCR conditions and adopted primers were described previously.¹² A 219-bp PCR product was digested with the restriction enzyme BsrB I (New England BioLabs, Beverly, MA, USA) and separated on a 3% agarose gel. The -77T allele has two restriction sites and produces three fragments of 116, 57, and 46 bp, and the -77C allele has only one restriction site, resulting in two fragments of 173 and 46 bp (Fig. 1). The accuracy of the assay was checked by direct sequencing of samples from -77T, -77T>C, and -77C polymorphism variant carriers.

Hardy–Weinberg Equilibrium and Statistical Analysis

The Hardy–Weinberg equilibrium in the control group was calculated with the formula:

$$p^2 - 2pq + q^2 = 1$$

where p is the frequency of dominant allele and q the frequency of the recessive allele. Deviation between

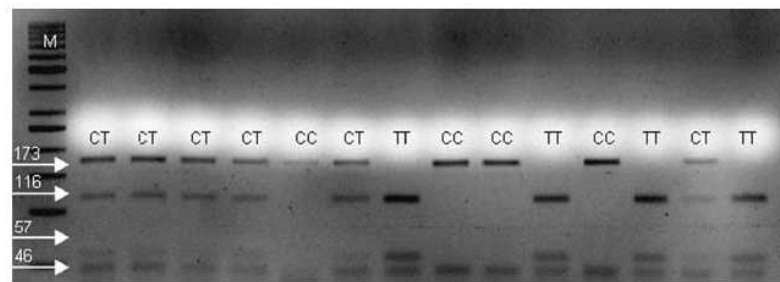


Figure 1 Genotype analysis at -77 promoter polymorphic site of *XRCC1* by digestion of PCR product with BsrB I restriction endonuclease. Band patterns of heterozygote with CT, homozygote with CC and TT.

expected and observed values was analyzed by means of Pearson's chi-square test. Comparison between genotype frequencies in the two groups under study was evaluated by means of Pearson's chi-square test. The genotypic specific risks were estimated as odds ratios with associated 95% confidence intervals (CI) by using correlation analysis and compared with Fisher's exact test. This analysis was repeated by stratifying for age (≤ 50 / >50), gender (male/female), and area of residence (high-risk versus low-risk areas). Furthermore, the genotypic specific risks were also estimated separately for cardia/noncardia tumor location, intestinal/diffuse histotype, tumor advancement (early GC versus advanced GC), and family history for lung cancer. For statistical analysis, SPSS statistical package (version 15.0) was used.

Results

Table 1 summarizes the genotype frequencies for the *XRCC1* -77T>C polymorphism among the 456 controls and among the 507 patients with primary GC. Considering both

populations, the polymorphisms did not deviate significantly from those expected under Hardy–Weinberg equilibrium. In controls, the frequency of T allele was 0.61, and the frequency of the C allele was 0.39 (χ^2 0.3; $p=0.96$). In the cancer group, the allelic frequency was 0.58 for the T allele and 0.42 for the C allele (χ^2 0.07; $p=0.96$). The frequencies of the TT, TC, and CC genotypes were 34%, 48%, and 18% in the cancer group versus 37%, 47%, and 16% in normal healthy individuals, respectively. The distribution was not statistically different between the two groups ($p=0.577$). Odds ratios (95% confidence interval) associated with TC, CC, and C carrier were 1.05 (0.93–1.19), 1.13 (0.88–1.45), and 1.05 (0.96–1.15), respectively; no significant cancer risk appeared to be associated with the distinct genotypes. Further, in Table 1, we stratified the genotype frequencies for the *XRCC1* -77T>C polymorphism and the genotype-specific risk according to age of onset (before and after 50 years), gender, and geographic risk areas (high-risk and low-risk areas). No significant difference in genotype frequencies and corresponding odds ratios was found.

The analyses between the genotype frequencies for the *XRCC1* -77T>C polymorphism and pathological character-

Table 1 *XRCC1* Genotype Frequencies in Cancer and Control Groups with the Correlated Genotypic Specific Risks (Odds Ratios and 95% Confidence Limits)

	Number of cases	Cancer group (%) $n=456$			Control group (%) $n=507$			p value	Odds ratio (95% CI)		
		TT	CT	CC	TT	CT	CC		CT	CC	C carrier
Age											
≤50	405	39	47	14	39	47	14	1.000	1.00 (0.71–1.40)	0.99 (0.45–2.17)	1.01 (0.76–1.33)
>50	558	34	48	18	33	45	22	0.612	1.02 (0.85–1.23)	0.88 (0.63–1.23)	1.01 (0.77–1.33)
Gender											
Male	658	35	47	18	37	47	16	0.779	1.01 (0.97–1.17)	1.12 (0.82–1.51)	0.96 (0.78–1.18)
Female	305	33	50	17	40	44	16	0.487	1.14 (0.91–1.43)	1.17 (0.74–1.86)	0.83 (0.62–1.12)
Risk areas											
High-risk	748	35	47	18	39	44	17	0.465	1.09 (0.95–1.26)	1.13 (0.84–1.50)	0.89 (0.74–1.07)
Low-risk	215	32	50	18	28	57	15	0.522	0.91 (0.73–1.13)	1.07 (0.63–1.84)	1.13 (0.75–1.72)
Total	963	34	48	18	37	47	16	0.577	1.05 (0.93–1.19)	1.13 (0.88–1.45)	1.05 (0.96–1.15)

Table 2 Stratification According to Lauren Histotype, Tumor Site and Stage, Family History, and Smoking Status

	No. of cases	Cancer group (%) n=456			<i>p</i> value ^a	Odds ratio (95% CI)		
		TT	CT	CC		CT	CC	C carrier
Lauren								
Intestinal	303	34	47	19	0.473	1.05 (0.92–1.21)	1.19 (0.90–1.56)	1.06 (0.95–1.17)
Diffuse	153	35	49	16	0.863	1.05 (0.88–1.24)	1.02 (0.70–1.48)	1.03 (0.90–1.18)
Tumor site								
Cardia	70	26	48	26	0.059	1.18 (0.95–1.46)	1.65 (1.14–2.4)**	1.18 (1.02–1.38)
Noncardia	386	36	48	16	0.897	1.03 (0.91–1.17)	1.03 (0.79–1.36)	1.02 (0.93–1.13)
Tumor advancement								
Early	61	43	46	11	0.554	0.93 (0.71–1.22)	0.70 (0.35–1.38)	0.92 (0.73–1.15)
Advanced	395	33	48	19	0.334	1.07 (0.95–1.21)	1.20 (0.93–1.55)	1.07 (0.97–1.18)
Family history for LC								
Positive	43	21	51	28	0.043	1.28 (1.01–1.63)	1.89 (1.25–2.85)***	1.26 (1.07–1.49)*
Negative	400	36	48	16	0.893	1.03 (0.91–1.17)	1.05 (0.80–1.37)	1.02 (0.93–1.13)
Smoking status								
No	185	34	52	14	0.679	1.14 (0.77–1.67)	0.83 (0.53–1.43)	1.08 (0.76–1.53)
Yes	196	34	43	23	0.0038	1.11 (0.75–1.62)	1.78 (1.12–2.8)	1.28 (0.90–1.82)

LC lung cancer

p*=0.032; *p*=0.023; ****p*=0.015, Fisher's exact test^a Comparison with 507 cases of the control group

istics of the tumors is presented in Table 2. No significant associations were found between the distinct genotypes and Lauren's classification of the tumors and wall invasion (early versus advanced). In contrast, when GC patients were divided according to tumor site (cardia and noncardia cancer), a significant difference in *XRCC1* -77T>C genotype frequencies was observed. In individuals with cardia carcinoma, the risk was higher for *XRCC1* -77C homozygote [odds ratio (OR)=1.65, 95% CI 1.14 to 2.4; *p*=0.015 (Fisher's exact test)] (Table 2). Interestingly, *XRCC1* heterozygous individuals did not show an increased risk of cardia cancer (OR=1.18 CI 1.02–1.38).

Additionally, we investigated the family history of cancer taking in account, specifically, the presence of family members affected with lung carcinomas, since *XRCC1* -77T>C polymorphism has been previously associated with lung cancer. We verified a significant association between GC cases with *XRCC1* -77C homozygote genotype and -77C carriers (*p*=0.043) and familial aggregation of lung cancer.

Since tobacco exposure is a well-known carcinogen involved in lung cancer development, we analyzed, whenever described in the clinical reports, the smoking habits of the patients enrolled in the study. Considering the smoking habits (smoker versus nonsmoker), we found a significant association between GC smokers and the genotype -77C>C (*p*=0.038; Table 2).

Discussion

XRCC1 protein is required for maintenance of DNA repair activity during single-strand break events caused by damaging agents. Human cell lines carrying *XRCC1*-deficient function are hypersensitive to DNA-damaging agents.¹⁶ The -77C>C promoter polymorphism of *XRCC1* induces a functional deficiency in the gene, and its presence has been associated to human cancer, namely, with non-small cell lung cancer.¹³ The association between the presence of the -77C>C promoter polymorphism of *XRCC1* and increased risk of lung cancer was verified by case control studies.^{12,17,18}

Recently, we verified that some of the families with clustering of GC also show members affected with lung cancer,¹⁵ leading us to hypothesize that, in this particular subset of GC, a susceptible genetic factor and/or a particular environmental factor are likely to play a pivotal role. In accordance to this hypothesis and as the first step of our analyses, we studied the putative relationship between *XRCC1* -77T>C polymorphisms and familial aggregation of GC, in particular familial cases of GCs also harboring lung cancer in direct relatives. Regarding family history of GC patients, we analyzed 43 GC cases with positive family history for lung cancer and demonstrated a significant correlation with *XRCC1* -77C allele and the presence of lung cancer in first-degree relatives. This data confirms that

familial aggregation of gastric and lung cancer is associated to susceptible genetic risk factors that do not occur in familial forms of stomach cancer associated to germ line defects of tumor suppressor genes, namely, E-cadherin and p53, as previously suggested by Pedrazzani and colleagues.^{15,19,20} In addition, we analyzed the association between the -77C>C promoter polymorphism of *XRCC1* and the clinicopathologic characteristics of the patients and tumors. Since in GC clear differences exist concerning the epidemiological data, etiological factors, and clinical differences, between cardia and noncardia tumors²¹ and between intestinal and diffuse histotypes,²² a stratified analysis was performed.

We found an association between the *XRCC1* -77C>C and increased risk for developing cardia carcinoma. According to our findings, GC cases with cardia location and homozygous genotype for the *XRCC1* -77C allele show an increased risk for GC. As reported previously, cardia adenocarcinoma is considered a distinct clinical entity with different prognostic factors. In accordance to this, it has been proposed that cardia cancer needs a different classification considering the different characteristics of cardia cancer since the current tumor–node–metastases staging system has been considered inadequate.^{23,24} Moreover, the association between *XRCC1* -77C allele and cardia carcinoma is novel, and it is one of few genetic alterations described in this subtype of GC since till now no specific molecular phenotypes are related with this carcinoma. In contrast, no associations were found between *XRCC1* -77C allele and other clinicopathologic features of the patients and tumors.

Further, it has been suggested that exposure to various environmental factors represent essential components of GC tumorigenesis. Factors such as tobacco consumption,²⁵ dietary habits,²⁶ and *H. pylori* infection²⁷ have been demonstrated to be involved in the multifactorial process of gastric carcinogenesis. Our results show that GC patients carrying the genetic susceptibility for some DNA repair genes, as the promoter *XRCC1* -77T>C polymorphism, concomitantly are exposed to tobacco consumption. Our results allow us to speculate that gastric cardiac carcinoma is associated to deficient activity of the *XRCC1* gene due to -77T>C polymorphism and a concomitant history of smoking habits.

Our study reports some limitations that should be explained. Firstly, because our study was a hospital-based study, with cases recruited from hospitals and controls recruited from the community population as blood donors, the study subjects may not be representative of the general population. However, we believe that our results are unlikely to be attributable to selection bias because we used a relatively large number of incident cases and matched the controls to the cases on age, sex, and

residential area. Second, because both populations, cancer and control groups, were recruited from different incidence area, Southern and Central Italy (respectively, low and high incidence areas), the -77T>C polymorphism may not explain a common risk factor. Nevertheless, our results did not identify significant correlation between stratified populations and -77T>C polymorphism, and both groups were in Hardy–Weinberg equilibrium. Third, smoking status and family history were evaluated only in cancer groups without correlation in respect to control group; we will provide to assess a complete smoking status also in control group in a further study to quantify the risk of cardia GC development in -77C allele carriers. This is the first study reported in literature; furthermore, other case control studies from different population in multicenter groups should confirm our results to improve the increased risk of cardia GC in subjects carrying this single nucleotide polymorphism.

Conclusion

In conclusion, we identified a novel biomarker involved in DNA repair for assessing cardiac GC risk that occurs in patients with a positive history of tobacco consumption. In familial clustering of GC, the presence of lung cancer in relatives may indicate that these patients can be carriers of *XRCC1* - 77T>C polymorphism risk allele.

Acknowledgments This work was supported by Istituto Toscano Tumori (“Gene expression profile and therapeutic implication in gastric cancer. From the clinical overview to the translational research”; Grant ITT-2007) and by Fundação para a Ciência e a Tecnologia (“Exploring the role of E-Cadherin-HER interaction in the search of molecular biomarkers for the clinical management of gastric cancer patients”; grant ref PIC/IC/82923/2007 and PhD grant ref SFRH/BD/40090/2007).

Competing interests The author(s) declare that they have no competing interests.

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PAPER II

Corso, G., *et al.*, E-cadherin genetic screening and clinico-pathologic characteristics of early onset gastric cancer. Eur J Cancer 47:631-639; 2011

available at www.sciencedirect.comjournal homepage: www.ejconline.com

E-cadherin genetic screening and clinico-pathologic characteristics of early onset gastric cancer

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ARTICLE INFO

Article history:

Received 6 September 2010

Received in revised form 12 October 2010

Accepted 13 October 2010

Available online 22 November 2010

Keywords:

Early onset gastric cancer

E-cadherin

Pathology

Diffuse gastric cancer

Hereditary and sporadic cancer

Germline mutations

Family history

Genetic screening

Second hit

Promoter methylation

ABSTRACT

Aim: CDH1 germline alterations occur in about 40% of hereditary diffuse gastric cancer (HDGC) families. CDH1 germline mutations are also documented in few early onset diffuse gastric cancer patients (EODGC) without family history, but the real frequency in this setting is unknown. In these patients, the advanced stage at the time of diagnosis remains a clinical burden due to the poor long term survival.

Methods: The entire coding region and exon flanking sequences of the CDH1 gene was analysed by direct sequencing in 21 EODGC patients aged ≤ 50 years. The potential deleterious nature for a new CDH1 missense variant was assessed by cell–cell aggregation and invasion assays. Somatic CDH1 mutation, loss of heterozygosity (LOH) and promoter hypermethylation was explored in the tumour from one CDH1 germline mutation carrier.

Results: Two novel CDH1 germline variants were identified in 21 EODGC cases, c.670C>T and –63C>A. Functional analysis of the c.670C>T missense variant classified this mutation as non-pathogenic. The analysis of CDH1 somatic second hits failed to demonstrate E-cadherin structural and epigenetic alterations in the tumour sample.

Conclusion: Data from the present work and a systematic review of the literature revealed that CDH1 germline mutations occurred in 7.2% of EODGC patients invariably with diffuse of mixed histology. From these, proved CDH1 mutation pathogenicity has been assigned only to 2.3% of the cases who were recurrently diagnosed before 35 years old. Germline CDH1 mutation remain the only germline genetic defect described in this type of patients and CDH1 mutation screening should be recommended for patients with these characteristics.

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1. Introduction

Gastric cancer (GC) represents the second most frequent cause of cancer related death and the 4th most common malignancy worldwide.^{1–4}

Several genetic, epigenetic and environmental factors interact simultaneously in the early steps of gastric carcinogenesis. Amongst these, human genetic polymorphisms of some inflammatory-related genes are thought to be responsible for an increased risk of GC.^{5,6} Other factors such as

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doi:10.1016/j.ejca.2010.10.011

tobacco consumption, dietary habits and *Helicobacter pylori* infection^{7–9} have been demonstrated to be involved in the multifactor process of gastric carcinogenesis.

Over the last years, the incidence of GC, especially the intestinal histotype, has been decreasing in older patients, remaining otherwise stable in young patients and in cases with diffuse histotype.¹⁰ This would suggest that the aetiology of GC in young people may be different compared to older subjects. In the older population, the association of different environmental factors and epigenetic changes frequently relates to GC development. In young patients, the role of genetics is presumably greater, environmental carcinogens playing a less significant role.¹¹

EOGC is defined as any GC presenting at the age of 45 or earlier and represents approximately 10% of all patients with stomach cancer with reported frequencies varying between 2.7% and 15% according to different populations studied.^{12,15–17} GC patients younger than 40–45 years old are believed to develop GCs involving molecular pathways different from those of sporadic carcinomas that occur later in life.^{12–14} GC that occurs in patients younger than 30 years is very rare (1.1–1.6%) and most of EOGCs are diagnosed in patients older than 35.^{18,19} GC's diagnosed before 20 years of age are exceptional and current literature is limited to a small number of cases.^{16,20,21}

EOGC may occur in sporadic or hereditary forms. Germline inactivating mutations and deletions of the E-cadherin gene (*CDH1*) are a well-documented genetic factor associated with diffuse histotype of EOGC (EODGC) or with the HDGC syndrome.^{22–24} HDGC is an autosomal dominant cancer disease and represents 1% of all GCs and notably, germline alterations of the *CDH1* gene are detected in about 40% of families that fulfil the clinical criteria for the HDGC syndrome.^{24–26}

Germline mutations of *CDH1* gene have also been documented in diffuse EOGC with and without inherited predisposition for the HDGC syndrome.^{22,23,27} Considering the apparently sporadic cases, about 6% of diffuse EOGCs are described to carry a constitutional potentially deleterious sequence variant of E-cadherin gene.²⁸

In this study, we screened for the presence of *CDH1* germline alterations in 21 EOGC patients with diffuse histotype and without family history of GC. Additionally, we revised the clinico-pathologic and molecular features presented by EOGC described in the literature, namely in those patients carrying *CDH1* germline alterations.

2. Methods

2.1. Patients and sample preparation

From 1993 to 2005, 26 patients with diagnosed sporadic diffuse GC and age at onset ≤ 50 years were admitted at the Department of Human Pathology and Oncology, Section of Surgical Oncology, of the University of Siena, Italy; 5 patients were excluded since they not have stored biological material. Twenty-one GC patients younger than 50 years old ($n = 11$ younger than 45 and $n = 10$ older than 45) were therefore included in the study. Family histories were obtained with informed consent and pedigree analysis revealed that all GC cases were apparently sporadic forms of GC. Selected patients

presented diffuse histotype of GC according to Laurén classification.²⁹

Genomic constitutional DNA was extracted from peripheral whole blood using a standard protocol. To test the frequency of identified alterations, a population of 224 blood donors was used as negative control. The study protocol was reviewed and approved by the local ethical committee.

In one case, DNA was extracted from macrodissected tumour areas with at least 80% of neoplastic cells from paraffin-embedded tissue using Invisorb Spin Tissue Mini Kit (Inviteck, Berlin, Germany). This material was used to characterise molecular alterations in tumour DNA in comparison to constitutional DNA.

2.2. Germline and somatic *CDH1* mutation screening, somatic analysis of *CDH1* promoter hypermethylation and LOH

CDH1 germline mutations were searched for in 21 EODGC patients. Briefly, each exon and intron–exon boundary of the *CDH1* gene was amplified by PCR and directly sequenced. Primers and conditions were as previously reported²⁹ (primer sequences available upon request).

Somatic *CDH1* alterations, which could work as *CDH1* inactivating second hits, were screened for in macrodissected tumour material from one EODGC patient that displayed a germline *CDH1* alteration. Somatic mutations were analysed in tumour DNA following the same strategy used for the above mentioned germline mutation screening. Promoter hypermethylation was evaluated using 200 ng/ μ l of bisulphite treated (EpiTect Bisulfite Kit, Qiagen, Valencia, California, USA) tumour DNA. An essential region of 221 base pairs of the *CDH1* promoter was amplified using primers flanking but not encompassing CpG sites (primer sequences available upon request). PCR products were purified and directly sequenced. Detailed methods were described earlier.³¹ To rule out the possibility of LOH in the tumour, known intragenic single nucleotide polymorphisms (SNPs) in the *CDH1* promoter region [–161 C/A (rs16260)], exon 13 [2076 T/C (rs1801552)] and 3'-UTR SNP (rs1801026), as well as the germline mutation found in one patient, were amplified by PCR and sequenced from normal and tumour material. When for a specific marker, a sample harboured a reduction in the peak area of one allele equal or greater than 90%, in comparison to the other allele, LOH was considered for that specific marker.

2.3. Characterisation of the impact in splicing for the mutation c.670C>T (p.Arg224Cys)

Netgene2 software (<http://www.cbs.dtu.dk/services/netgene2>) was used to predict the impact of *CDH1* missense mutations in creating cryptic splice sites. In light of such predictive results, RNA was isolated from frozen white peripheral blood lymphocytes (PBLs) from the patient of interest as well as from *CDH1* mutation negative controls using Trizol Reagent (Life Technologies, Inc., Paisley, Scotland) following the manufacturer's instructions. Total RNA was used to synthesise first-strand cDNA (Invitrogen SuperScript[®] III Reverse Transcriptase, Milan, Italy) according to the manufacturer's protocol. *CDH1* transcripts were amplified using a primer set

designed to amplify CDH1 exons 4–6 that flank the c.670C>T sequence variant (cDNA_Ex4_F: 5'-GCT CAC ATT TCC CAA CTC CTC T-3'; cDNA_Ex6_R: 5'-ATT CGG GCT TGT TGT CAT TCT-3'). To investigate the generation of cryptic splice sites induced by the presence of the missense mutation c.670C>T, RT-PCR products from the carrier and from a control were run in a 2% agarose gel. Band size and migration patterns were compared in both samples.³²

2.4. Characterisation of the effect of the p.Arg224Cys germline missense mutation on E-cadherin function

The theoretical impact of p.Arg224Cys mutation was firstly evaluated *in silico* by using the *Sorting Intolerant From Tolerant* (SIFT) software.³³ *In vitro* functional relevance was assessed on the basis of its impact on cell–cell adhesion and cell invasion as described earlier.²³ For this purpose, a construct was generated using a pLenti virus based expression vector (pLenti6/V5 Directional TOPO[®] Cloning Kit; Invitrogen) with an inserted full cDNA sequence of CDH1 that previously underwent site-directed mutagenesis to include the mutation c.670C>T (that give rise to the mutation p.Arg224Cys in the E-cadherin protein). Chinese Hamster Ovary (CHO) cells were then stably transduced with the following expression constructs: empty vector (mock cells) and wild-type CDH1 cDNA vector (previously generated in the lab) as well as with the CDH1 cDNA construct including the mutation p.Arg224Cys. Protein expression levels and localisation were evaluated by Western Blot and immunocytochemistry using the human E-cadherin monoclonal antibody HECD-1 (R&D Systems, 1/200 dilution). For the slow aggregation assay, after trypsinization of each stable cell line, 2×10^4 cells were transferred to an agar gel (0.66%, w/v) in a 96-well plate and then incubated at 37 °C under 5% CO₂ in humidified air. Aggregate formation was evaluated qualitatively after 24 and 48 h using an inverted microscope. The formation of compact cellular aggregates was considered to be a feature of normal cell–cell adhesion. In contrast the presence of small and loose cell clusters was considered as suggestive of impaired cell adhesion.

The same cell lines were tested in the Matrigel invasion assay. Matrigel invasion chambers were purchased from BD (Bedford, USA) and the assay was performed following manufacturer's instructions. In brief, 1×10^5 cells were seeded on top of the Matrigel layer inside the plastic chambers and incubated for 22 h at 37 °C. Invasion indices were expressed as ratios between the number of invasive cells (cells able to degrade the Matrigel layer and cross the semi-permeable membrane at the bottom of the chamber) and the number of cells initially seeded. Each functional assay was repeated at least three times in the same conditions.

3. Results

3.1. CDH1 genetic screening revealed two novel germline potentially deleterious sequence variants

Table 1 encompasses the main clinico-pathologic features of the 21 gastric carcinoma patients considered in this study.

We performed CDH1 germline mutation screening in our series of 21 EOGC patients and 2/21 (9.5%) were found to dis-

play previously unidentified germline CDH1 sequence variants. None of the remaining 19 patients displayed other potentially deleterious sequence variants of CDH1.

The first patient, a 46 years old female with diffuse GC without further history of gastric cancer in the family, presented a heterozygous missense c.670C>T alteration that affects codon 224 of CDH1 exon 5 and leads to a substitution of an Arginine to a Cysteine (p.Arg224Cys) (Fig. 1A). This sequence variant was identified in a single blood donor subject from our series of 244 cancer free individuals used as controls, indicating a population frequency of 0.4%.

In a second patient with 42 years old male with diffuse GC without further history of gastric cancer in the family, a new CDH1 sequence variant was identified at the at the 5'UTR region, position -63 from the ATG (Fig. 1B). This sequence alteration was never found in the control population used (0/244).

3.2. The c.670C>T germline sequence variant does not induce cryptic splicing of CDH1

Netgene2 software was used to predict, *in silico*, the potential for the c.670C>T CDH1 missense mutation in inducing cryptic splicing in the vicinity of the canonical donor splice-site of CDH1 exon 5. By applying this tool, we verified that a new potential cryptic splice-site was expected to be created 42 base pairs upstream of the canonical splice-site (data not shown). We then tested this possibility by analysing whether this would occur *in vivo* using cDNA produced from PBLs retrieved from the patient harbouring the c.670C>T mutation. In contrast to the *in silico* prediction, the PCR fragment generated using primers flanking the mutation in the mutation carrier and in the control sample did not show differences when run in a 2% agarose gel. This result was indicative that a potential cryptic splicing was not likely to be occurring as a consequence of the c.670C>T missense mutation.

3.3. The c.670C>T (p.Arg224Cys) missense mutation does not impair E-cadherin induced cell adhesion and invasion *in vitro*

The theoretical impact of p.Arg224Cys mutation was firstly evaluated *in silico* by using the *Sorting Intolerant From Tolerant* (SIFT) software³³ that predicted a deleterious nature for this novel missense variant.

Two of the most relevant biological effects mediated by E-cadherin are the promotion of homotypic cell–cell adhesion and the ability of suppressing cell invasion. To assess these parameters *in vitro*, we measured slow aggregation in soft agar and cell invasion through a Matrigel matrix of CHO E-cadherin negative cells engineered to stably express the c.670C>T (p.Arg224Cys) mutant, the wild-type CDH1 cDNA, as well as a negative control (empty vector). We verified that cells expressing the p.Arg224Cys sequence variant maintained the ability to form compact aggregates at a similar level to that of the wild-type CDH1 expressing cells, while the negative control cells (transduced with the empty vector or mock cells) were not able to form compact cell aggregates (Fig. 2). Moreover, when seeded on Matrigel chambers, the p.Arg224Cys expressing cells behaved in a similar manner to cells transduced with the wild-type CDH1, retaining the

Table 1 – Clinico-pathologic characteristics and CDH1 alterations identified in 21 patients with sporadic diffuse gastric cancer.

ID code	Age onset	Gender	Tumour location	Làuren classification	Depth invasion (pT)	Lymph node involvement (pN)	Stage grouping	CDH1 variants
MF164	49	Female	Non-cardia	Diffuse	pT 2	pN1	II	–
ZM227	46	Male	Non-cardia	Mixed	pT 2	pN2	III	–
MP245	42	Male	Non-cardia	Diffuse	pT 1	pN0	I	–63C>A
FA286	30	Male	Cardia	Diffuse	pT 3	pN2	III	–
DA290	30	Female	Non-cardia	Diffuse	pT 1	pN0	I	–
PB311	50	Female	Non-cardia	Diffuse	pT 3	pN2	III	–
PA320	38	Male	Non-cardia	Diffuse	pT 2	pN2	III	–
DA328	45	Male	Non-cardia	Diffuse	pT 2	pN3	III	–
PP382	38	Male	Non-cardia	Diffuse	pT 2	pN2	III	–
CS425	48	Male	Cardia	Mixed	pT 3	pN3	IV	–
TF432	50	Female	Linitis plastica	Diffuse	pT 4	pN3	IV	–
SG450	49	Male	Linitis plastica	Diffuse	pT 3	pN1	III	–
DE462	43	Female	Linitis plastica	Diffuse	pT 4	pN3	IV	–
CA471	46	Female	Non-cardia	Diffuse	pT 3	pN3	IV	c.670C>T
ZG1	33	Male	Non-cardia	Diffuse	pT 3	pN3	IV	–
PD8	46	Female	Non-cardia	Diffuse	pT 1	pN1	I	–
CS96	40	Male	Non-cardia	Diffuse	pT 1	pN0	I	–
CC442	46	Male	Non-cardia	Diffuse	pT 2	pN2	III	–
BG414	43	Male	Non-cardia	Diffuse	pT 3	pN1	III	–
MF24	45	Female	Linitis plastica	Diffuse	Not resected	–	–	–
NC410	31	Female	Non-cardia	Diffuse	pT 4	pN2	IV	–

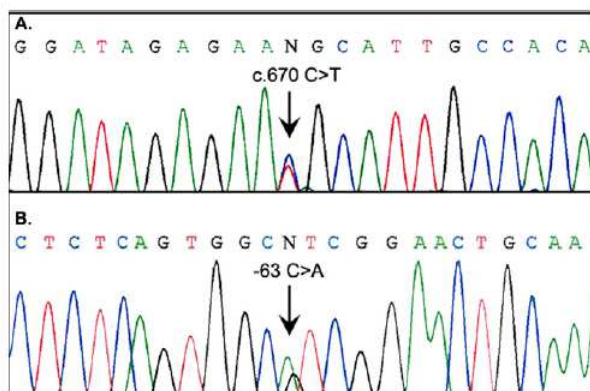


Fig. 1 – Sequencing chromatogram representing novel CDH1 germline mutations identified in EODGC patients. (A) mis-sense mutation c.670C>T that results in the aminoacid substitution p.Arg224Cys, and (B) promoter substitution –63C>A from the ATG.

capacity to prevent invasion in clear contrast to what was observed for mock cells. These data suggest that the p.Arg224Cys missense mutation is most likely a non-pathogenic CDH1 sequence variant.

3.4. The tumour from the c.670C>T CDH1 germline sequence variant carrier did not display additional CDH1 somatic mutations, promoter hypermethylation nor LOH as second hits

In order to determine whether the tumour from the c.670C>T CDH1 sequence variant carrier harboured a second somatic hit, inactivating the remaining germline unaltered allele, we analysed DNA extracted from an area enriched in neoplastic

cells. We initially screened for somatic mutations and verified that no somatic mutations were detectable in the tumour DNA. Moreover, neither CDH1 promoter hypermethylation nor LOH were also detected. We further verified that heterozygosity at SNPs either in the CDH1 promoter region (–161C/A [rs16260]) or in the mRNA sequence (2076T/C [rs1801552] and 3'-UTR [rs1801026]) as well as at the site of the germline sequence variant was retained in the tumour.

Due to lack of tumour material, we were not able to characterise the second hit inactivating the remaining germline unaltered allele in the patient carrying the –63C>A sequence variant.

4. Discussion and review of the literature

4.1. CDH1 germline sequence variants and EOGC

CDH1 encodes the E-cadherin tumour suppressor protein that acts as a trans-membrane calcium dependent glycoprotein and plays an essential role in the intercellular adhesion between epithelial cells.³⁴

Decreased E-cadherin expression is often found in several epithelial neoplasias during tumour progression and is correlated with a higher infiltrative and metastatic ability of the tumour³⁵ due to loss of cell adhesion and increased cell motility.³⁶

Typically, inactivating E-cadherin alterations are present in a fraction of both sporadic and hereditary diffuse GC.^{22,24,31,37,38} Moreover, several authors identified CDH1 germline alterations in apparently sporadic EOGC, always presenting diffuse or mixed histotype.^{23,39–42} The largest series of diffuse EOGC screened for CDH1 germline mutations was reported by Bacani and colleagues who identified eight germline sequence variants in 81 (11.1%) patients younger

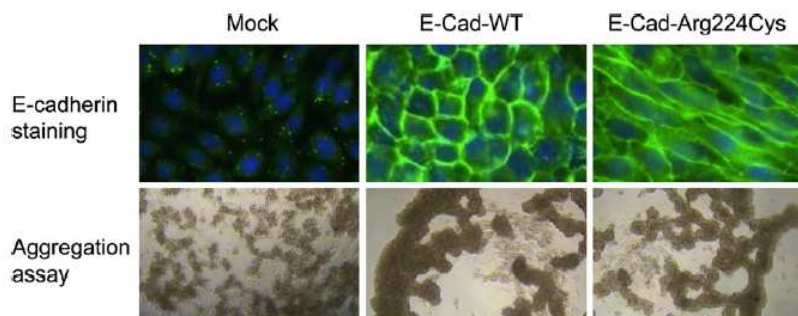


Fig. 2 – Functional analysis of *CDH1* missense mutation p.Arg224Cys. The upper panel displays representative images of the E-cadherin localisation in CHO transduced cells (Mock; E-cad-WT and p.Arg224Cys) by immuno-fluorescence analysis. The lower panel displays representative images obtained after the aggregation assay performed in soft agar with the same cell lines.

than 50 year old, in the Central-East Ontario region, Canada, a region of low incidence of GC.⁴⁰ Suriano and Oliveira also described a large series of 54 diffuse or mixed type EOGC patients for which five (9.3%) germline sequence variants were reported.³⁰

We selected a series of diffuse EOGC patients younger than 50 years old with apparently negative GC family history, from a region in Italy with high incidence of GC (4.2/10000 inhabitants; ISTAT 2002), and analysed the presence of *CDH1* germline sequence variants. Considering that Bacani⁴⁰ identified *CDH1* germline mutations also in sporadic DGC patients with age of onset of 50 years, we have established the 50 years at diagnosis as the cut-off limit. Herein, we report the presence of *CDH1* germline sequence variants in 2/21 cases (9.5%). This frequency is similar to that obtained when combining all reports on the same issue available in the literature, in which 7.2% of the EOGC cases were reported to harbour germline *CDH1* sequence variants, most of them with unknown functional significance (Table 2).

Apart from analysing the frequency of germline *CDH1* alterations in EOGC, we also addressed the potential *in silico* and *in vitro* functional impact of one missense sequence alteration (p.Arg224Cys) herein identified. Despite obtaining a deleterious nature for this alteration using *in silico* prediction, the E-cadherin protein function was preserved when *in vitro* functional analysis was performed, as ability to mediate cell-cell adhesion and suppress cell invasion was retained. This single nucleotide alteration (c.670C>T – p.Arg224Cys) was searched for in a control population and its frequency was proved to be very low (0.4%). Moreover, the other identified alteration also found in the present report (–63C>A) was never found in the population and its functional effect was not possible to address. The C-nucleotide at this position is conserved in *Homo sapiens*, *Pan troglodytes* and *Gorilla gorilla* but is not conserved in other species (data not shown) and therefore an alteration at this position may not have a great deleterious effect. Therefore, it is likely that both alterations are new rare polymorphic variants rather than germline sequence variants associated with increased susceptibility of GC. Another possibility, that was not addressed in this report, is the putative role of these two sequence variants in the activation of other E-cadherin

related pathways such as the EGFR pathway. Although not likely for the –63C>A, as it localizes at the promoter region and does not impact the protein sequence, the c.670C>T mutation may have a role in the modulation of E-cadherin downstream effector pathways.

To date, 264 apparently sporadic diffuse or mixed EOGC patients aged 51 years old or less have been screened for the presence of *CDH1* germline sequence variants (for references see Table 2). From these 264 EOGC cases, 19 (7.2%) carried *CDH1* constitutional germline sequence variants but only 2.3% (6/264) of them did in fact represent variants with a proven potentially deleterious effect (Table 2). This predicted pathogenicity that is depicted in Table 2 was based on the type of mutation (frameshift) or on the results obtained from *in vitro* functional analysis.

Five of the six germline pathogenic *CDH1* mutations were detected in EOGC patients from low or moderate incidence GC areas (Table 2), whereas only one arose in a patient from Portugal, a high incidence GC area. Although *CDH1* germline mutations have been searched for in EOGC patients from other areas where high incidence rates of GC are verified, namely in countries like China, Korea, Japan, and even Italy, no deleterious *CDH1* germline variants have been identified (Table 2).

Gathering all data on germline sequence variants reported in diffuse and mixed EOGC, both from the literature and herein presented, functional assessment is the only currently available mean to shed light into the role of *CDH1* missense mutations. Although *in silico* tools for prediction of cryptic splicing, protein function and deleterious effects, as well as for conservation analysis may be used to weigh up missense, intronic and promoter mutation impact, such an evaluation frequently fails when used on its own.

The presence of genetic and epigenetic second hit events affecting the remaining *CDH1* wild-type allele, in tumour DNA from EOGC patients carrying *CDH1* germline variants, may also help in interpreting the deleterious effect of a given sequence variant. In line with our previous results, we were unable to detect *CDH1* somatic mutation, promoter methylation or LOH in the tumour from the p.Arg224Cys mutation carrier, re-enforcing the thesis that this alteration is most probably not deleterious.

Table 2 – Summary of reported diffuse and mixed histotype EOGC cases screened for CDH1 germline sequence variant.

Ref.	Total EOGC screened n (age setting)	CDH1 Sequence variants (%)	Ethnicity	Histotype	Age onset	Population frequency	CDH1 sequence variant	Mutation Type	Gene position	Predicted protein change	Predicted Pathogenicity
39	1	1	New Zealand	Diffuse	31	Nd	c.1487del7	Frameshift	Exon 10	PTC 556	Yes
30	54 ^b (age<51)	5 (7.6%)	Africa/USA	Diffuse	43	1%	c.1849G>A	Missense	Exon 12	p.Ala617Thr	No
			Africa/USA	Diffuse	43	1%	c.1849G>A	Missense	Exon 12	p.Ala617Thr	No
			Portugal	Diffuse	30	0%	c.1901C>T	Missense/splice-site	Exon 12	p.Ala634Val	Yes
			England	Diffuse	51	0%	c.532-18C>T	Intronic	Intron 4	Nd	No
			Portugal	Mixed	42	0%	c.532-18C>T	Intronic	Intron 4	Nd	No
23	10 (age<35)	2 (20%)	USA	Diffuse	27	Nd	c.1285C>T	Missense	Exon 9	p.Pro429Ser	Yes
41	15 (age<45)	1 (6.7%)	USA	Diffuse	27	Nd	c.1063delT	Frameshift	Exon 8	PTC 355	Yes
40	81 (age<50)	8 (9.9%)	Europe	Diffuse	29	Nd	c.1619_1620insG	Frameshift	Exon 11	PTC 546	Yes
			USA	Diffuse	Na	Nd	-117G>A	5'-UTR substitution	Promotor	Nd	Nd
			USA	Diffuse	Na	3.90%	-71C>G	5'-UTR substitution	Promotor	Nd	Nd
			Europe/USA	Diffuse	30	Nd	c.41delT	Frameshift	Exon 1	PTC 55	Yes
			USA	Diffuse	Na	0%	c.48-5G>C	Intronic	Intron 1	Nd	Nd
			USA	Diffuse	Na	Nd	c.48-15C>G	Intronic	Intron 1	Nd	Nd
			USA	Diffuse	Na	Nd	c.387+26C>T	Intronic	Intron 3	Nd	Nd
			USA	Diffuse	Na	Nd	c.2295+53G>A	Intronic	Intron 14	Nd	Nd
			USA	Diffuse	Na	Nd	c.2439+31G>A	Intronic	Intron 15	Nd	Nd
Present report	21 (age<50)	2 (8.9%)	Italy	Diffuse	50	0%	-63C>A	5'-UTR substitution	Promotor	Nd	Nd
			Italy	Diffuse	45	0.4%	c.670C>T	Missense	Exon 5	p.Arg224Cys	No
63	9 (age<35)	-	Japan	Diffuse	<35	-	-	-	-	-	-
43	9 (age<50)	-	USA	Diffuse	<50	-	-	-	-	-	-
64	24 (age<50)	-	China	Unknown	<50	-	-	-	-	-	-
13	40 (age<45)	-	Europe and USA	Diffuse 9 mixed	<45	-	-	-	-	-	-
Total	264	19 (7.2%)	-	-	-	-	-	-	-	-	6/264 (2.3%)

Note: PTC, premature termination codon; Na, not available; Nd, not determined.

^a This mutation also generates a cryptic splice-site at the site of the missense change leading to the generation of premature termination at codon 653.

^b The whole series encompassed 66 EOGC cases, from which only 54 were of diffuse or mixed histology; bold, predicted pathogenicity based on mutation type (frameshift) or in vitro functional analysis.

The most interesting finding from the previous analysis is the fact that the 2.3% EOGC cases displaying deleterious *CDH1* germline alterations were always younger than 35 years old and their tumours always presented diffuse histology (Table 2). These results are important as they impact the clinical management, counselling and molecular diagnosis to be offered to EOGC patients in the near future. This observation has been also previously advanced in smaller series of cases^{42,43} and based on these strong evidences, *CDH1* mutation screening is now recommended for young patients with diffuse GC and the cut-off for the age of diagnosis of EOGC was accepted to be decreased from 50 to 35 years.²⁶

4.2. Clinical and pathological features of EOGC and older GC patients

EOGC patients and patients with GC at older ages clearly display very different clinical and pathological features. Those younger than 30 years are most commonly females,^{17,44,45} while older ones are most frequently male patients.⁹ Based on these findings, it has been hypothesised that the development of GC in young women may be significantly influenced by natural, biological and hormonal factors,⁴⁶ nevertheless this has never been proven.

Positive family history of GC has been reported in about 10% of young patients with diffuse histotype of GC,⁴⁷ and therefore these patients are frequently considered strong candidates to be part of HDGC families. In such cases, families should be offered genetic counselling and should be tested for the presence of germline alterations at the *CDH1* gene.^{25,48}

EOGC occur more frequently in the proximal stomach at the gastroesophageal junction^{49,50} with diffuse histotype and signet ring cell features while these features are less frequent in older patients.^{49–53}

Patients with EOGC are frequently reported to display a worse long-term prognosis due to a delay in diagnosis and a more aggressive course of the disease,^{44,54,55} but this view is debatable as long-term results have also been reported to be similar to those of the older population with GC,^{56–61} instead stage of disease was the most important prognostic factor.⁴⁹

The advanced stage of the tumour in many young patients represents a clinical burden for the physician who has little if no therapeutic options for patients. The lack of symptoms in the early stages of GC may be the main cause of the delayed diagnosis with the subsequent poor clinical outcome and the cause of the advanced disease in young patients.^{62,63}

5. Conclusion

Approximately 2.3% of EOGC cases, without family history of GC, display deleterious *CDH1* germline alterations. These tumours invariably present partial (mixed) or complete diffuse histology and occur in patients younger than 35 years old. Germline *CDH1* mutation screening is now recommended for patients with these characteristics.

From the clinico-pathologic stand point, EOGC preferentially occur in female gender presenting tumours with diffuse histology, multifocal appearance, proximal location and poor

outcome. In EOGC patients, the advanced stage at the time of diagnosis remains a clinical burden due to the poor long term survival.

Aetiological factors of EOGC remain unknown as environmental agents represent a minor component and therefore a high impact of genetic causes is predicted. *CDH1* germline mutations in well documented diffuse EOGC cases remain the only germline genetic defect described in this type of patients.

Conflict of interest statement

None declared.

Acknowledgements

This work was supported by Grants from: Istituto Toscano Tumori ("Gene expression profile and therapeutic implication in gastric cancer. From the clinical overview to the translational research"; Grant ITT-2007) and The Portuguese Foundation for Science and Technology (FCT) [Grant number: PIC/IC/82923/2007, PTDC/SAU-GMG/72168/2006, PTDC/SAU-GMG/110785/2009, PTDC/SAU-ONC/110294/2009; PhD grant numbers: SFRH/BD/40090/2007-GC and SFRH/BD/41223/2007-HP; and Salary support from Program Ciência 2007 to CO).

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PAPER III

Corso, G., *et al.*, **Familial Gastric Cancer: update for practice management.** Familial Cancer 10:391-396; 2011

Familial gastric cancer: update for practice management

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Published online: 24 December 2010
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Abstract About 90% of gastric carcinoma presents a sporadic setting and only 10% shows a familial cluster; among this group, 1–3% are considered as hereditary syndromes, with a clear genetic pathway. The most important genetic mechanisms are associated with *CDH1* germline mutations, causing the hereditary diffuse gastric cancer syndrome. Other inherited predispositions with gastric carcinoma are the hereditary nonpolyposis colorectal cancer, Li-Fraumeni and Peutz-Jeghers syndromes. In this brief update, we described these principal hereditary syndromes offering a simple management to physicians where are these diseases diagnosed.

Keywords E-cadherin · Mismatch repair genes · *TP53* · *STK11*

Introduction

Detailed family history contributed greatly to the understanding of hereditary cancer syndromes as well as to the evolution of molecular genetic of these familial diseases. Cancer family history investigation and genetic counselling

have an unquestionable role in selecting families with suspicious hereditary cancer syndromes [1].

In particular, the recent molecular discoveries in gastric cancer (GC), where better defined when this disease occurs as sporadic, familial or hereditary. The knowledge of genetic basis is necessary because it opens different approaches for genetic testing and for clinical management of an asymptomatic population.

Among the hereditary forms, the main genetic mechanism is related with the E-cadherin (E-cad) germline mutation, causing the hereditary diffuse GC (HDGC) syndrome [2, 3]. Moreover, GC can participate in other inherited predispositions as a component of the hereditary nonpolyposis colorectal cancer (HNPCC), Li-Fraumeni (LF) disease and Peutz-Jeghers syndrome (PJS). In this brief review, we described these principal hereditary syndromes offering a simple management to physicians where are these diseases diagnosed.

Familial gastric cancer and environmental factor exposure

About 10% of GC shows a familial clustering and of these only 1–3% constitutes a clear hereditary form [4]; conversely, the majority of GC patients (90%) presents a sporadic tumor. To assess the diagnosis of GC with an hereditary setting, pedigree should present multiple cases of diagnosed GC and members should carry at last a documented germline mutation with high penetrance genotype, otherwise, we could define the form as familial cluster or aggregation. It seems that in a restricted geographic area, environmental factors without a clear genetic cause, such as diet and particular traditional habits contribute to the familial GC development. Specific environmental factors

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may play a role in generating mismatch repair (MMR) system deficiency leading to microsatellite instability (MSI) phenotype in some GC with familial cluster. Indeed, Palli et al. showed in a study performed in Tuscany, Italy, a geographical region with high rate for GC, an association between MSI, positive family history of GC and high consumption of red meat and nitrates, suggesting that environmental factors, such as nutritional habits, may play a key role in inducing genomic instability and an increased risk for GC with familial clustering [5, 6]. Aggressive and continuous general environmental exposures, such as these particular diets, are considered as potential generators of increased microsatellite alterations in tumors with an increased tolerance to DNA damage associated with reduced MMR activity [7]. Tobacco consumption is also considered another risk factor for gastric carcinoma development [8], leading to a functional deficiency of the MMR system, as for example the *XRCC1* gene; we demonstrated recently that the $-77T > C$ *XRCC1* promoter polymorphism is related with tobacco consumption and GC with familial aggregation for lung cancer [9].

E-cadherin and hereditary diffuse gastric cancer

HDGC is an autosomal dominant cancer disease caused by germline mutation of E-cad gene (*CDH1*) [10] that maps to chromosome 16q22.1 and encodes for the protein E-cad, a member of the transmembrane glycoprotein family expressed on epithelial tissue, responsible for calcium-dependent, cell-to-cell adhesion [11, 12]. In 1998 Guilford et al. firstly identified *CDH1* germline mutations in Maori kindreds from New Zealand with a strong familial cluster for DGC [2]; subsequently the International GC Linkage Consortium (IGCLC) in 1999 defined the clinical criteria to select pedigree for candidate to the E-cad genetic screening [3]. The clinical criteria were subsequently revised by Brooks-Wilson et al. in 2004 in which patients with sporadic early onset DGC with age at diagnosis ≤ 35 years were also considered for the genetic screening. In few cases, germline mutations of E-cad can be identified also in pedigree showing DGC and lobular breast cancer and/or mucinous colon cancer [13]. Families fulfilling the clinical criteria for HDGC established by the IGCLC showed a high penetrance of *CDH1* gene mutations, with an estimated risk of $>80\%$ and an individual *CDH1* germline mutation carrier presents more than 80% lifetime risk of developing GC. About 25–30% of families fulfilling the criteria for HDGC have germline mutations of the E-cad gene. Clinical criteria for HDGC definition revised by the IGCLC consensus are the following: (a) two GC cases in a family, one confirmed <50 ; (b) three confirmed DGC cases in 1st or 2nd degree relatives independent of age; (c) age

<40 ; (d) personal or family history of DGC and lobular breast cancer, 1 < 50 [14].

To date, about 100 *CDH1* germline mutations have been reported; from these the most frequent are insertion/deletion, nonsense, splice site (72%), instead missense variations occurred in about 28% of DGC patients [15]. To assess the pathogenic role of *CDH1* missense mutations, in silico and in vitro analysis is required [16].

Pathology of HDGC typically presents a diffuse histotype with signet ring cells; tumoral foci are multiple and spread through the entire gastric mucosa with a particular concentration in the antrum and in the transition zone between antrum and body [17]; frequently conventional endoscopy fails to detect these foci at early stage, and addition of chromoendoscopy improves the detection of early HDGC. *CDH1* germline mutations are documented only in diffuse GC cases, as well as in sporadic early onset cancers and in hereditary tumors; 37 cases it has been screened showing aggregation for intestinal GC and no E-cadherin constitutional alterations have been identified [18].

DNA mismatch repair genes and hereditary nonpolyposis colorectal cancer

HNPCC or Lynch syndrome is an autosomal dominant human cancer syndrome related with constitutional mutations in the DNA MMR genes, namely *MLH1*, *MSH2*, *PMS1*, *PMS2* or *MSH6* with a MSI pattern in tumoral samples [19]; among these, germline mutations of *MLH1* and *MSH2* MMR genes are more frequently detected in HNPCC [20]. MMR system is involved in DNA repair during genomic replication; mutations of MMR genes causes the “mutator phenotype” that leads to the MSI phenotype, due to the accumulation of errors for incorporation of number of bases during replication of long repetitive DNA sequences [21].

Aside from colon and rectum cancer, many other type of cancers can occur in HNPCC pedigree and GC represents a common extra-colonic tumor of this hereditary disease [22, 23]. In GC cases with MMR genes carriers, cumulative incidence is 13% by 70 years of age [24]. HNPCC gastric carcinomas carrying MMR genes germline mutations (as *MLH1* or *MSH2*), show prevalently intestinal histotype (more than 90% of the cases), and only a small percentage are diffuse. This correlation is due to the strong association between MSI tumor phenotype and intestinal GC [25].

In 1991, the International Collaborative Group on HNPCC developed the original Amsterdam Criteria for recruiting HNPCC patients in which performing the genetic testing; in this initial meeting GC was excluded. Subsequently, criteria were modified in 2004 with a revision of

the Bethesda guidelines [26], due to the identification of some GC cases in HNPCC family. The novel criteria recommended MSI testing and considered gastric carcinoma as a spectrum of HNPCC [27]. A complete description of clinical criteria was reported by Vasen et al. [28]. Whenever GC patient is component of HNPCC, and fulfills to the restrict described criteria [27], the probability to find a MMR gene mutation is extremely high (>80%) [25].

***TP53* gene and Li-Fraumeni syndrome**

LF syndrome is a rare autosomal dominant hereditary disease associated with *TP53* (chromosome 17p13) germline mutation. Germline carriers of the *TP53* mutation show increased risk for multiple primary tumours, such as sarcoma, breast cancer, brain tumours and adrenocortical carcinoma. Other less common tumours have also been associated with LF syndrome, including leukaemia, lung cancer, melanoma, pancreatic cancer, prostate cancer and GC [29, 30].

Clinical criteria were firstly described by Li and Fraumeni in 1988 [31] and subsequently in 1994, Birch et al. introduced a new definition of Li-Fraumeni-like syndrome (LFL). It was based on more extensive and updated information of the type of tumours and the ages of onset in families [32, 33].

The International Agency for Research on Cancer database reports that GC frequency is up to 2.8% of LFS families. *TP53* germline mutations have been identified in about 70% of patients with clinical criteria of LF syndrome; constitutional *TP53* alterations can appear in both GC histotype with similar frequency, as well as in intestinal and in diffuse forms. In particular, diffuse GC patients with *TP53* mutation, in some cases overlap the HDGC clinical criteria [30, 34–38]. However, *TP53* constitutional mutations are very rarely documented in the overall GC spectrum [39, 40].

***STK11* gene and Peutz-Jeghers syndrome**

The PJS is a rare autosomal dominant inherited disease associated with the germline mutations in the tumor suppressor gene namely *STK11*. This syndrome relates with gastrointestinal hamartomas, polyposis and mucocutaneous pigmentations; criteria were described first by Peutz in 1921 [41] and subsequently by Jeghers in 1949 [42]. Typically, initial morphology of GC with the PJS is a gastric mucosa polyposis. Among gastrointestinal cancers, GC is the third most frequent tumor in PJS, preceded by small intestine and colorectal carcinoma [43], with a cumulative cancer risk of 47% at the age of 65 years [44].

Screening guidelines and surveillance

The correct management of hereditary cancer syndrome requires genetic counselling, information on three generation family pedigrees, histopathological confirmation of gastric carcinoma and informed consent.

- (a) *Hereditary diffuse gastric cancer.* The IGCLC guidelines were established in the Cambridge consensus [14]; confirmation of diffuse histotype is mandatory. Genetic testing should be initiated in affected proband and performed on blood, the recommended age for genetic testing is 16 versus 18 years; in *CDH1* positive cases, prophylactic gastrectomy or endoscopic surveillance should be proposed. Prophylactic gastrectomy should be considered in truncating *CDH1* germline mutations, conversely in subject carriers *CDH1* missense mutation prophylactic surgical approach is debateable. Firstly, it is important to define the pathogenicity of the identified missense variation [45]; also in cases of deleterious missense mutation it seems that endoscopic surveillance is preferable [18]. In *CDH1* positive cases with deleterious missense mutations or in cases that declined the surgery, endoscopic screening should be performed with chromoendoscopic technique, with a minimum of 30 biopsies and with an annual frequency.

Prophylactic surgical treatment should be proposed in *CDH1* truncating carriers, with a total gastrectomy with Roux-en-Y reconstruction and performed in a high volume cancer centre.

- (b) *Hereditary nonpolyposis colorectal cancer.* Management of MMR gene mutation carriers is focused on endoscopic colon surveillance or prophylactic surgery; considering GC screening, data are inconsistent because the cancer risk is not extremely high and no studies have been conducted. However, with an increased rate of GC cluster, upper gastrointestinal tract endoscopy could be proposed periodically; in any case, the number of affected members is not a target of predisposition to the Lynch syndrome and the adequate surveillance for GC in Lynch syndrome remains undefined and controversial [46, 47].
- (c) *Li-Fraumeni syndrome.* Considering the low incidence rate of GC in pedigree with LF or LFL syndrome, the cumulative risk is unknown and no guidelines for management of *TP53* gene mutation carriers have been proposed; it is reasonable to suggest an annual endoscopy in pedigree with multiple cases of GC. Certainly, detection of *TP53* mutations contributes considerably to increase GC risk in family with LF or LFL syndrome [31].

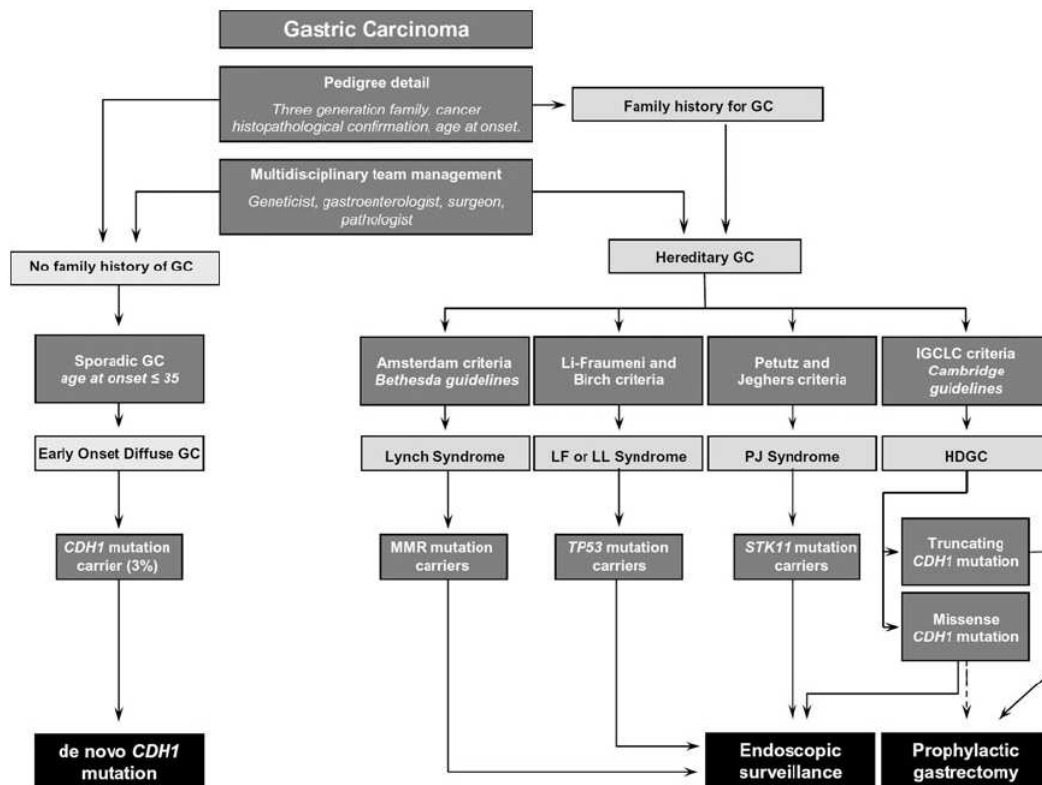


Fig. 1 Algorithm for familial gastric carcinoma management starting from clinical overview, pedigree data collection, clinical criteria, genetic testing, surveillance with endoscopy and gastrectomy

- (d) *Peutz-Jeghers syndrome*. Considering the relative high cancer risk (47%), surveillance for PJS patients is recommended; in the specific for the gastrointestinal tract, the Dutch surveillance suggests a gastro-duodenoscopy starting from 20 years with a frequency of 2–5 years, depending on findings and on the number of cases of GC [42].

Conclusion

Physicians should know GC with familial cluster through simple information as reported in this brief review. Clinical approach to patients with a suspicious inherited predisposition for gastric tumors requires a strict collaboration between geneticist for testing, surgeon, gastroenterologist and pathologist for a correct clinical management and treatment.

In the Fig. 1, we propose a flow-chart for management of familial and hereditary GC patients. The first approach claims a clinical method, providing the correct description

of a detailed pedigree, with all familial information, as number of patients, sex, age at onset and at the end of the follow-up, cancer diagnosis, histotype confirmation and outcome. Detection of clinical criteria can help to understand when GC is sporadic or familial or hereditary; in the specific, management of GC with suspicious familial or hereditary firstly requires a pedigree with at least knowledge of three member generations [48, 49], and when analysed pedigree overlap the clinical criteria, genetic testing is mandatory. The identification of a germline alteration requires a specific treatment in asymptomatic patients, in particular in *CDH1* mutation carriers in the HDGC syndrome. In a very low percentage of sporadic early onset DGC (3%), it has been documented *CDH1* germline alterations, presenting as de novo mutations. E-cad genetic testing should be proposed also in cases of sporadic DGC with age at onset of ≤ 35 years (Fig. 1) [50].

Unfortunately, cases with familial aggregation and without specific gene involvement are the majority and represent an urgent clinical burden considering that familial GC, unlike its sporadic form, shows an increasing trend, in particular, in cases with diffuse gastric carcinoma.

Acknowledgments This work was supported by Istituto Toscano Tumori ("Gene expression profile and therapeutic implication in gastric cancer. From the clinical overview to the translational research"; Grant ITT-2007) and PhD grant (ref. SFRH/BD/40090/2007).

Conflict of interest The author(s) declare that they have no competing interests.

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ABSTRACT I and II

- I. Corso, G., *et al.*, **CDH1 structural alteration as novel prognostic biomarker in gastric cancer patients.** J Clin Oncol Suppl 4:42; 2011
- II. Corso G., *et al.*, **Oncogenic mutations in MAPK cascade as novel molecular biomarkes for treatment of gastric cancer patients with EGFR inhibitors.** J Clin Oncol Suppl 4:39;2011

***CDH1* structural alterations as novel prognostic biomarker in gastric cancer patients.**

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Abstract

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Background: Loss of *CDH1* function occurs in sporadic gastric cancer (GC) progression by different molecular mechanisms. Hypermethylation and somatic mutations are considered the most frequent events. About 50% of sporadic diffuse GC shows *CDH1* promoter of hypermethylation and 30% harbor somatic mutations; loss of heterozygosity (LOH) is documented in only 5% of diffuse GC cases. The present study aims to assess *CDH1* alterations in 246 GC and study associated clinicopathologic features and family history of GC.

Methods: Bisulphite treated tumoral DNA was used for E-cad promoter methylation. A region of 221 bps was amplified using flanking primers and direct sequencing. To search for somatic mutations the hotspot region of E-cad was amplified and directly sequenced. For LOH analysis tumor and matched normal mucosae were amplified in three microsatellite markers (D16S3025, D16S496, D16S3067) flanking the *CDH1* locus.

Results: Promoter hypermethylation was the most frequent event occurring in 20.7% (51 of 246) of the cases. We found 26 (10.6%) cases affected by structural alterations (three with somatic mutations and 23 with LOH). Promoter *CDH1* methylation is associated with the diffuse type of GC ($p = 0.017$) and the presence of distant lymph node metastasis ($p = 0.043$). We identified association between male gender and structural *CDH1* alterations ($p = <0.001$), R1/2 radicality of resection ($p = 0.015$). We verified a correlation between advanced stages and *CDH1* promoter methylation ($p = 0.033$). Kaplan-Meier survival analysis estimates that *CDH1* structural alterations participate as poor prognostic factor in GC patients ($p = 0.017$).

Conclusions: Considering the reversible nature of hypermethylation phenomena, demethylating drugs have emerged as possible chemopreventive strategies in solid tumors. Moreover, we verify that epigenetic changes of E-cadherin leading to its decrease protein expression induce an invasive behaviour to neoplastic cells; as structural alterations of *CDH1* gene is assessed as predictive of worse prognosis we found a new molecular biomarker of prognosis in GC that can be used to predict the clinical prognosis of the patients.

No significant financial relationships to disclose.

Oncogenic mutations in *MAPK* cascade as novel molecular biomarkers for treatment of gastric cancer patients with *EGFR* inhibitors.

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Abstract

ASCO Merit Award Recipient

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Background: Mitogen-activated protein kinase (*MAPK*) cascade and phosphatidylinositol 3-kinase (*PI3K*) survival pathways are frequently activated in the progression of gastrointestinal malignancies. In this study, we aimed to determine the frequency of gene mutations in members of these pathways-*EGFR*, *KRAS*, *BRAF*, *PIK3CA* and *MLK3* in a series of 63 gastric carcinomas with high levels of microsatellite instability.

Methods: Gene mutation analysis was performed by PCR amplification followed by direct sequencing. In selected tumor cases, *EGFR* expression was evaluated by immunohistochemistry. Association studies between molecular data and clinicopathologic characteristics were performed.

Results: Mutations in *EGFR* (3'-UTR polyA repeat), *KRAS*, *PIK3CA* and *MLK3* genes occurred in 30 (47.6%), 11 (17.5%), 9 (14.3%) and 2 (3.2%) of the MSI gastric cancer (GC) cases, respectively. No *BRAF* or *EGFR* hotspot mutations were identified. Overall, mutations in at least one of these genes were found in 55.6% (35/63) of gastric carcinomas. From those mutant cases 40.0% (14/35) of them had concomitant gene mutations, always involving *EGFR* polyA deletions. Interestingly, we observed significant associations between oncogenic mutations and female gender ($p=0.046$) old age of diagnosis ($p=0.001$) and intestinal subtype ($p=0.043$).

Conclusions: Our results show that MSI gastric carcinoma frequently shows activation of *EGFR*-*MAPK* and *PI3K* pathways. Within all alterations found, deletions of the A13 repeats of *EGFR* were common, suggesting this molecular event as an important biomarker for stratification of GC patients for treatment with *EGFR* inhibitors.

No significant financial relationships to disclose.

PAPER IV

Leite, M., *et al.*, **MSI phenotype and MMR alterations in familial and sporadic gastric cancer**. Int J Cancer 128:1606-1613; 2011

MSI phenotype and MMR alterations in familial and sporadic gastric cancer

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Microsatellite instability (MSI) is a major pathway involved in gastric carcinogenesis occurring in 20% of gastric cancer (GC). However, it is not clear whether MSI phenotype preferentially occurs in the sporadic or familial GC, when stringent inclusion criteria are used. The aim of this study was to compare the frequency of MSI and hypermethylation of *MLH1* promoter in a large series of familial GC patients (non-HNPCC and non-*CDH1*-related) and sporadic cases. Additionally, we analysed the immunoexpression of MMR proteins in a fraction of cases. Overall, the frequency of familial GC was 7.1%, and the frequency of hereditary tumours was 4.6%. MSI phenotype and *MLH1* hypermethylation frequencies were not statistically different between familial and sporadic GC settings. Further, the MSI phenotype was not associated with any clinico-pathological features studied in the familial GC setting, whereas in the sporadic setting, it was associated with older age, female gender and intestinal histotype. Using our stringent Amsterdam-based clinical criteria to select familial GC (number of cases, age of onset), we verified that sporadic and familial cases differed in gender but shared histopathological features. We verified that the frequency of MSI was similar in familial and sporadic GC settings, demonstrating that this molecular phenotype is not a hallmark of familial GC in contrast to what is verified in HNPCC. Moreover, we observed that the frequency of *MLH1* hypermethylation is similar in sporadic and familial cases suggesting that in both settings MSI is not associated to MMR genetic alterations but in contrast to epigenetic deregulation.

Key words: gastric cancer, MSI, MMR, HDGC, HNPCC

Abbreviations: CRC: colorectal cancer; FGC: familial gastric cancer; GC: gastric cancer; HDGC: hereditary diffuse gastric cancer; HE: hematoxylin-eosin; HGC: hereditary gastric cancer; HNPCC: hereditary non-polyposis colorectal cancer; IHC: immunohistochemistry; MMR: DNA mismatch repair; MSI: microsatellite instability; MSS: microsatellite stable; SGC: sporadic gastric cancer

Grant sponsor: Sixth Framework Programme; **Grant number:** EU-FP6 (LSHC-CT-2005-018754); **Grant sponsor:** Portuguese Foundation for Science and Technology; **Grant numbers:** PTDC/SAU-OB/68310/2006, PIC/IC/82923/2007, SFRH/BD/40090/2007; **Grant sponsor:** Program Ciência 2007 and Portuguese Foundation for Science and Technology (FCT)

DOI: 10.1002/ijc.25495

History: Received 3 Mar 2010; Accepted 17 May 2010; Online 7 Jun 2010

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Gastric cancer (GC) is one of the leading causes of cancer death, and despite the decreasing trend in worldwide incidence, there are still high-incidence regions.¹ The majority of GCs are sporadic, yet familial aggregation occurs in about 10%. Of these, only 1–3% clearly constitutes hereditary forms.² So far, a single gene has been identified with germline alterations in families with clustering of early onset diffuse GC (HDGC, Hereditary diffuse GC); the *CDH1* gene (OMIM +192090) encoding for E-cadherin.^{3,4} Families from countries with high incidence of GC display the lowest frequency *CDH1* alterations (~20%), whereas those from low incidence countries display the highest rates of alterations (~50%).⁵ So far, no other germline defects have been discovered in *CDH1* negative HDGC families neither in families not fulfilling the strict HDGC criteria.

As familial clustering of GC, independently of histologic type, persists among GC cases from high GC incidence countries, it is important to understand whether other “causative” molecular alterations, so far unidentified, occur in these cases, or else whether this reflects a clustering of sporadic cases due to shared environmental factors.

Lessons from GC revealed that two main genomic instability pathways are involved in gastric tumourigenesis: the

chromosomal instability pathway characterised by gross copy number changes with largely unknown causal molecular mechanisms that is believed to occur in at least 60% of cases,⁶ and the microsatellite instability (MSI) pathway that characterise 10–20% of cases^{7–9} and induces alterations in the length of repetitive microsatellite sequences. In GC, MSI is reported to be mainly caused by hypermethylation of the *MLH1* promoter, one of the genes from the DNA mismatch repair (MMR) system.^{6,10} Also important for GC tumorigenesis is the recently described CpG islands methylator phenotype (CIMP), characterised by the simultaneous hypermethylation of several tumour-related genes, and for which no driving molecular mechanism or definitive frequency has been yet identified.^{11–13}

Moreover, in the tumour spectrum of hereditary nonpolyposis colorectal cancer (HNPCC) families, the syndrome caused by germline alteration in MMR genes and the hallmark of MSI phenotype, GC is a common neoplasia.^{14–17} It is then conceivable to hypothesise that at least a small fraction of GC families may also be associated with inherited MMR deficiency.

Current data concerning the association of MSI phenotype and/or *MLH1* methylation with familial clustering are scarce and whenever data are available these studies analyse a low number of patients.^{9,18–23} Thus, in this study, we analysed the frequency of MSI in a series of 410 non-HNPCC and non-*CDH1* related GCs classified as familial and sporadic using a strict classification criteria (Table 1). *MLH1* hypermethylation was assessed in 89 MSI tumours, and immunorexpression of *MLH1*/*PMS2* ($n = 22$) and *MSH2*/*MSH6* ($n = 3$) pairs of proteins was also assessed in a fraction of MSI GC samples. In two MSI familial GC cases that displayed loss of *MLH1* immunorexpression, germline *MLH1* promoter hypermethylation, mutations and deletions were also analysed.

Material and Methods

Patients and tumours

A total of 410 GC (GC) patients from high GC incidence countries (Portugal and Italy) from which frozen and formalin-fixed paraffin-embedded tumours were collected and included in this study. Information about family history of cancer was obtained from the patients' clinical reports. Ethics approval was obtained from the Ethics Committee of Hospitals involved in this study.

Patients enrolled in this study were classified as belonging to familial or sporadic (non-familial) GC subsets using the criteria depicted in Table 1. Patients from the familial subset were further classified as having a complete criteria (hereditary HGC) or incomplete criteria (familial aggregation FGC), using a stringent clinical criteria adapted from the Amsterdam guidelines used for hereditary colorectal cancers.²⁴

Two-hundred and forty nine tumours were collected from patients from the Department of General Surgery and Surgi-

Table 1. Criteria for definition of hereditary or familial gastric cancer

Hereditary gastric cancer (HGC)—complete criteria
Three or more cases of histological documented gastric cancer in first/second degree relatives (negative for <i>CDH1</i> mutations)
At least one should be diagnosed before age 50
Familial gastric cancer (FGC)—incomplete criteria
Two histological confirmed cases of gastric cancer in first/second degree relatives (negative for <i>CDH1</i> mutations)
At least one should be diagnosed before age 50

cal Oncology of University of Siena (Italy). One-hundred and sixty one tumours were collected from 4 Portuguese institutions (IPO-Porto ($n = 15$), HSJ-Porto ($n = 126$), HGSA-Porto ($n = 5$) and IPATIMUP ($n = 15$)).

Clinicopathological features

The histopathological classification of each specimen was studied by hematoxylin and eosin-stained tissue sections by board-certified pathologist from each of the Institutions participating in this study. For the analysis herein performed, clinical parameters such as patient's age and gender as well as family history of cancer were collected with informed consent and ethical approval. Pathological features such as tumour spectrum within GC families and the tumour histological type according to Lauren classification²⁵ were also gathered. These features were used to determine potential associations with all molecular parameters analysed in this study.

DNA extraction

High-molecular-weight genomic DNA from tumour and normal gastric mucosa was extracted either from frozen material using standard protocols or from paraffin-embedded material using the Invisorb Spin Tissue Mini Kit (Invitex, Berlin, Germany) following the manufacturer's instructions. In every case, hematoxylin and eosin-stained, 4 μ m sections were used to discriminate tumour and normal mucosa before DNA extraction. For paraffin-embedded samples, 10–20 μ m sections were used for macrodissection of areas with at least 75% of neoplastic cells (tumour) and normal for DNA extraction. Germline high -molecular-weight genomic DNA was isolated from blood in two patients with positive family history using standard methodologies.

MSI analysis

From the 410 tumours, 249 were already characterised for MSI phenotype.⁹ MSI status was evaluated on genomic DNA extracted from 161 tumour samples from Portuguese origin, using the single fluorescent multiplex PCR comprising five quasimonomorphic mononucleotide repeat markers (BAT-25, BAT-26, NR-21, NR-24 and NR-27) described by Buhard and collaborators.²⁶ Tumours were classified as MSI when

two or more of the five markers showed instability, otherwise were classified as microsatellite stable (MSS) tumours.

MLH1 promoter hypermethylation analysis

Somatic promoter hypermethylation status of *MLH1* was analysed in 91 tumours by bisulphite-specific PCR, using primers flanking CpG sites located in the promoter region and spanning from -336 bp to -48 bp relative to the transcription start site. Bisulphite conversion of tumour genomic DNA was performed using the EpiTect[®] Bisulfite Kit (QIAGEN). The modified DNA was amplified for the *MLH1* with the forward 5'-TTTTGTTTATTGGTTGGATATTT-3' and reverse 5'-CCTTCAACCAATCACCTCAATACCT-3' primers, using the QIAGEN[®] Multiplex PCR Kit (QIAGEN). The PCR product was purified with illustra[™]GFX PCR DNA and Gel Band Purification kit (GE Healthcare, Buckinghamshire, UK) and sequenced on 3130XL Genetic Analyser AB (Applied Biosystems/Hitachi) using the BigDye[®] Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing data were analysed using the AB DNA Sequencing Analysis software v5.2 (Applied Biosystems).

Germline promoter hypermethylation analysis of *MLH1* was performed using the same strategy in bisulphite converted DNA extracted from peripheral blood lymphocytes from two patients with HGC.

Immunohistochemistry staining for MMR proteins

Immunohistochemical staining was performed on 3 µm sections of formalin-fixed paraffin-embedded tumour samples. Heat-induced antigen retrieval was performed in microwave using 10 mM citrate buffer, pH 6.0 for MLH1 and MSH2 proteins, and 1 mM EDTA buffer, pH 8.0 for MSH6 and PMS2 proteins. Human anti-MLH1 (clone G168-15, BD Pharmingen[™], San Diego, California, USA), anti-MSH2 (clone FE11, Calbiochem[®], Cambridge, Massachusetts, USA), anti-MSH6 (clone 2D4B5, Zymed[®] Laboratories, Inc., San Francisco, California, USA), and anti-PMS2 (clone A16-4, BD Pharmingen[™]) monoclonal antibodies were used. Antibody reactions were detected and developed by SuperPicture[™] Polymer Detection Kit from Zymed[®] Laboratories, Inc. or Dako REAL[™] EnVision[™] Detection system, Dako Denmark, Glostrup, Denmark. Tissue sections were counterstained with Mayer's Hematoxylin. Tumours were classified as negative when nuclear staining was absent in neoplastic cells and positive when more than 90% of neoplastic showed nuclear staining. Cases that had inadequate internal control tissue or scarce tumour in the section or in which the staining was technically inadequate were eliminated.

Germline analysis of MMR genes

Germline mutations of *MLH1*, *MSH2* and *MSH6* were analysed using genomic DNA isolated from peripheral blood from two patients with HGC by denaturing gradient gel electrophoresis (DGGE), followed, in the case of aberrant band patterns, by direct sequencing of independently amplified

PCR products. Multiplex ligation-dependent probe amplification (MLPA) was used to detect large rearrangements of *MLH1* and *MSH2* following manufactures instructions (MRC-Holland[®], Amsterdam, The Netherlands) in the same two patients.

Statistical analysis

Fisher's exact test and Chi-square, as well as t test were used as appropriate. Sample sizes in each analysis varied according to the samples available in each setting. Statistically significant values were considered for *p* values less than 0.05.

Results

Familial and non-familial GC patient's clinicopathological features

From the 410 patients with GC enrolled in this study, 29 (7.1%) patients belong to families with a proven family history of GC (familial GC subset). Nineteen of these twenty-nine patients belong to families that fulfill hereditary GC criteria (HGC) (19/410, 4.6%), while the remaining 10 (10/410, 2.4%) GC patients belong to families with incomplete criteria, thus classifying as familial aggregation of GC (FGC) (Table 1). Three-hundred and eighty one (381/410, 92.9%) GC patients did not report a family aggregation of GC, thus were considered to be patients with a sporadic form.

The comparison between sporadic (*n* = 381) and familial GC subset (*n* = 29) concerning the clinicopathologic features, such as age of diagnosis, gender, Lauren's histotype, showed that the mean age of patients with sporadic GC was significantly higher than that of familial GC patients (67.7 ± 11.7 years vs. 54.4 ± 14.0 years, respectively; *p* < 0.0001). Moreover, while sporadic GC was more frequent in males than in females (*p* = 0.0034), the opposite was observed in the familial GC setting (Table 2). Within the familial GC subset, no significant differences were observed between patients with hereditary GC (HGC) and patients with familial aggregation of GC (FGC) regarding the clinico-pathological characteristics studied, namely age of diagnosis, gender, Lauren's histotype (data not shown).

MSI in familial and sporadic GC

Ninety-five of 410 (23.2%) GC tumours analysed were identified as MSI. The overall frequency of MSI in the familial setting was 24.1% (7/29) and was not different from that of the sporadic setting, where MSI was found in 23.1% (88/381) of the tumours. Specifically within the familial setting, 26.3% (5/19) of HGC patients and 20.0% (2/10) of FGC displayed tumours with MSI phenotype. Overall, no statistically significant differences were detected in the different settings.

Associations between patients' age and gender, and tumours' Lauren histotype with the MSI status of GC were analysed for the two subsets of GC cancer (familial and sporadic GC). Although no associations were detected for familial GC associated features and MSI (Table 3), in sporadic GC, the MSI phenotype was associated with older age at

Table 2. Comparison of clinical, histological and molecular parameters of sporadic and familial gastric cancer

	Sporadic GC (n = 381)	Familial GC (n = 29)	p value
Age (years, mean $\pm$ sd)	67.7 $\pm$ 11.7	54.4 $\pm$ 14.0	<0.0001
Gender			0.0034
Female	156 (41.1%)	20 (69%)	
Male	224 (58.9%)	9 (31%)	
Lauren's histotype			0.1805
Intestinal	214 (56.2%)	11 (37.9%)	
Diffuse	119 (31.2%)	11 (37.9%)	
Unclassified	48 (12.6%)	7 (24.2%)	
MSI status			0.8981
MSI	88 (23.1%)	7 (24.1%)	
MSS	293 (76.9%)	22 (75.9%)	
MLH1 methylation			0.6384
Met MLH1	65 (79.3%)	5 (71.4%)	
UnMet MLH1	17 (20.7%)	2 (28.6%)	

diagnosis (72.8 $\pm$ 10.5 in MSI vs. 66.2 $\pm$ 11.6 in MSS, p < 0.0001), with female gender (56.3% in MSI vs. 36.5% in MSS, p = 0.0012) and intestinal histological type of tumours (71.6% in MSI vs. 51.5% in MSS, p = 0.0030) (Table 4). Further, we compared the age of onset between sporadic and familial MSI GC cases and verified that familial GCs are statistically significant younger (72.8 $\pm$ 10.5 vs. 57.9 $\pm$ 16.5, p = 0.0276). Also, the age of onset comparison between sporadic and familial MSS GC cases raised a similar finding (66.2 $\pm$ 11.6 vs. 53.3 $\pm$ 13.4, p < 0.0001).

MLH1 promoter methylation in MSI GC

Overall, *MLH1* promoter hypermethylation was found in 78.7% (70/89) of the MSI cases analysed. The frequency of *MLH1* promoter methylation was similar in familial (5/7, 71.4%) and in sporadic (65/82, 79.3%) GC forms (Table 2).

Immuno-expression of MMR proteins in MSI GC

Smaller series of cases were used for immunohistochemistry analysis of MMR proteins (*MLH1*, *PMS2*, *MSH2* and *MSH6*) due to lack of biological material. Twenty-two MSI tumours were analysed: two HGC for all proteins and, 20 sporadic GC for *MLH1* and *PMS2*. Three MSS cases were also analysed for *MLH1*/*PMS2* as a control for immunoexpression in proficient MMR tumours.

From the 22 MSI cases: 20/22 (90.9%) (2 HGC and 18 sporadic) displayed decreased or loss of both *MLH1* and *PMS2* protein expression in tumour cells; 1/22 (4.5%) displayed normal *MLH1* expression and loss of *PMS2* expression and 1/22 (4.5%) displayed normal *MLH1* and *PMS2* immunoexpression.

Both HGC tumours displayed loss of *MLH1* and *PMS2* immunoexpression, one of the cases displayed *MLH1* pro-

Table 3. Comparison of clinical and histological parameters of MSI and MSS familial gastric cancer

	Familial gastric cancer (n = 29)		
	MSI	MSS	p value
Age (years, mean $\pm$ sd)	57.9 $\pm$ 16.5	53.3 $\pm$ 13.4	0.4614
Gender			0.3816
Female	6 (85.7%)	14 (63.6%)	
Male	1 (14.7%)	8 (36.4%)	
Lauren's histotype			0.0568
Intestinal	4 (57.1%)	7 (31.8%)	
Diffuse	0 (0%)	11 (50%)	
Unclassified	3 (42.9%)	4 (18.2%)	

Table 4. Comparison of clinical and histological parameters of MSI and MSS sporadic gastric cancer

	Sporadic gastric cancer (n = 381)		
	MSI	MSS	p value
Age (years, mean $\pm$ sd)	72.8 $\pm$ 10.5	66.2 $\pm$ 11.6	< 0.0001
Gender			0.0012
Female	49 (56.3%)	107 (36.5%)	
Male	38 (43.7%)	186 (63.5%)	
Lauren's histotype			0.0030
Intestinal	63 (71.6%)	151 (51.5%)	
Diffuse	16 (18.2%)	103 (35.2%)	
Unclassified	9 (10.2%)	39 (13.3%)	

moter methylation, whereas the other did not display this epigenetic feature (Fig. 1). Within the MSI sporadic setting, 90% (18/20) of the cases with loss or decreased expression of these proteins also displayed *MLH1* promoter hypermethylation in 83.3% (15/18) of the cases. Therefore, only in three cases of the MSI sporadic setting, the cause of *MLH1*/*PMS2* loss of expression was not associated with *MLH1* methylation.

The three MSS cases, used as a control for integrity of expression of *MLH1*/*PMS2*, displayed, as expected, normal expression of both proteins.

Additionally, and as *MSH6* dysfunction has been found associated with the MSS phenotype, we also analysed 115 MSS cases for *MSH2* and *MSH6* immunoexpression: 15 MSS familial cases (11 HGC and 4 FGC) and 100 SGC cases. We verified that only 1/115 (0.9%) of these cases displayed loss of the *MSH2*/*MSH6* complex, demonstrating the rarity of this phenomena in GC. Further, as this was a GC case without familial history, we did not proceed with other analysis.

MMR germline alterations analysis in familial

MSI GC cases

One of the two HGC tumours displayed simultaneously loss of *MLH1* and *PMS2* protein expression and MSI phenotype

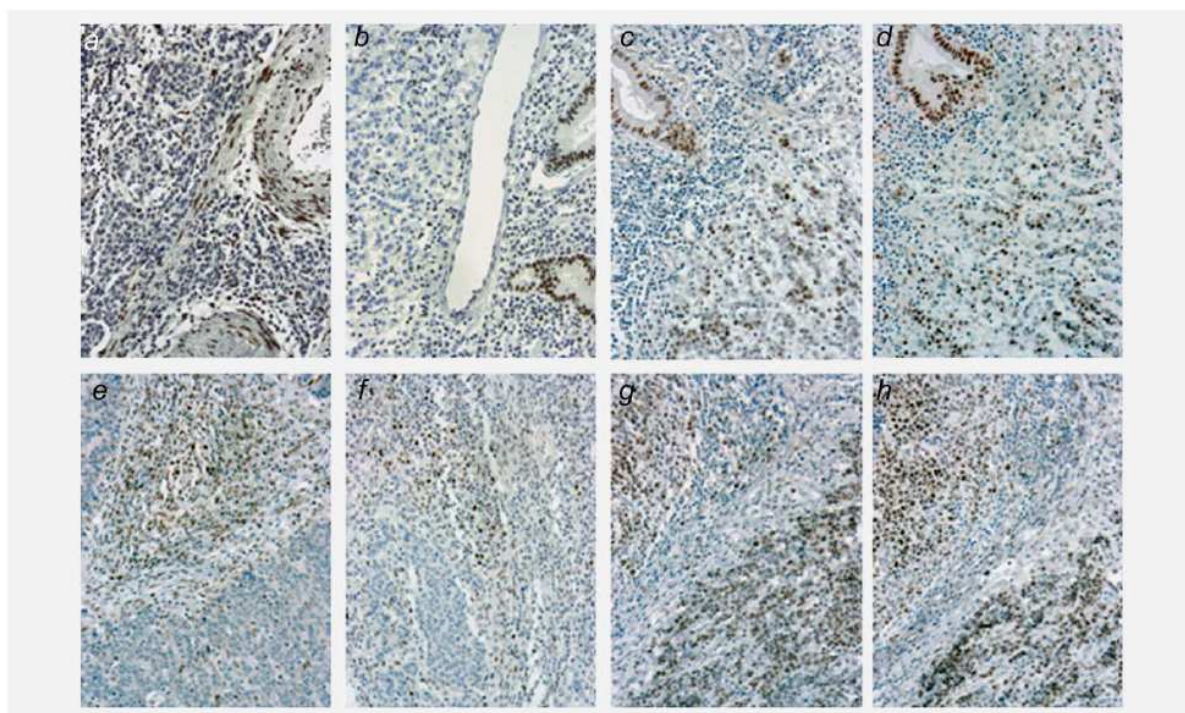


Figure 1. Immunohistochemistry of MLH1, PMS2, MSH2 and MSH6 proteins in MSI gastric carcinomas from two familial gastric cancer patients, one with (panel 1, *a* to *e*) and other without (panel 2, *e* to *h*) promoter hypermethylation of *MLH1* in tumour cells. The MLH1 (*a* and *e*) and PMS2 (*b* and *f*) immunoexpression is lost in tumor cells, whereas their expression is preserved in the adjacent mucosa, (scattered) lymphoid cells or blood vessels (internal controls). MSH2 (*c* and *g*) and MSH6 (*d* and *h*) immunoexpression are preserved in tumour cells. $\times 200$ Magnification.

but lacked *MLH1* promoter hypermethylation. This case was regarded as a potential germline MMR related case. Although the other HGC tumour did present features similar to those displayed by the sporadic MSI tumours, we performed, for both patients, germline screening of *MLH1* and *MSH2* mutations and genomic rearrangements as well as germline methylation of *MLH1* gene. No alterations were detected in any of the cases.

Discussion

MSI phenotype is the hallmark of HNPCC and is caused by germline mutations in MMR genes in $\sim 60\%$ of the cases.^{27,28} In the sporadic setting of MSI cases, both colorectal and gastric, somatic promoter *MLH1* methylation rather than somatic mutations at MMR genes is the most prevalent mechanism leading to DNA repair impairment.^{13,29–37} The frequency of MSI phenotype and *MLH1* methylation in tumour from families with clustering of GC is poorly understood as well as the potential role of germline MMR deficiency as the cause of this familial aggregation.^{22,35}

To shed light into this issue, we analysed a large series of unselected GCs from two high-incidence countries, Portugal and Italy (Tuscany), that we initially characterised for familial aggregation using stringent clinical criteria based not only on

the number of GC cases per family but also on the age of onset, by selecting only those families presenting at least one GC case aged less than 50 years old. Moreover, to prevent an over-inclusion of GC families with already described genetic causes, we excluded familial cases belonging to families harboring *CDH1* alterations (HDGC) and with undoubted HNPCC criteria, and adopted for the classification of familial GC cases a clinical criteria analogous to the Amsterdam criteria used to select HNPCC.

We verified that 7.1% of the cases in our series displayed familial aggregation of GC. This frequency is slightly lower than the overall accepted of 10%^{38,39}; nevertheless, one has to consider that HDGC and HNPCC cases were excluded, and therefore, this lower frequency is not unexpected.

Supporting the criteria herein chosen to select familial cases was the fact that patients with familial GC were significantly younger than those with sporadic GC. Moreover, in the familial setting females were significantly more affected than in the sporadic setting. This finding is inharmonious with reports in the literature that address the same issue. All reports refer to a high male-to-female ratio either in families with positive history of GC and or in early onset GC patients, although without statistical significance.^{9,20,21,35,40–42} Interestingly, among *MLH1* and *MSH2* mutation carriers

belonging to HNPCC families, GC also affected preferentially males than females (male:female ratio 1.9:1)⁴³; therefore, the reason behind our result (a higher female-male ratio) remains unknown.

Among familial cases, HGC and FGC were not significantly different regarding all the clinico-pathological characteristics analysed (age, gender, Lauren's histotype). This finding is possibly demonstrating that both criteria are adequate to select GC familial aggregation.

The MSI frequency reported in the literature for familial and sporadic GCs is highly divergent.^{18,19,21,23,40} Although some reports described the absence of a relationship between MSI and family history of GC,^{19,20,23} others report the opposite.^{9,18,21} This divergence is most probably related to clinical criteria used to select family history of GC. Using our strict clinical criteria, we showed a similar overall frequency of MSI both in familial and sporadic settings of GC, and even in HGC and FGC. These results demonstrate that the MSI phenotype cannot be used to distinguish familial and sporadic GC, in contrary to colorectal cancer where MSI phenotype is preferentially associated with HNPCC in comparison to sporadic CRC (90% vs. 15%, respectively).^{16,28,44,45} In any case, it would be interesting to analyse a larger series of familial GC cases selected under the same stringent clinical criteria that were herein used to support our results.

Within the series of sporadic GC cases, MSI was significantly more frequent in intestinal type tumours, in older patients and in female gender. Similar associations have been previously described in the literature.^{7,18,46}

Importantly, when we compared the mean age of onset of GC between MSI and MSS cases in the subsets of sporadic and familial GC, we verified that while patients with sporadic MSI cancers were statistically older than those with sporadic MSS cancers, in the familial subset, no differences in age of onset were found between MSI and MSS cases. This last observation is consistent with increased susceptibility in patients from the familial setting that can be related or not with the MSI phenotype. A similar finding has not been reported so far in the literature, probably because the criteria to select familial cases infrequently used age of onset as a strong selector; therefore, this result re-enforces again the value of our clinical criteria. In fact, since our cohort of patients was first selected based on the presence of GC as sole criteria and only after information on family history of GC (and other types of cancer) and the age of onset was obtained, our finding regarding the differences between age of onset of familial and sporadic cases, independently of MSI status, should not be biased. Moreover, all families where large numbers of GC were identified without at least one family member aged 50 or less were excluded. In these excluded families we believe that there is a higher probability of clustering of sporadic cases due to the same environment.

To disclose the mechanism by which MSI occurs in our series of GC, we analysed the status and frequency of *MLH1* promoter methylation in all available tumours. We confirmed

the importance of aberrant *MLH1* promoter hypermethylation in the majority of MSI GC cases as 78.7% of the tumours displayed this mechanism of inactivation. This frequency was similar to that described in literature,^{8,22,32} and established this epigenetic alteration as the major mechanism triggering MSI in GC. The frequency of *MLH1* hypermethylation was not significantly different between familial (71.4%) and sporadic (79.3%) GC subsets, thus excluding a differential contribution of *MLH1* hypermethylation in tumours from different MSI settings. The low number of familial gastric MSI tumours precluded any further considerations.

We used immunohistochemistry (IHC) to access the expression of MLH1 protein and verified that in our series of MSI cases, those tumours harbouring *MLH1* hypermethylation also displayed loss of MLH1 protein immunoreactivity. These results confirmed reported data both in gastric and colorectal cancers.^{22,30,32,47} All MSI tumours with *MLH1* promoter hypermethylation lacked not only MLH1 but also PMS2 protein expression, consistent with the destabilization of PMS2 protein in the absence of its heterodimer partner MLH1.⁴⁸ We further identified lack of MLH1 immunoreactivity in a few MSI GC cases without *MLH1* promoter hypermethylation. In these cases, other mechanisms of *MLH1* inactivation may be involved, such as somatic mutations or deletions. Additionally, the effect of hypoxia in cancer cell lines has also been reported as a mechanism responsible for the downregulation of *MLH1* mRNA and protein expression.^{49,50}

Interestingly, MSH2 and MSH6 immunoreactivity was retained in tumour cells both from MSI GC and MSS GC demonstrating that the MSI phenotype in GC is majorly associated with the impairment of MLH1 (data not shown).

To complete the study and determine whether at least a small fraction of GC families would be associated with inherited MMR deficiency, we tested germline mutations and gene rearrangements at *MLH1* and *MSH2* MMR genes, as well as germline methylation of the *MLH1* promoter in the probands of two GC families with tumours lacking MLH1 immunoreactivity. At least in these two cases, we were not able to detect germline alterations that could explain familial aggregation of early onset MSI GC.

In conclusion, we defined two clinical criteria to select familial GC based on number of GC cases and early onset of disease and independent from the tumour histotype based on Amsterdam Criteria. Noteworthy, sporadic and familial cases differed only in age of onset and gender, while sharing other clinicopathological features. We verified that the frequency of MSI caused by *MLH1* hypermethylation is similar in familial and sporadic GC settings, demonstrating that MSI phenotype is not a hallmark of familial GC, in contrast to HNPCC. Moreover, although in familial cases, the MSI phenotype was not associated with any clinico-pathological features, in sporadic GC, the MSI phenotype was associated with older age, female gender and intestinal histotype of tumours, as verified in other series of cases, re-enforcing again the value of the clinical criteria used in this report.

Acknowledgements

This study was supported by the Portuguese Foundation for Science and Technology (PhD grant to G.C. reference SFRH/BD/40090/2007; and salary support from the program Ciência 2007 to C.O.). IPA-

TIMUP is an Associate Laboratory of the Portuguese Ministry of Science, Technology and Higher Education and is partially supported by FCT, the Portuguese Foundation for Science and Technology.

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PAPER V

Pedrazzani, C., et al., Evidence of tumor microsatellite instability in gastric cancer with familial aggregation. *Familial Cancer* 8:215-220; 2009.

Evidence of tumor microsatellite instability in gastric cancer with familial aggregation

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Published online: 17 January 2009
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Abstract About 90% of gastric cancer (GC) cases appear in a sporadic setting. Nonetheless, in high incidence areas high familial aggregation rates have been recently described. Microsatellite instability (MSI) is thought to be an important molecular phenotype both in sporadic GC and in tumors of the HNPCC spectrum. The aim of this study was to assess the frequency of MSI in GC with familial aggregation. Five quasimonomorphic mononucleotide repeats (BAT-26, BAT-25, NR-24, NR-21 and NR-27) were analyzed in 250 GC patients. Seventy-five patients (30%) had at least one first-degree family member affected by GC and 63 patients (25.2%) showed MSI. The frequency of MSI was significantly higher in patients with a positive family history of GC (38.7%) compared to patients with other tumor types within the family (15.7%) or with a negative oncological familial history (21.9%, $P = 0.004$). Within cases with a positive familial oncological history,

the MSI frequency in families with GC only was similar to the one observed in families with GC and colon cancer ($P = 0.96$). Nonetheless, in families with GC and lung cancer, the frequency of MSI was significantly lower (5.6%, $P = 0.007$). MSI occurs in GCs with familial aggregation. Similar MSI rates have been observed in GC patients with other family members affected by GC or colon cancer. The same does not occur in families with other members affected by lung cancer. Our data seem to suggest that familial aggregation for either GC alone or gastric and colon cancer share common etiological factors in contrast to families with gastric and lung cancers.

Keywords Gastric cancer · Family history · Microsatellite instability

Introduction

Most gastric cancer (GC) cases are known to present in a sporadic form, whereas familial clustering is observed only in a small proportion of cases (10%) [1–3]. Recently, familial aggregation of GC has been reported to occur at a rate of 33–46% in certain high incidence areas of GC in Italy and Japan [4, 5].

It has been suggested that both genetic and environmental factors represent essential components of GC tumorigenesis in cases with familial aggregation. Factors such as dietary habits and *H. pylori* infection have been demonstrated to be involved in the multifactorial process of gastric carcinogenesis and they may partially explain familial clustering [6, 7]. Recently, it has been demonstrated that diffuse GC can develop in a hereditary form named hereditary diffuse gastric cancer (HDGC) caused by germ line mutations of *CDH1* gene [8–10]. Furthermore, genetic predisposition plays a

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key role in cancer hereditary syndromes where GC is part of the tumor spectrum. GC occurs in family members with Li-Fraumeni syndrome [11] and hereditary non-polyposis colorectal cancer (HNPCC) [12].

Microsatellite instability (MSI) is the hallmark of HNPCC tumors and it also occurs in about 15% of sporadic carcinomas of the HNPCC spectrum [13–15]. In stomach cancer, MSI occurs in 15–30% of cases and it tends to occur preferentially in intestinal tumors of the antrum of elderly patients with a relatively good prognosis [16–19]. Data concerning MSI and familial aggregation of GC are scarce due to the low number of families studied to date [20–23].

In the present study, we analyzed a large cohort of GC patients ($n = 250$) using a panel of five quasimonomorphic mononucleotide repeats (BAT-26, BAT-25, NR-24, NR-21 and NR-27) [24] in order to assess the frequency of MSI phenotype. Moreover, the familial history of cancer was analyzed and association studies were performed.

Materials and methods

Patients under study

The study enrolled 250 patients affected by primary GC and operated on at the Division of General Surgery and Surgical Oncology, University of Siena, Italy. The cohort under study considered 193 patients from a previously described group of 238 patients with known oncological familial history [4], whose frozen samples of normal mucosa and tumor tissue were available in our biological material bank. In addition, we prospectively included 57 new cases with known familial history.

Oncological familial history

Oncological familial history was collected as previously described in detail [4]. In summary, the patient or his/her closest relative were asked, in a counseling session, to report the total number of first- and second-degree relatives in the patient's family, their age of onset, the living status and whether anyone in the family had been diagnosed with cancer. All types of tumors were considered, including hematological malignancies. According to the purpose of this study only first-degree family history was taken into account.

Samples and DNA extraction

Tumoral mucosa and corresponding normal mucosa of the stomach were flash-frozen in liquid nitrogen. Tumoral and

constitutional DNA were extracted after histopathological confirmation, to exclude the possibility that MSI status reflects the mixture of neoplastic and stomal cells, microdissection was performed in order to enrich the percentage of tumor cells in the sample.

Pentaplex PCR and microsatellite analysis

Microsatellite analysis was evaluated using five quasimonomorphic mononucleotide repeats named BAT-26, BAT-25, NR-24, NR-21 and NR27 as previously described [19]. The 5' anti-sense primer was labeled with a fluorescent dye using FAM for BAT-26 and NR-21, NED for BAT-25 and NR-27 and VIC for NR-24 (ABI PRISM® Primer Pairs Applied Biosystems, Milan, Italy).

The five mononucleotide repeat were co-amplified in one multiplex PCR (pentaplex PCR) using the protocol for amplification of microsatellite loci (Quiagen® Multiplex PCR, Milan, Italy). The pentaplex PCR was performed on tumoral and matched constitutional DNA of the same GC patient. The allelic profile of these five mononucleotides was detected on an automated DNA sequencer ABI PRISM 3100 Genetic Analyser (Applied Biosystems, Milan, Italy) according to the protocol of the manufacturer.

Scoring of microsatellite instability

According to the definition of the National Cancer Institute Workshop on Microsatellite Instability for Cancer Detection and Familial Predisposition [15], we considered a MSI tumor whenever two or more markers showed instability on five loci considered. Tumors with instability at one marker and tumors without instability were pooled together and defined as MSS [19]. DNA samples showing an abnormal allelic shift were confirmed in a second PCR and GeneScan run.

Statistical analysis

Analyses were performed using the Statistical Product and Service Solutions, SPSS 14.0 for Windows, 2006, SPSS Inc., Chicago, IL, USA. Statistical associations between the presence of family history of GC and clinico-pathological characteristics as well as between family history of GC and MSI status (MSI vs. MSS) were assessed by chi-square test for categorical variables and Student's *t*-test or ANOVA test for continuous variables. The association between family history of GC and MSI was also assessed separately for intestinal and non-intestinal Lauren histotypes. A *P* value less than 0.05 was considered significant.

Results

Family history of cancer

Among the 250 patients affected by GC, 145 (58%) had at least one first-degree relative affected by cancer of any type. A negative oncological family history was reported by 105 patients (42%). Of the 145 patients with a history of neoplasia in their family, 74 (51%) had only one member affected by cancer, 41 (28.3%) had two other members and 30 (20.7%) had three or more other relatives diagnosed with cancer.

Family history of gastric cancer

Within the 145 patients with a history of neoplasia in their family, 75 (51.7%) patients showed at least another relative affected by GC. From these 75 families with GC, 59 families (78.7%) had only another member affected, 12 families (16%) had 2 other members with GC and 4 families (5.3%) had more than 3 members with GC. Familial aggregation of GC was positively associated to an older age of patients ($P = 0.033$) and showed a trend to a higher rate of intestinal histotype tumors ($P = 0.051$) (Table 1).

Positive family history of cancer other than gastric

Among the 145 patients with aggregation of cancer, 21 (14.5%) had a positive family history of colon cancer (two relatives in two cases), whilst 18 (12.4%) had a history of lung cancer (two relatives in two cases; three relatives in one case). In 31 families, GC patients reported various types of cancer in their family, namely breast, liver, pancreas, prostate cancer or other non-epithelial tumors.

MSI status and positive family history of gastric cancer

Among the 250 GC patients, MSI was identified in 63 GCs (25.2%). Table 2 describes the status of the five analyzed loci (BAT-26, BAT-25, NR-24, NR-21 and NR-27) according to the family history of GC. The frequency of MSI was significantly higher in patients with a positive family history of GC (29/75; 38.7%) in comparison to families with other types of tumors (11/70; 15.7%) or with a negative oncological familial history (23/105; 21.9%) ($P = 0.004$). Out of the 75 patients with a history of GC, cases with another member affected showed a higher frequency of MSI (24/59; 40.7%) compared to patients with two or more members (5/16; 31.3%).

Table 1 Clinico-pathological characteristics and MSI status according to the family history of GC

Variable	Positive family history of GC (75 cases)		Negative family history of GC (175 cases)		P
Mean age (SD)	70.5 (9.7)		67.3 (11.3)		0.033
Gender					0.678
Male	42 (56)		103 (58.9)		
Female	33 (44)		72 (41.1)		
Tumor location					0.069
Antrum	32 (42.7)	32 (42.7)	94 (53.7)	94 (53.7)	0.129
Cardia	8 (10.7)	43 (57.3)	26 (14.9)	81 (46.3)	
Fundus/ Corpum	35 (46.7)		55 (31.4)		
Lauren classification					0.051
Intestinal	55 (73.3)		108 (61.7)		
Non intestinal	20 (26.7)		67 (38.3)		
Other tumors in family					0.014
Yes	42 (56)		70 (40)		
No	33 (44)		105 (60)		
Microsatellite instability					0.001
MSS	46 (61.3)		141 (80.6)		
MSI	29 (38.7)		34 (19.4)		

Numbers in parentheses are percentages

Table 2 Status of the five quasimonomorphic mononucleotide repeats (BAT-26, BAT-25, NR-24, NR-21 and NR-27) according to the family history of GC

Numbers in parentheses are percentages

Repeat	Positive family history of GC (75 cases)		Negative family history of GC (175 cases)	
	Stable	Instable	Stable	Instable
BAT-26	49 (65.3)	26 (34.7)	140 (80)	35 (20)
BAT-25	36 (48)	39 (52)	119 (68)	56 (32)
NR-24	52 (69.3)	23 (30.7)	152 (86.9)	23 (13.1)
NR-21	48 (64)	27 (36)	144 (82.3)	31 (17.7)
NR-27	51 (68)	24 (32)	142 (81.1)	33 (18.9)

MSI status and lauren histotype

Considering intestinal type, MSI was found in 23 (41.8%) cases with familial aggregation of GC and in 28 (25.9%) without it ($P = 0.049$). In non-intestinal type, MSI was demonstrated in 6 (30%) cases with GC family history and in 6 (9%) without it ($P = 0.027$).

MSI status and positive family history of colon and lung cancer

In GC patients with positive family history of colon cancer, MSI was observed in 8 out of 21 cases (38.1%). Within this type of families, the frequency of MSI is similar to that observed in patients with familial aggregation of GC ($P = 0.96$). Nonetheless, in patients with positive family history of lung cancer, the frequency of MSI is significantly lower (1/18; 5.6%) in comparison to patients with familial aggregation of GC ($P = 0.007$).

Discussion

GC is thought to present mostly as a sporadic tumor. Recently, very high familial aggregation rates (33–46%) have been described in high incidence areas, such as Japan and Tuscany, a region of central Italy [4, 5]. Familial clustering represents a biological and clinical burden because of the unknown etiological factors and underlying molecular mechanisms, as well as uncertain practical management. In a small percentage of cases (1–3%) familial aggregation of GC is due to genetic inherited factors mostly represented by E-cadherin germ line mutations (HDGC) [8, 10].

Germ line defects in mismatch repair genes (MMR), such as *hMSH2*, *hMLH1* and *MSH6*, have been described in HNPCC. Tumors with MMR defects show an increased rate of instability that can lead to the inactivation of specific target genes [15] which play a role in MSI tumorigenesis. Very few cases of sporadic GC show mutations of MMR genes [25, 26], whilst MSI phenotype is observed in 15–30% of the cases. In our study no HNPCC cases were included in the study and from the cases herein

analyzed only 14 families fulfilled the HDGC clinical criteria and were reported elsewhere [4].

A correlation between MSI and familial aggregation of GC has been reported [7, 22, 23, 27]. Keller et al. reported MSI in 46% of patients with positive family history of GC in first- or second-degree relatives [25]. In our study, MSI status has been analyzed in a large population of GC patients in which a complete oncological familial history was carefully collected during a counseling session.

Consistent with data published to date, MSI has been demonstrated in one out of 4 GC patients. Within cases with a positive family history of GC, we verified that cases with two members affected showed a higher frequency of MSI (24/59; 40.7%) in comparison to families with three or more members affected (5/16; 31.3%). We can speculate that within the former group, exposure of similar environmental carcinogens induces genomic instability. The latter group includes families that fulfill the criteria for HDGC and families harboring germline defects in still unidentified genes.

Considering MSI status and family history of GC, the frequency of MSI was significantly higher in patients with familial clustering of stomach cancer in comparison to GC cases harboring other types of cancer within the family or cases with a negative oncological familial history (38.7 vs. 21.9 vs. 19.4%, $P = 0.004$). It is interesting to note that the frequency of MSI in patients with other GC cases in their family was similar to GC cases with family members affected by colon cancer (38.7 vs. 38.1%, $P = 0.96$). These results suggest that both types of familial clusters are likely to share common molecular mechanisms inducing MSI phenotype. Probably, environmental factors may play a role in generating MMR deficiency leading to MSI phenotype. Interestingly, Palli et al. showed in another study performed in the same geographical region an association between MSI, positive family history of GC and high consumption of red meat and nitrates, suggesting that environmental factors, such as nutritional habits may play a key role in inducing genomic instability and an increased risk for GC with familial clustering [7]. General environmental exposures, such as diet, were considered as potential generators of increased microsatellite alterations in tumors with an increased tolerance to DNA damage

associated with reduced MMR activity [28]. It is tempting to speculate that familial groups with aggregation of stomach or colon cancer in our study were exposed to the same environmental factors. Such factors, as opposed to highly penetrant genetic defects, need a long time of exposure before tumor develops which may explain the higher age of cancer onset of MSI patients compared to MSS ones [mean (SD): 72.8 (10.7) and 66.7 (10.6) years, respectively; $P < 0.001$, data not shown].

Our results also evidenced that GC patients with positive family history of GC have a considerably higher frequency of MSI in comparison to GC patients with positive family history of lung cancer (38.7 vs. 5.6%, $P = 0.007$). This finding suggests that the underlying molecular mechanisms and the etiological factors responsible for these two familial clusters are likely to be distinct. Further epidemiological studies are needed to identify putative environmental factors associated to families with aggregation of GC and lung cancer.

In conclusion, our data confirm that MSI occurs in GC patients with familial aggregation. This association is likely to be related to the common exposure of environmental factors. Similar frequencies of MSI have been observed in GC patients with other family members affected by gastric or colon cancer. The same does not occur in families with other members affected by lung cancer. Our data seem to suggest that familial aggregation for either GC alone or gastric and colon cancers share common etiological factors in contrast to families with gastric and lung cancers.

Acknowledgments This work was supported by PRIN 2005 “Fattori genetici ed ereditari nel carcinoma gastrico” Grant number: 2005064071_004, by Istituto Toscano Tumori (Grant ITT-2007 “Gene expression profile and therapeutic implication in gastric cancer. From the clinical overview to the translational research”) and Sixth Framework Programme from EU-FP6 (LSHC-CT-2005-018754)

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PAPER VI

Corso, G., *et al.*, **Correlation of microsatellite instability at multiple loci with long-term survival in advanced gastric carcinoma.** Archives of Surgery 144:722-727; 2009.

Correlation of Microsatellite Instability at Multiple Loci With Long-term Survival in Advanced Gastric Carcinoma

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Hypothesis: Microsatellite instability (MSI) correlates with clinicopathologic characteristics and long-term prognosis in patients having gastric carcinoma.

Design: Analysis of prospectively collected data and biologic material.

Setting: Tertiary University Hospital, Policlinico "Le Scotte," Siena, Italy.

Patients: Two hundred fifty patients with gastric carcinoma.

Main Outcome Measures: Five mononucleotide repeats (BAT-26, BAT-25, NR-24, NR-21, and NR-27) were analyzed in these patients.

Results: An MSI phenotype was identified in 63 patients (25.2%) and correlated with specific clinicopathologic characteristics. Favorable prognosis was confirmed for patients with an MSI phenotype in univariate ($P < .001$) and multivariate ($P = .05$) analyses. Signifi-

cant differences in clinicopathologic characteristics and long-term prognoses were observed among patients with microsatellite-stable tumors, tumors having instability at 2 to 4 markers, and tumors having instability at all 5 markers (MSI/5). The MSI/5 phenotype was associated with older age ($P < .001$), female sex ($P = .001$), antral tumor location ($P = .04$), intestinal histotype ($P = .003$), and less infiltration of the serosa ($P = .006$); lymph node involvement was rare ($P < .001$) and was limited to few (median, 3) metastatic lymph nodes ($P = .001$). Long-term survival of patients with the MSI/5 phenotype is favorable and was confirmed in multivariate analysis (relative risk vs patients with stable tumors, 0.32; 95% confidence interval, 0.16-0.63; $P = .002$).

Conclusions: Compared with stable tumors, MSI tumors have distinct clinicopathologic features and are associated with a better prognosis. Patients with the MSI/5 phenotype have a very good prognosis.

Arch Surg. 2009;144(8):722-727

DESPITE A DECREASING incidence during the last few decades, gastric carcinoma (GC) remains the fourth most common cancer worldwide and the second leading cause of cancer-related death.¹ The overall mortality has decreased during the same period, although the long-term prognosis remains poor, with 5-year survival rates that rarely exceed 40%.²

See Invited Critique at end of article

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As with most other tumors, GC develops through the accumulation of genetic and epigenetic alterations affecting oncogenes and tumor suppressor genes. It is widely accepted that microsatellite instability (MSI) and chromosome instability are 2 major genomic instability pathways in-

involved in GC pathogenesis.³ Microsatellite instability has been described as a frequent genetic alteration in colon cancer⁴ and in several other cancer types.^{5,6} Although microsatellite alterations have been widely investigated in familial and sporadic colon cancer,⁷ limited and inconclusive data are available for stomach cancer.

In the present study, we analyzed a large cohort of patients with GC using a panel of 5 quasimonomorphic mononucleotide repeats (BAT-26, BAT-25, NR-24, NR-21, and NR-27).⁸ We assessed clinicopathologic characteristics and long-term prognoses of patients having MSI tumors.

METHODS

STUDY POPULATION

The study analyzed 250 patients with primary GC and operated on at the Division of Surgical Oncology, Tertiary University Hospi-

tal, Policlinico "Le Scotte," University of Siena, Siena, Italy, between January 1, 1990, and December 31, 2004. All patients underwent resection, including 183 patients with potentially curative (R0) tumors and 35 patients with microscopic (R1) and 32 patients with macroscopic (R2) residual tumors; 20 patients (17 with R1 and R2 residual tumors) had metastatic disease at the time of operation. Clinicopathologic and follow-up data were prospectively collected for the whole cohort. The median follow-up period for surviving patients was 92.1 months (range, 36.3-260.9 months).

Informed consent was obtained from all patients. The study was approved by the appropriate local ethics committees.

SURGERY

The objective of surgery was complete resection of the tumor, although gastrectomy was performed even in patients with untreatable disease when required for symptom palliation. Gastric resection was performed based on the extent of tumor in the stomach. Distal subtotal gastrectomy was preferred for tumors located in the lower and middle thirds of the stomach provided that the proximal resection margin remained at least 5 cm from the tumor edge; otherwise, total gastrectomy was performed.⁹ Extended (D2) and superextended (D3) lymphadenectomy (in 157 patients [62.8%]) was performed in most R0 resections and has been routinely used since 1995, except for patients deemed unfit for this procedure.¹⁰ None of the patients received preoperative chemotherapy or chemoradiotherapy.

HISTOPATHOLOGIC CLASSIFICATION AND STAGING

Tumor size was calculated by measuring the largest diameter of the neoplasm. The most recent version of the TNM classification by the American Joint Committee on Cancer¹¹ and the International Union Against Cancer was used for histopathologic staging. Level and number of metastatic lymph nodes were also recorded for each patient. Histologic classification followed the criteria by Lauren¹²; mixed-type tumors were considered together with diffuse (nonintestinal) type. Lymphatic and vascular invasion was assessed for 176 and 184 patients, respectively.

SAMPLES AND DNA EXTRACTION

Frozen samples of normal mucosa and tumor tissue were available for each patient in our biologic material bank. Tumoral mucosa and corresponding normal mucosa of the stomach were flash-frozen in liquid nitrogen. Tumoral and constitutional DNA were extracted after histopathologic confirmation using a commercially available DNA purification kit (Puregene; Gentra Systems, Inc, Minneapolis, Minnesota) and following the manufacturer's instructions.

PENTAPLEX POLYMERASE CHAIN REACTION AND MICROSATELLITE ANALYSIS

Microsatellite analysis was evaluated using 5 quasimonomorphic mononucleotide repeats, namely, BAT-26, BAT-25, NR-24, NR-21, and NR-27.⁸ The 5' antisense primer was labeled with a fluorescent dye using FAM for BAT-26 and NR-21, NED for BAT-25 and NR-27, and VIC for NR-24 (ABI PRISM Primer Pairs; Applied Biosystems, Foster City, California).

The 5 mononucleotide repeats were coamplified in one multiplex (pentaplex) polymerase chain reaction (PCR) using the protocol for amplification of microsatellite loci (Multiplex PCR; Qiagen, Studio City, California). The pentaplex PCR was performed on tumoral and matched constitutional DNA of the same

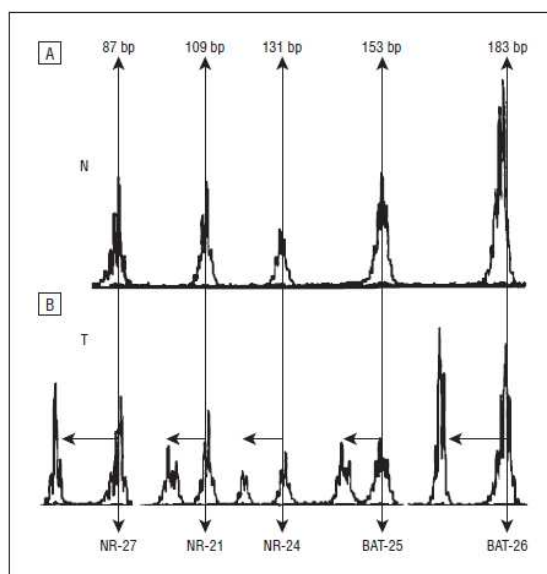


Figure 1. Typical allelic profiles of microsatellite stability in constitutional DNA samples (A) and microsatellite instability in tumoral samples (B). bp Indicates base pair; N, lymph nodes; and T, tumor size.

patient with GC. The allelic profiles of these 5 mononucleotides were detected on an automated DNA sequencer (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems) according to the manufacturer's protocol (**Figure 1**).

SCORING OF MSI

According to the definition of the National Cancer Institute⁵ workshop on MSI for cancer detection and familial predisposition, we considered a tumor as having MSI whenever 2 or more markers showed instability on 5 loci. The clinicopathologic characteristics and survival rates associated with tumors having low instability (one locus involved) are similar to those of stable tumors^{5,13} and were considered together (microsatellite-stable [MSS] phenotype). DNA samples showing an abnormal allelic shift were confirmed by a second PCR and gene scan.

STATISTICAL ANALYSIS

Analyses were performed using commercially available statistical software (SPSS 14.0 for Windows; SPSS Inc, Chicago, Illinois). Statistical associations between clinicopathologic characteristics and MSI status were assessed by χ^2 test for categorical variables and by *t* test or analysis of variance for continuous variables. Survival curves were estimated using the Kaplan-Meier method and were compared using log-rank test. Multivariate analysis was performed using a Cox proportional hazards regression model by considering the following risk factors: sex, age (older vs the median or younger), tumor location (other vs antrum), Lauren¹² histotype (nonintestinal vs intestinal), depth of tumor invasion (pT2, pT3, or pT4 vs pT1), lymph node involvement (pN1, pN2, or pN3 vs pN0), presence of systemic metastasis (M1 vs M0), and R category (R1 or R2 vs R0). Postoperative mortality was assessed, with deaths unrelated to tumor recurrence considered censored observations at the time of death. Because differences in clinicopathologic features and survival rates were observed within the MSI phenotype for tumors with instability at 2 to 4 markers (MSI/≤4) vs tumors with instability at all 5 markers (MSI/5), an analysis was performed

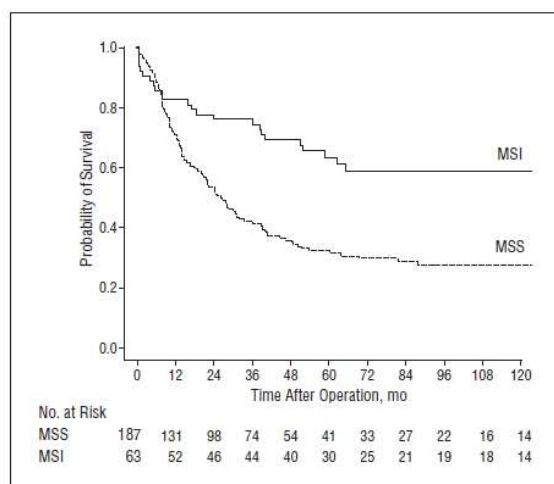


Figure 2. Kaplan-Meier survival estimates according to microsatellite instability (MSI) status in 250 patients who underwent gastrectomy. The median (95% confidence interval) survival was 26.7 (20.8-32.5) months for patients with microsatellite-stable (MSS) tumors, and the median was beyond the observation period for patients with MSI tumors ($P < .001$).

to separately investigate MSS, MSI/<5, and MSI/5 tumors. $P < .05$ was considered statically significant.

RESULTS

MSI STATUS AND CLINICOPATHOLOGIC FACTORS

Among 250 patients with GC, MSI was identified in 63 GCs (25.2%). The MSI phenotype was significantly associated with older age (mean [SD], 72.8 [10.7] vs 66.7 [10.6] years; $P < .001$), and it was more frequent in women (55.6% vs 37.4%, $P = .02$) with intestinal histotype tumors (81% vs 59.9%, $P = .002$). The MSI phenotype was rarely associated with systemic metastases (1.6% vs 10.2%, $P = .03$) or with pT3 or pT4 tumors (38.1% vs 60.4%, $P = .002$). Compared with the MSS phenotype, pN+ lymph node involvement was less frequent in patients having the MSI phenotype (57.1% vs 75.9%, $P < .001$), with fewer metastatic lymph nodes (mean [SD], 3.6 [7.1] vs 9.4 [12.1]; $P = .001$). Conversely, no differences in tumor size (mean [SD], 58.8 [2.9] vs 57.8 [3.2] mm; $P = .82$) or percentage of R1 or R2 resections (19% vs 29.4%, $P = .14$) were observed.

The presence of lymphovascular invasion was also evaluated. Both vascular invasion (21.4% vs 53.5%) and lymphatic invasion (33.3% vs 69.3%) were rarely associated with MSI tumors compared with MSS tumors ($P < .001$).

MSI STATUS AND SURVIVAL

Kaplan-Meier survival estimates according to MSI phenotype are plotted in **Figure 2**. In patients with M0 and R0 disease, 5-year survival was 46% (median, 48.9 months; 95% confidence interval, 31.0-66.8 months) for patients with MSS tumors and 71% (the median was beyond the observation period) for patients with MSI tumors ($P = .002$).

Multivariate analysis of Cox proportional hazards regression models confirmed MSI as an independent predictor of

survival (relative risk vs patients with MSS, 0.62; 95% confidence interval, 0.38-1.00; $P = .05$). The models controlled for age ($P = .74$), sex ($P = .09$), tumor location ($P = .70$), Lauren¹² histotype ($P = .10$), depth of tumor invasion ($P < .001$), lymph node involvement ($P < .001$), presence of systemic metastasis ($P = .005$), and R category ($P < .001$).

MSI AT ALL 5 MARKERS

Table 1 shows differences in clinicopathologic characteristics associated with the types of tumors. These include MSS, MSI/<5, and MSI/5 tumors.

The MSI/5 phenotype correlated with older age ($P < .001$), female sex ($P = .001$), antral tumor location ($P = .04$), and intestinal histotype ($P = .003$). Invasion of the serosa ($P = .006$) and lymph node metastasis ($P < .001$) were rarely observed. Among 22 patients having MSI/5 tumors with lymph node involvement, deposits were observed in few (median, 3; range, 1-8) metastatic lymph nodes ($P = .001$).

Lymphovascular MSI invasion was rare compared with that in MSS tumors ($P < .001$). Vascular invasion was observed in 33.3% of MSI/<5 tumors and in 14.8% of MSI/5 tumors ($P = .24$). Lymphatic invasion was equally frequent (33.3%) in MSI/<5 and MSI/5 tumors.

Figure 3 shows Kaplan-Meier curves for patients with MSS, MSI/<5, and MSI/5 tumors ($P < .001$). Survival rates for patients with M0 disease who underwent potentially curative (R0) resections are shown in **Figure 4** ($P = .004$). Limiting the analysis to patients who underwent noncurative (R1 or R2) resections, the median (95% confidence interval) survival was 9.0 months (5.6-12.4 months) for 55 patients with MSS tumors, 5.2 months (2.1-8.3 months) for 7 patients with MSI/<5 tumors, and 58.4 months (0.0-132.5 months) for 5 patients with MSI/5 tumors ($P < .009$).

Table 2 summarizes results of the multivariate analyses using Cox proportional hazards regression models controlling for other risk factors. The MSI/5 phenotype was confirmed to be an independent predictor of long-term survival (relative risk vs patients with the MSS phenotype, 0.32; 95% confidence interval, 0.16-0.63; $P = .002$).

COMMENT

Microsatellite instability is defined as the presence of replication errors resulting in insertions or deletions of bases within nucleotide repeats, known as microsatellite regions. Although a consensus panel for determination of MSI phenotype has been proposed and accepted for colorectal cancer,³ several markers with differing results have been used for determination of MSI in GC.¹⁴⁻¹⁹ Based on evaluation using several markers, the MSI phenotype generally characterizes 15% to 25% of patients with GC tumors, more frequently in older women.^{3,18} Most MSI tumors are of intestinal histotype located in the distal part of the stomach, with limited lymph node involvement. Our data regarding MSI phenotype are consistent with other findings reported to date.^{3,18}

Several observations of unexpectedly good prognoses in patients with advanced tumors and instability at all 5 markers led us to look at this subset of patients. The case of a 31-year-old woman who had undergone palliative sub-

Table 1. Clinicopathologic Characteristics of the Study Cohort According to Microsatellite Instability (MSI) Status

Characteristic	MSS (n=187)	MSI/<5 (n=22)	MSI/5 (n=41)	P Value ^a
Age, mean (SD), y	66.7 (10.6)	69.3 (9.0)	74.7 (11.0)	<.001 [.06]
Sex, No. (%)				
Male	117 (62.6)	15 (68.2)	13 (31.7)	.001 [.008]
Female	70 (37.4)	7 (31.8)	28 (68.3)	
Tumor location, No. (%)				
Antrum	89 (47.6)	9 (40.9)	28 (68.3)	.04 [.06]
Other	98 (52.4)	13 (59.1)	13 (31.7)	
Lauren ¹² histotype, No. (%)				
Intestinal	112 (59.9)	15 (68.2)	36 (87.8)	.003 [.09]
Nonintestinal	75 (40.1)	7 (31.8)	5 (12.2)	
Tumor size, mean (SD), mm	57.8 (3.2)	60.8 (3.2)	57.9 (2.8)	.92 [.72]
Depth of tumor invasion, No. (%)				
pT1	14 (7.5)	1 (4.5)	2 (4.9)	.006 [.36]
pT2	60 (32.1)	10 (45.5)	26 (63.4)	
pT3 or pT4	113 (60.4)	11 (50.0)	13 (31.7)	
Lymph node involvement, No. (%)				
pN0	45 (24.1)	8 (36.4)	19 (46.3)	<.001 [.003]
pN1	60 (32.1)	6 (27.3)	21 (51.2)	
pN2	48 (25.7)	6 (27.3)	1 (2.4)	
pN3	34 (18.2)	2 (9.1)	0	
No., mean (SD)	9.4 (12.1)	6.9 (10.9)	1.7 (2.1)	.001 [.005]
Presence of metastasis, No. (%)				
M0	168 (89.8)	21 (95.5)	41 (100.0)	.08 [.35]
M1	19 (10.2)	1 (4.5)	0	
R category, No. (%)				
R0	132 (70.6)	15 (68.2)	36 (87.8)	.07 [.09]
R1 or R2	55 (29.4)	7 (31.8)	5 (12.2)	

Abbreviations: MSS, microsatellite-stable tumors; MSI/<5, MSI tumors having instability at 2 to 4 markers; MSI/5, MSI tumors having instability at all 5 markers.
^aBrackets indicate *P* value differences between MSI/<5 and MSI/5 tumors.

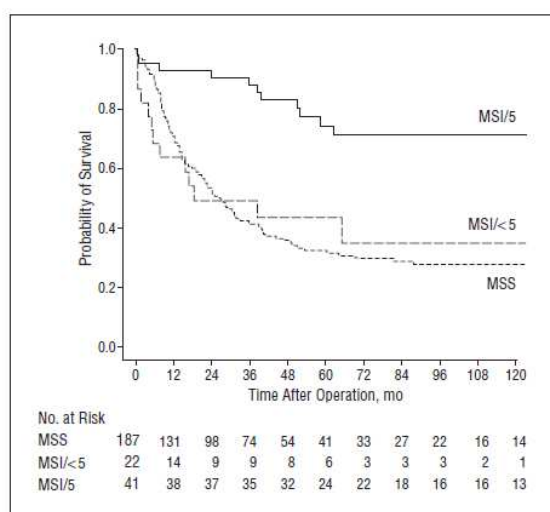


Figure 3. Kaplan-Meier survival estimates according to microsatellite instability (MSI) in 250 patients who underwent gastrectomy. The median (95% confidence interval) survival was 26.7 (20.8-32.5) months for patients with microsatellite-stable (MSS) tumors and 18.5 (0.0-50.1) months for patients with MSI tumors having instability at 2 to 4 markers (MSI/<5), and the median was beyond the observation period for patients with MSI tumors having instability at all 5 markers (MSI/5) (*P* < .001).

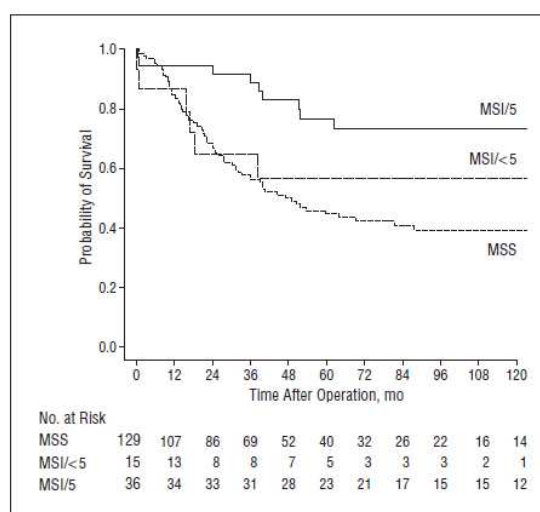


Figure 4. Kaplan-Meier survival estimates according to the number of microsatellite instability (MSI) markers in 180 patients with M0 disease who underwent potentially curative (R0) gastrectomy. The median (95% confidence interval) survival was 48.9 (31.0-86.8) months for patients with microsatellite-stable (MSS) tumors, and the median was beyond the observation period for patients with MSI tumors having instability at 2 to 4 markers (MSI/<5) or with MSI tumors having instability at all 5 markers (MSI/5) (*P* = .004).

total gastrectomy with macroscopic residual (R2) tumor 63 months previously for a nonintestinal pT4N2 tumor is still alive. The favorable survival in patients with the MSI/5

phenotype was also evident among patients who underwent palliative resection (median survival, 58.4 months; 95% confidence interval, 0.0-132.5 months).

Table 2. Relative Risks of Death From Gastric Carcinoma as a Function of Microsatellite Instability (MSI)^a

Variable	Relative Risk (95% Confidence Interval)	P Value
Age, y		.76
> vs ≤ Median	1.06 (0.74-1.52)	
Sex		.31
Male vs female	1.22 (0.83-1.80)	
Tumor location		.36
Antrum vs other	0.85 (0.61-1.20)	
Lauren ¹² histotype		.04
Intestinal vs nonintestinal	0.66 (0.45-0.98)	
Depth of tumor invasion		<.001
pT2 vs pT1	5.16 (0.69-38.79)	
pT3 or pT4 vs pT1	12.14 (1.61-91.23)	
Lymph node involvement		<.001
pN1 vs pN0	2.40 (1.36-4.24)	
pN2 vs pN0	3.68 (1.98-6.82)	
pN3 vs pN0	3.67 (1.89-7.10)	
Presence of metastasis		.008
M1 vs M0	2.13 (1.22-3.72)	
R category		<.001
R1 or R2 vs R0	2.37 (1.61-3.47)	
MSI status		.002
MSI/<5 vs MSS	1.43 (0.79-2.59)	
MSI/5 vs MSS	0.32 (0.16-0.63)	

Abbreviations: MSS, microsatellite-stable tumors; MSI/<5, MSI tumors having instability at 2 to 4 markers; MSI/5, MSI tumors having instability at all 5 markers.

^aRelative risks and P values are derived from Cox proportional hazards regression analyses controlling for all other variables.

Older age, female sex, antral tumor location, intestinal histotype, less invasion of the serosa, and involvement of fewer lymph nodes significantly correlated with MSI/5 phenotype. The number of involved lymph nodes was particularly low in patients having tumors with instability at all 5 markers. Among 22 patients with MSI/5 pN+ disease, 21 had fewer than 6 positive lymph nodes, while the 31-year-old patient already mentioned had 8 positive lymph nodes.

Significantly lower rates of lymphovascular invasion were consistently observed for MSI/5 and MSI/<5 tumors compared with MSS tumors. These data should correlate with less tumor aggression, lower disease stage, and older age in patients with MSI/5 tumors. Some of these characteristics have been reported in MSI tumors,¹³ but they seem to be particularly evident in MSI/5 tumors.

The MSI/5 phenotype delineates a subset of tumors with a similar growth pattern but with less propensity to invade serosal layers and spread systemically or via lymph nodes. Considering the locoregional growth of this class of tumors, one could argue for an aggressive surgical approach even in very advanced cases.

Survival analysis confirms high survival rates for patients with MSI/5 tumors (Figures 3 and 4). Survival of patients with the MSI/5 phenotype was significantly better than that of patients with the MSI/<5 phenotype, and this difference was confirmed in multivariate analysis. These novel findings identify a particular subset of MSI tumors with less biologic aggression and with a favorable prognosis. Other findings similarly correlate tumor characteristics and differentiation with tumor invasion and genetic changes.²⁰

In conclusion, our data confirm previous evidence that tumors with MSI demonstrate distinct clinicopathologic features and better prognosis compared with patients having stable tumors. A subset of tumors was observed with instability at 5 quasimonomorphic mononucleotide repeats (BAT-26, BAT-25, NR-24, NR-21, and NR-27). These tumors have a low predilection to spread systemically or to involve lymph nodes, and they are associated with favorable long-term prognosis even in patients with advanced disease. Further studies among more patients are necessary to confirm our results and to render them reproducible and useful in clinical practice.

Accepted for Publication: May 28, 2008.

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Author Contributions: All authors take responsibility for the integrity of the data and the accuracy of the data analysis. *Study concept and design:* Corso, Pedrazzani, Pinto, and Roviello. *Acquisition of data:* Marrelli. *Analysis and interpretation of data:* Corso, Pedrazzani, Marrelli, and Pascale. *Drafting of the manuscript:* Corso, Pedrazzani, Marrelli, and Pascale. *Critical revision of the manuscript for important intellectual content:* Pedrazzani, Pinto, and Roviello. *Statistical analysis:* Pedrazzani and Marrelli. *Obtained funding:* Pinto and Roviello. *Administrative, technical, and material support:* Corso, Marrelli, and Pascale. *Study supervision:* Roviello. *Financial Disclosure:* None reported.

Funding/Support: This study was supported by grant 2005064071_004PRIN (Progetti di Ricerca di Interesse Nazionale) 2005, "Fattori genetici ed ereditari nel carcinoma gastrico," from Ministero Università e Ricerca. Additional Contributions: Lorenzo Garosi provided valuable help in data collection, material storage, and technical support.

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INVITED CRITIQUE

The future in staging of cancer is molecular. Molecular markers will ultimately replace pathologic findings in our staging of gastrointestinal cancer. Corso et al from Italy show that genomic MSI in gastric adenocarcinoma is useful in determining the prognosis of patients. DNA from tumor and from adjacent normal tissue was amplified by pentaplex PCR, and DNA fragments were sequenced, which determined the presence of microsatellites.

DNA microsatellites are small (1-3 bp) repetitive DNA sequences, and instability results from the presence of these, which are absent from normal parental DNA.¹ They are the result of mismatch repair gene mutations and have been implicated in hereditary nonpolyposis colorectal cancer²; the present study confirms the utility of MSIs in gastric cancer as well. The authors showed that patients having tumors with all 5 MSI markers did better than those who did not.

Articles describing the use of molecular markers are being published frequently, and the names, functions, and utility of the markers are confusing. The first genetic investigations focused on mutations or deletions in host genomic DNA. A classic study by Shibata et al³ in 1996 demonstrated that patients with stage II colon cancer who harbored the deleted in colorectal carcinoma (*DCC*) gene mutation had prognoses similar to those of patients with stage III *DCC*-positive disease. In the *Archives*, I recently commented about the utility of cyclooxygenase 2 gene expression as a molecular predictor of the response of colon cancer to chemoradiotherapy.⁴

Newer molecular markers focus not on DNA mutations but on molecular changes in genetic expression. Epigenetics includes the study of DNA methylation and of micro RNA inhibition of genetic expression.⁵ The former represents inhibition of tumor suppressor gene expression by changes in DNA methylation or enzymatic modification of histone proteins that lead to inhibited DNA repair, replication, and gene expression. The latter comprises inhibition of messenger RNA expression of tumor suppressor genes by small 22-bp endogenous anti-

sense micro RNAs that destroy the message and inhibit expression of tumor suppressor genes. Each molecular marker is complicated to understand and requires extensive study to determine its utility.

Corso et al studied MSI in genomic DNA of gastric cancers and found that patients having tumors with 5 MSI markers have better survival than patients having tumors without MSI markers. This is potentially an important observation because it was true for lymph node-positive gastric cancers with MSI. It is critical to determine whether this is clinically important. One simple test would have been to determine whether survival by stage changes is based on MSI, as assessed by Shibata et al,³ but no TNM staging was presented in the current article. Similarly, Corso et al could have built on their finding that MSI was more frequent in older women with antral lesions. For example, it could have been determined whether younger men with MSI have survival similar to that of older women without MSI.

Molecular markers of cancer are the wave of the future. We need to determine which marker will be best for each type of cancer, and studies like this are important steps.

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Financial Disclosure: None reported.

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PAPER VII

Corso, G., et al., Oncogenic mutations in gastric cancer with microsatellite instability. Eur J Cancer 47:443-451; 2011

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Oncogenic mutations in gastric cancer with microsatellite instability

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ARTICLE INFO

Article history:

Received 16 July 2010

Received in revised form 20 August 2010

Accepted 2 September 2010

Keywords:

Gastric cancer

MAPK

Oncogenic mutations

MSI

KRAS

MLK3

PI3K

EGFR

BRAF

ABSTRACT

Aim: Mitogen-activated protein kinase (MAPK) cascade and phosphatidylinositol 3-kinase (PI3K) survival pathways are frequently activated in the progression of gastrointestinal malignancies. In this study, we aimed to determine the frequency of gene mutations in members of these pathways – Epithelial Growth Factor Receptor (EGFR), KRAS, BRAF, PIK3CA and MLK3 in a series of 63 gastric carcinomas with high levels of microsatellite instability (MSI).

Methods: Gene mutation analysis was performed by PCR amplification followed by direct sequencing. In selected tumour cases, EGFR expression was evaluated by immunohistochemistry. Association studies between molecular data and clinicopathologic characteristics were performed.

Results: Mutations in EGFR (3'-untranslated region [UTR] polyA repeat), KRAS, PIK3CA and MLK3 genes occurred in 30 (47.6%), 11 (17.5%), 9 (14.3%) and 2 (3.2%) of the MSI gastric cancer (GC) cases, respectively. No BRAF or EGFR hotspot mutations were identified. Overall, mutations in at least one of these genes were found in 55.6% (35/63) of gastric carcinomas. From those mutant cases 40.0% (14/35) of them had concomitant gene mutations, always involving EGFR polyA deletions. Interestingly, we observed significant associations between oncogenic mutations and female gender ($p = 0.046$) old age of diagnosis ($p = 0.001$) and intestinal subtype ($p = 0.043$).

Conclusion: Our results show that MSI gastric carcinoma frequently shows activation of EGFR-MAPK and PI3K pathways. Within all alterations found, deletions of the A13 repeats of EGFR were common, suggesting this molecular event as an important biomarker for stratification of GC patients for treatment with EGFR inhibitors.

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doi:10.1016/j.ejca.2010.09.008

1. Introduction

EGFR is a transmembrane protein that homo- or heterodimerizes with other EGFR family members at the cell membrane.¹ Receptor dimerisation causes activation of the intrinsic cytoplasmic kinase domain, resulting in the phosphorylation of several tyrosine residues.² The active EGFR stimulates the MAPK cascade and PI3K survival pathways.¹ In some neoplasias, such as in non-small cell lung cancer, it has been demonstrated that patients with tumours harbouring structural alterations on the EGFR kinase domain could benefit from the pharmacological treatment with EGFR inhibitors.³ However, it is well known that in lung and also colon cancer, the clinical response to EGFR inhibitors depends on the tumour genetic profile. Moreover, it has been clearly demonstrated that patients with metastatic colon cancer harbouring mutations in EGFR downstream molecules, namely in KRAS or BRAF genes, are resistant to EGFR inhibitors, specifically to the anti-EGFR monoclonal antibody cetuximab.^{4–7}

Recently, Yuan¹⁴ found a novel mechanism for EGFR activation occurring in colon carcinomas with MSI phenotype; mutations in an A13 repeat located at the 3' (UTR) of gene. Further, mutations in this region of EGFR were found to be associated with EGFR overexpression.¹⁴

In GC, and in particular in the MSI subset, data on EGFR alterations as well as mutations on its downstream targets, namely those belonging to the MAPK and PI3K pathways, are very limited. Some authors reported that EGFR is over-expressed in a maximum frequency of 38% of GC^{8–10} and very few cases were reported to harbour gene structural alterations like gene amplification or mutations.^{11–13}

In GC, the KRAS gene mutation frequency varies between 3 to 8% and whenever present, KRAS mutations normally cluster in the MSI subset (~30% of MSI cases).^{15–19} In contrast, others and we found that BRAF mutations rarely occur in this type of epithelial cancer.^{15,20–23} We have previously reported mutations in PIK3CA gene in MSI GC¹⁸ and recently, our group have also reported mutations in the MLK3 gene, which is a component of the multiprotein BRAF/RAF1 complex, in MSI gastric and colorectal tumours.^{24,25} MLK3 oncogenic mutations were found in 21% of the MSI gastrointestinal cases and were described to be functionally relevant.²⁶

In the present study, we aimed to: (1) determine the frequency of activating oncogenic gene mutations in the 3'-UTR A13 repeat of EGFR in mutation hotspots from EGFR, KRAS, BRAF and PIK3CA, as well as in the full coding region of MLK3, in a series of 63 MSI GC, and (2) to analyse the pattern of these oncogenic mutations to understand the role played by EGFR and its downstream targets, namely those belonging to the MAPK and PI3K pathways in GC progression. Mutations were screened in all cases and associations between the molecular data and the clinicopathologic features of the patients and tumours were also studied.

2. Patients and methods

2.1. Gastric cancer patients and genomic DNA extraction

To assess MSI frequency, 250 GC patients were analysed.^{27–29} In total, we selected a series of 63 MSI GC well characterized in

terms of clinicopathologic features and geographic area of origin. Microsatellite analysis was evaluated using five quasi-monomorphic mononucleotide repeats BAT-26, BAT-25, NR-24, NR-21 and NR27 cases were considered MSI whenever two or more markers showed instability on five loci considered.²⁸ The study population was stratified according to area of residence into Central Italy, representing a GC high-risk area, and Southern Italy, representing a GC low-risk area. Tumour and constitutional DNA were extracted from fresh frozen sample tissues using a standard protocol (Gentra Systems, Minneapolis, USA). Pathological examination allowed the selection of areas of neoplastic cells of more than 80%.

2.2. Somatic mutation analysis of EGFR, KRAS, BRAF, PIK3CA and MLK3 oncogenes

For the EGFR gene, direct sequencing of the kinase domain (exons 18, 19, 20 and 21) was performed, using a detailed protocol described by Moutinho and colleagues.¹³ Structural alterations on the A13 repeat within the 3'-untranslated region of EGFR (3'-UTR polyA repeat) gene were also searched, according with the protocol recently described by Yuan¹⁴ in MSI colon cancer. The 3'-UTR polyA repeat was evaluated in normal, as well as, in GC samples. Mutation analysis of KRAS codons 12 and 13 and BRAF V600E hotspot mutation were performed by PCR amplification and direct sequencing using the protocol used by Oliveira.³⁰ To search for somatic alterations of PIK3CA gene, exons 9 and 20 were sequenced according to the protocol described in detail by Velho.¹⁸ All exons and intron-exon boundaries of MLK3 gene were screened for mutations. Primer sequences and PCR conditions adopted were recently described.²⁶ Except for exon 9, a multiplex PCR approach was used to amplify MLK3 sequence using the QuantiTect Multiplex PCR Kit (Multiplex PCR, Qiagen, Studio City, CA) and following the manufacturer instructions. Purified PCR products were directly sequenced. All sequence alterations in EGFR, KRAS, BRAF, PIK3CA and MLK3 genes were validated with a second independent PCR.

2.3. EGFR immunohistochemistry

EGFR immunohistochemistry was evaluated on 3 µm sections from formalin-fixed, paraffin-embedded tissue in only two cases with A13 repeat deletion and in one wild-type sample due to the lack of good quality paraffin material for analysis. Epitope retrieval for EGFR was performed by proteolytic enzyme digestion (pepsin A, 4 g/l; Sigma-Aldrich, Germany) at 37 °C. After the antigen retrieval procedure, the slides were washed in a phosphate buffer solution (PBS), and submitted to blockage of the endogenous peroxidase activity by incubation of the slides in a 3% hydrogen peroxide (Panreac, Spain) in methanol (Sigma-Aldrich). The slides were further incubated with a blocking serum (LabVision Corporation kit) for 15 min and then incubated with the primary antibody anti-EGFR (Zymed, San Francisco, CA, USA, dilution: 1/100; Clone: 31G7) during 60 min. The secondary antibody was associated with HRP labelled polymer (DakoCytomation) and, after that, the slides were immediately revealed with DAB. Tissues were then counterstaining with Mayer's haematoxylin, dehydrated and coverslipped using a permanent mounting solution

(Zymed, San Francisco, CA, USA). Positive and negative controls were included in order to guarantee the reliability of the assay.

2.4. Statistical analysis

Analyses were performed using the Statistical Product and Service Solutions, SPSS 14.0 for Windows, 2006, SPSS Inc., Chicago, IL, USA. Statistical associations between the presence of GC oncogenic mutations and clinicopathologic characteristics was assessed by chi-square test for categorical variables and Student's t-test or ANOVA test for continuous variables. A *p* value lower than 0.05 was considered significant.

3. Results

Thirty-five of 63 (55.6%) MSI GC showed oncogenic mutations in at least one of the screened genes (EGFR, KRAS, BRAF,

PIK3CA and MLK3) (Table 1). Representative images of tumour specific oncogenic mutations are shown in Fig. 1. Table 2, summarizes the association studies performed between the presence of oncogenic mutations (independently of the number and type of genes mutated per case) and the clinicopathologic features of patients and tumours.

3.1. EGFR screening

We did not find pathogenic mutations in the hotspot regions of EGFR (exons 18, 19, 20 and 21). Heterozygous polymorphic variants of EGFR were detected in exon 20 (G>A; rs1050171) and in exon 18 (G>A; rs55959834) in 54 and in 2 samples, respectively. However, deletions at the A13 repeat localized into the 3' UTR of EGFR were found in 30/63 (47.6%) of MSI GC. Within the 30 mutated carcinomas, 15 (50%) showed a mononucleotide A deletion, 12 (40%) had a dinucleotide A deletion and, 3 (10%) a trinucleotide A deletion. All 63 normal

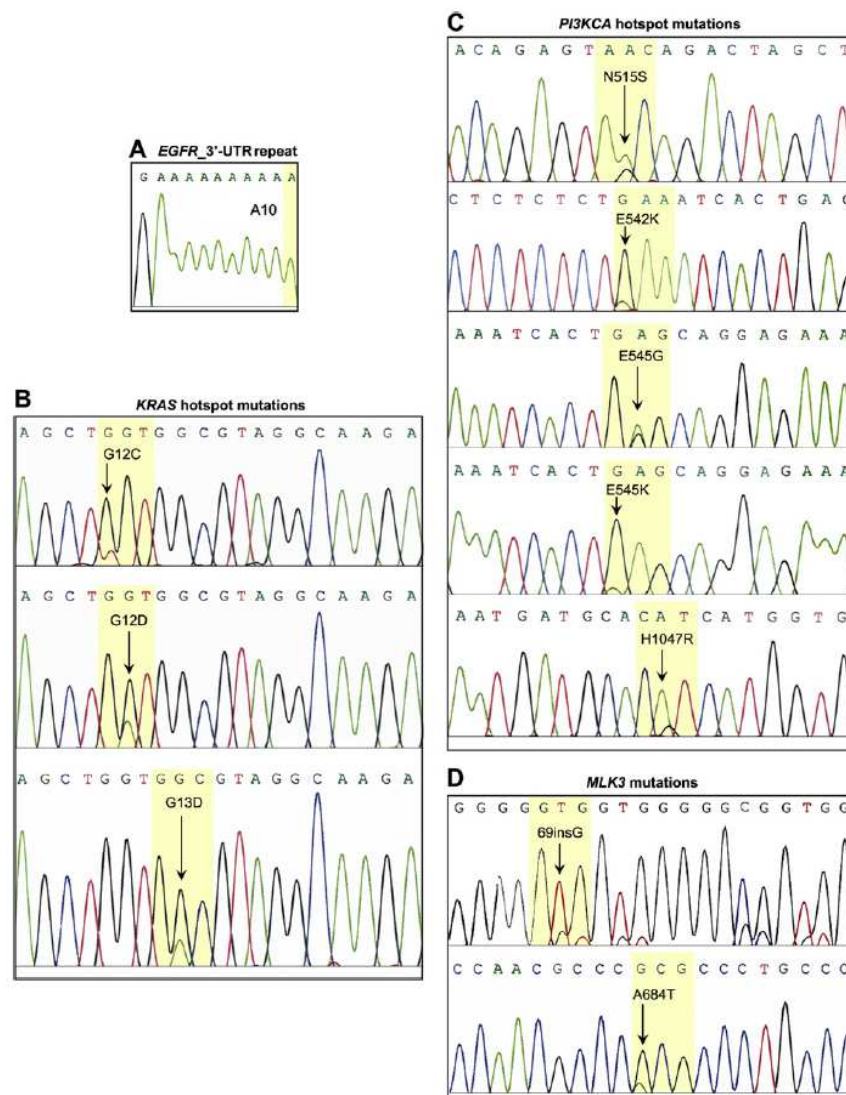


Fig. 1 – Representative images of all oncogenic mutations identified. (A) A10 tumour deletion localized into the 3' UTR of EGFR; (B) KRAS hotspot mutations (reverse sequence); (C) PIK3CA tumour specific alterations; and (D) MLK3 gene alterations.

Table 1 – Detailed description of oncogenic mutations identified in the 63 MSI gastric carcinomas.

	Sample code	MLK3 mutation	KRAS mutation	PIK3CA mutation	BRAF mutation	EGFR hotspot mutation	EGFR 3'-UTR polyA deletion
1	AU415	wt	wt	N515S	wt	wt	(A) ₁₃ /(A) ₁₃
2	AB248	wt	wt	wt	wt	wt	(A) ₁₃ /(A) ₁₄
3	BG191	wt	wt	wt	wt	wt	(A) ₁₃ /(A) ₁₃
4	BF070	wt	wt	wt	wt	wt	(A) ₁₃ /(A) ₁₃
5	BM175	wt	wt	wt	wt	wt	(A) ₁₃ /(A) ₁₄
6	BL276	wt	wt	wt	wt	wt	delA(A) ₁₂ /(A) ₁₃
7	BE163	wt	G12D	H1047R	wt	wt	delAA(A) ₁₁ /delAA(A) ₁₂
8	BM213	wt	G12D	wt	wt	wt	delA(A) ₁₃ /delA(A) ₁₃
9	BC355	wt	G13D	wt	wt	wt	delA(A) ₁₂ /(A) ₁₃
10	BM406	wt	wt	wt	wt	wt	(A) ₁₃ /(A) ₁₄
11	BP280	wt	wt	wt	wt	wt	delA(A) ₁₂ /delA(A) ₁₃
12	CD361	wt	wt	wt	wt	wt	(A) ₁₃ /(A) ₁₃
13	CE120	wt	wt	H1047R	wt	wt	delAAA(A) ₁₀ /delAAA(A) ₁₁
14	CC442	wt	wt	wt	wt	wt	(A) ₁₃ /(A) ₁₃
15	CI362	wt	G12C	H1047R	wt	wt	delA(A) ₁₂ /(A) ₁₃
16	CG072	wt	wt	wt	wt	wt	(A) ₁₃ /(A) ₁₃
17	CI376	wt	wt	wt	wt	wt	delAA(A) ₁₁ /delA(A) ₁₂
18	DM272	wt	wt	wt	wt	wt	delAAA(A) ₁₀ /delAAA(A) ₁₀
19	DE226	wt	wt	E545K	wt	wt	(A) ₁₃ /(A) ₁₄
20	DM187	wt	wt	wt	wt	wt	(A) ₁₃ /(A) ₁₄
21	FF336	wt	wt	wt	wt	wt	(A) ₁₃ /(A) ₁₄
22	FL141	wt	wt	wt	wt	wt	delAA(A) ₁₁ /delAA(A) ₁₂
23	FL208	wt	wt	wt	wt	wt	delA(A) ₁₂ /delA(A) ₁₃
24	FF269	wt	wt	wt	wt	wt	(A) ₁₃ /(A) ₁₃
25	FP285	wt	wt	wt	wt	wt	(A) ₁₄ /(A) ₁₄
26	FD373	wt	wt	wt	wt	wt	(A) ₁ /(A) ₁₄
27	GA220	wt	wt	wt	wt	wt	delA(A) ₁₂ /delA(A) ₁₃
28	GE126	wt	G12D	wt	wt	wt	delA(A) ₁₂ /delA(A) ₁₃
29	GF364	wt	wt	wt	wt	wt	(A) ₁₄ /(A) ₁₄
30	JG153	wt	wt	wt	wt	wt	(A) ₁₃ /(A) ₁₃
31	LG445	wt	G13D	wt	wt	wt	delAA(A) ₁₁ /delA(A) ₁₂
32	LI266	wt	wt	wt	wt	wt	delA(A) ₁₂ /(A) ₁₃
33	MA155	wt	G12D	wt	wt	wt	(A) ₁₃ /(A) ₁₄
34	MG296	wt	wt	wt	wt	wt	delA(A) ₁₂ /(A) ₁₃
35	MI052	wt	wt	H1047R	wt	wt	delAA(A) ₁₁ /delA(A) ₁₂
36	MS399	wt	wt	wt	wt	wt	(A) ₁₃ /(A) ₁₃
37	MG359	wt	wt	wt	wt	wt	(A) ₁₃ /(A) ₁₄
38	ME196	wt	wt	wt	wt	wt	delAA(A) ₁₀ /delA(A) ₁₂
39	MM110	wt	wt	wt	wt	wt	(A) ₁₃ /(A) ₁₃
40	MM122	wt	G12D	wt	wt	wt	delAA(A) ₁₁ /delAA(A) ₁₂
41	NV424	wt	wt	wt	wt	wt	(A) ₁₃ /(A) ₁₃
42	NC410	wt	wt	wt	wt	wt	(A) ₁ /(A) ₁₃
43	OG249	wt	wt	E545G	wt	wt	delA(A) ₁₂ /(A) ₁₃
44	PA158	wt	wt	wt	wt	wt	delAA(A) ₁₁ /delA(A) ₁₂
45	PB114	wt	wt	wt	wt	wt	(A) ₁₃ /(A) ₁₃
46	PD192	wt	wt	wt	wt	wt	delAA(A) ₁₁ /delAA(A) ₁₁
47	PR209	wt	wt	wt	wt	wt	(A) ₁₃ /(A) ₁₃
48	PD214	wt	G12D	wt	wt	wt	delA(A) ₁₂ /delA(A) ₁₃
49	RF421	wt	wt	wt	wt	wt	(A) ₁₃ /(A) ₁₃
50	RA125	A684T	wt	E542K	wt	wt	delAA(A) ₁₁ /delA(A) ₁₂
51	RI107	wt	wt	wt	wt	wt	delAA(A) ₁₁ /delAA(A) ₁₂
52	RA393	wt	wt	wt	wt	wt	delA(A) ₁₂ /(A) ₁₃
53	RA270	wt	G13D	wt	wt	wt	(A) ₁₃ /(A) ₁₃
54	RC205	wt	wt	wt	wt	wt	delA(A) ₁₂ /(A) ₁₃
55	RL139	wt	wt	E542K	wt	wt	(A) ₁₃ /(A) ₁₄
56	SA13 5	wt	wt	wt	wt	wt	(A) ₁₃ /(A) ₁₃
57	SE315	wt	wt	wt	wt	wt	(A) ₁₃ /(A) ₁₃
58	SA259	wt	wt	wt	wt	wt	(A) ₁₃ /(A) ₁₄
59	TL130	wt	wt	wt	wt	wt	(A) ₁₃ /(A) ₁₄
60	TB146	wt	wt	wt	wt	wt	delA(A) ₁₂ /(A) ₁₃
61	VN174	wt	wt	wt	wt	wt	(A) ₁₃ /(A) ₁₄
62	VP199	69insG	wt	wt	wt	wt	delAA(A) ₁₁ /delAA(A) ₁₂
63	ZR195	wt	G13D	wt	wt	wt	delAAA(A) ₁₀ /delAAA(A) ₁₁
Total		2	11	9	0	0	30

Table 2 – Relation between clinicopathologic features of the 63 MSI gastric carcinomas and oncogenic mutations.

	Oncogenic mutation + (35/63; 55.6%) ^a	Oncogenic mutation – (28/63; 44.4%)	P value
Gender			
Male	10 (28.6)	18 (64.3)	0.046
Female	25 (71.4)	10 (35.7)	
Mean age (±SD)	76.7 ± 7.9	67.9 ± 11.4	0.001
Tumor location			
Cardia	4 (11.4)	1 (3.6)	ns
Non-cardia	31 (88.6)	27 (96.4)	
Lauren classification			
Intestinal	31 (88.6)	19 (67.9)	0.043
Non-intestinal	4 (11.4)	9 (32.1)	
Depth of invasion			
pT1–T2	25 (71.4)	14 (50)	ns
pT3–pT4	10 (28.6)	14 (50)	
Lymph node involvement			
pN0–N1	30 (85.7)	20 (71.4)	ns
pN2–pN3	5 (14.3)	8 (28.6)	
Extent of gastrectomy			
Partial	27 (77.1)	20 (71.4)	ns
Total	8 (22.9)	8 (28.6)	
Lymphadenectomy			
D1	15 (42.8)	12 (42.9)	ns
D2/D3	20 (57.2)	16 (57.1)	
Radicality of resection			
R0	32 (91.4)	19 (67.9)	0.017
R1–2	3 (8.6)	9 (32.1)	
Stage grouping			
I–II	23 (65.7)	14 (50)	ns
III–IV	12 (34.3)	14 (50)	
Geographic area			
High incidence	35 (100)	21 (75)	0.001
Low incidence	0 (0)	7 (25)	

Numbers in parentheses are percentage.

^a Fourteen GC patients carried concomitant somatic mutations.

gastric samples showed a wild-type A13 or A14 repeat of EGFR in a homozygous or heterozygous state. According with the findings from the EGFR 3'-UTR mutation screening, we verified, by EGFR immunohistochemistry, that while two EGFR mutant GC (deletion [delAA(A)11]) showed an increased expression of EGFR in tumour cells in comparison to the surrounding normal tissue, a wild-type EGFR GC case did not display EGFR expression in the tumour area (Fig. 2). Due to the lack of good quality paraffin tissue, we can only consider this data as preliminary. This result needs to be validated in a larger series of MSI GC in order to confirm the correlation between the EGFR expression and the mutations in A13 deletions in the 3'-UTR polyA repeat of EGFR.

3.2. KRAS and BRAF mutations

KRAS mutations were observed in 17.5% (11/63) of MSI GC cases. From these 11 cases harbouring somatic mutations,

we found that codon 12 was mutated in 63.6% (7/11) and codon 13 in 36.4% (4/11). All but one KRAS mutation localized in codon 12 were G12D. All mutations in codon 13 were G13D. KRAS mutations were more frequently found in elderly patients ($p = 0.006$), but no further significant associations were found between other clinicopathologic characteristics and the KRAS mutation status (data not show). None of the MSI GC cases under study showed BRAF somatic mutations in the hotspot codon previously associated to MSI colorectal carcinomas, the BRAF V600E.³¹

3.3. PIK3CA mutations

PIK3CA mutations were found in 14.3% (9/63) of the MSI GC studied. Among the mutated cases, eight somatic mutations were located in codons previously described as PIK3CA hotspots (codons 542, 545 and 1047). Five PIK3CA alterations occurred at the helical domain (codon 515, 542 and 545) and four mutations affected the kinase domain (codon 1047) (Table 1). A novel missense mutation (1544A>G) was identified within the helical domain at codon 515 (N515S). None of these mutations were present in the normal counterpart of these cases.

3.4. MLK3 mutations

MLK3 mutations were found in two carcinomas corresponding to a frequency of 3.2% (Table 1): one was a missense mutation (2052G>A) localized in proline-serine-threonine rich domain (A684T) and the other was a frameshift mutation (c.69insG). Besides these two somatic mutations, that were tumour specific, we identified one splice site alteration (c.1069+10C>T) and one missense mutation (2190G>A) (R730H) that were present in both tumour and constitutional DNA, both with unknown pathogenic function. Moreover, we observed two silent MLK3 sequence variants (225A>G and 2259A>T) that did not change the aminoacid residues of the MLK3 protein (A75A and P753P, respectively) and were previously described in the normal population.²⁶

3.5. Concomitant oncogenic mutations

We verified that within the 35 MSI GC harbouring oncogenic mutations, 14 (40.0%) of the mutant cases showed concomitant oncogenic alterations, always involving EGFR polyA mutations. From those cases with more than one mutation, seven had EGFR A13 repeat deletions and KRAS mutations (7/14 – 50%), three showed EGFR polyA deletion and a PIK3CA mutation (3/14 – 21.4%) and two had EGFR deletions and both KRAS and PIK3CA (2/14 – 14.3%) mutations. In one case we found an EGFR deletion and concomitant missense mutations in MLK3 and PIK3CA and in another case an EGFR deletion and a MLK3 mutation (Fig. 3).

4. Discussion

Despite the general advances in diagnosis, standard surgery and chemo- and radio-therapy regimens, the overall outcome of GC patients remains poor, with a 5-year global survival of

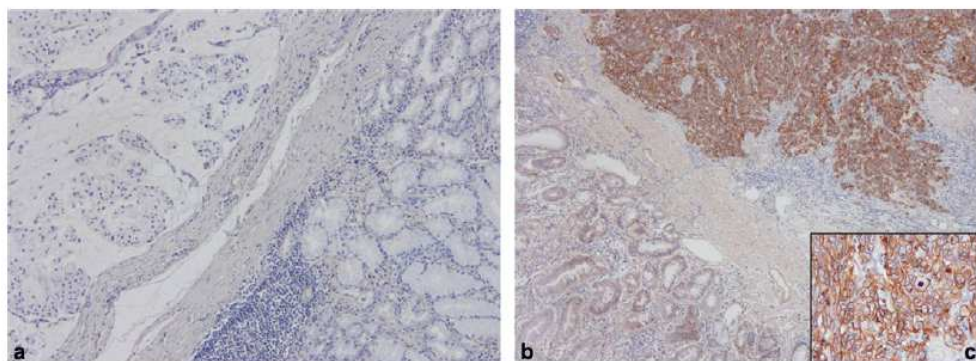


Fig. 2 – EGFR immunohistochemistry expression in gastric carcinoma: (a) example of a EGFR A13 tract wild-type GC case showing negative EGFR expression (amplification 100X); (b) EGFR overexpression in tumour area in a GC case displaying EGFR A10 deletion in 50X amplification and (c) in 400X amplification.

about 26%.³² Various multimodal therapy regimens are used to improve the prognosis of GC patients, but no single chemotherapy regimen is recognized as a global standard.³³ The high prevalence of incurable disease and the poor overall survival of GC patients create the urgent need to find new therapeutic tools for GC treatment.

In patients with advanced GC, several clinical trials were conducted, namely testing EGFR inhibitors. However, the overall response to EGFR tyrosine kinase inhibitors was poor. Among EGFR tyrosine kinase inhibitors, it has been demonstrated that gefitinib treatment showed a particularly low response rate (18%) in advanced GC while erlotinib was completely inactive.^{34,35} Recently, more encouraging results were obtained in GC treatment using a combination of multiple chemotherapies.^{36–41} As example, patients treated with cetuximab and FUFOX/FOLFOX (5-fluorouracil, oxaliplatin, folinic acid) or FOLFIRI (5-fluorouracil, irinotecan and folinic acid) had a significant higher response rate (62%) when compared with GC patients treated with cetuximab alone

(5%).^{36–38} Furthermore, when cetuximab was associated with oxaliplatin/leucovorin and 5-fluorouracil, 50% of the patients showed a positive response rate.^{39,40} A similar response rate (65%) has been reported in metastatic GC treated with cetuximab plus oxaliplatin/ folinic acid, in which EGFR overexpression has been documented.⁴¹ However, until now it was impossible to predict which GC patients will respond to anti-EGFR therapy. In other words, no predictive biomarkers are available for clinicians to use in the stratification of GC patients or to predict treatment benefit. Taking into account data from treatment of colon cancer patients, where the response to anti-EGFR therapy depends on the genetic make-up of the tumours, namely on the EGFR activation status and on the mutation profile of members of EGFR and MAPK signalling pathways,^{42,43} we decided to study these same players in MSI GC.

Different molecular mechanisms underlie EGFR protein activation, such as somatic mutations or gene amplification, both leading to an abnormal receptor function. In our series

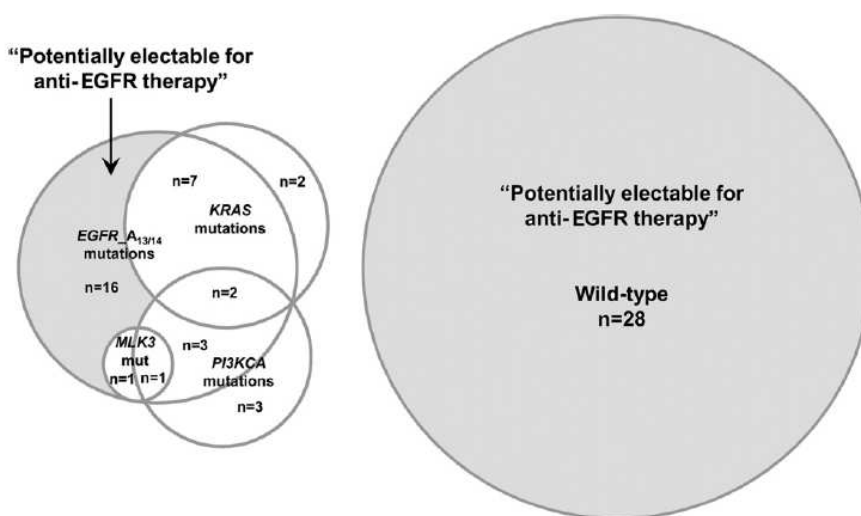


Fig. 3 – Schematic representation of the distribution of the oncogenic mutations identified in the present study (occurring as single events or as concomitant alterations). Oncogenic mutations were found in 35 GC cases but fourteen of them showed more than one mutagenic event in genes belonging to the EGFR signalling pathway.

of MSI GC, we did not find hotspot EGFR mutations. This finding was not surprising as EGFR mutations have been rarely described in GC and gene amplification was only described in a low frequency of cases.¹³ Therefore, the presence of other mechanisms directly or indirectly leading to EGFR activation were herein investigated.

Since it was recently demonstrated that a polyA tract at the 3'-UTR of EGFR was prone to harbour deletions in MSI colon cancer,¹⁴ we searched for this type of alterations in our series of MSI GC. We found alterations at this site in a high frequency (47.6%) of cases. A higher frequency (69%) was found for this type of alteration in MSI colon cancer.¹⁴ Furthermore, and similarly to EGFR 3'-UTR polyA tract mutant colon cancer cases, we also verified high level of EGFR expression in two tumour samples harbouring this type of alteration. These results suggest EGFR A13 repeat mutations as a putative molecular marker to select GC patients for anti-EGFR therapies, namely anti-EGFR monoclonal antibodies as verified in colon cancer.^{14,45}

As previously mentioned, in metastatic colorectal carcinomas, a response to anti-EGFR therapies was only observed in patients with tumours without KRAS or BRAF mutations.^{4,6,17,43–45} In particular, the presence of a KRAS mutation is well established as predictive of non-response to anti-EGFR antibody and shorter patient survival.^{5,6} In contrast, the clinical evidence concerning PIK3CA mutations is not yet straightforward in this cancer setting.^{46,47}

Given the similarities in the mutation spectrum of colorectal carcinomas and MSI gastric carcinomas,¹⁸ we hypothesize that MSI GC cases without alterations in KRAS and BRAF, and possibly in other members of the MAPK (MLK3) or PI3K (PIK3CA) pathways may benefit from this therapeutic approach. Taking in account this hypothesis, we determined the frequency of mutations in all these genes in order to verify their putative importance as biomarkers for therapy assessment.

In the present study, KRAS mutations were found in 17.5% of MSI GC cases, while no BRAF mutations were detected in these samples, in keeping with data on record.^{15,16,18,20–22} These data elicit KRAS but not BRAF activation as potential biomarkers for therapy assessment in MSI GC. Similarly to BRAF, somatic alterations at the MLK3 gene were very rarely found (3.2%), as previously described in another sample setting of MSI GC.²⁶ PIK3CA mutations were found in a non-negligible frequency of MSI GC cases (14.3%, respectively) and despite no proof exists showing that mutant PIK3CA tumours may not respond to anti-EGFR therapy, it is important to characterize its mutation status. The above presented data suggest, in light of the current knowledge on the treatment of gastrointestinal cancer with anti-EGFR therapy, that MSI GC patients: (1) can also be stratified according to the molecular profile of their tumours; (2) present, in over 45% of the cases mutations at the EGFR 3'-UTR that potentially lead to EGFR overexpression which turn these cases in a subset of potentially responsive tumours to anti-EGFR treatment; (3) display KRAS mutations in nearly 20% of the cases making these GC patients as potentially resistant to anti-EGFR therapies; (4) harbour PIK3CA mutations in almost 15% of the cases but the use of this information is of limited interest, and finally; (5) lack or display very low frequencies of BRAF and MLK3 mutations and therefore these markers will not improve a fu-

ture panel of genes to be tested in potentially eligible patients for anti-EGFR therapy. Another layer of information that was obtained in our multiple gene mutation screening approach, showed that over one third (40%) of the mutant MSI GC cases accumulate mutations in more than one gene, demonstrating, on one hand, that multiple molecules within or targeted by the EGFR pathway are involved in GC progression; but on the other hand, reducing the number of cases potentially benefiting from anti-EGFR therapy. In detail, as 30% (9/30) of EGFR mutated cases also display KRAS mutations, thus it is predictable that 70% (21/30) of mutant EGFR patients will benefit from EGFR inhibitors (Fig. 3). Furthermore, another set of the patients harbouring EGFR mutations also displayed a PIK3CA mutation which, although unproved, may also result in resistance to therapy (Fig. 3).

In GC, cases harbouring concomitant oncogenic mutations in the MAPK cascade is about 3%.¹⁸ Our data showed that 40% of cases have concomitant oncogenic mutations (Fig. 3). In our series, the higher percentage of cases with multiple oncogenic mutations is related to the additional screening of the EGFR polyA tract and MLK3 gene, never evaluated so far. In fact, considering only PIK3CA and KRAS alterations, the number of cases with concomitant mutations is much lower and similar to the frequency reported by Velho and colleagues.¹⁸ The accumulation of EGFR 3'-UTR and/or PIK3CA/KRAS/MLK3 mutations within MSI gastric carcinomas suggest a possible synergistic effect in the signalling pathways associated to the activation of these genes in GC development/ progression.

Our results support the proposal to implement a multiple gene screening approach to predict EGFR-targeted therapy. We show that within MSI GC three groups can be individualized: Group A: cases that are wild-type for all genes under screening (EGFR and genes of the MAPK and PI3K pathways) – 44.4% (28/63); Group B: cases with oncogenic alterations restricted to EGFR – 25.4% (16/63); Group C: cases with oncogenic mutations in genes of the MAPK and PI3K pathways with or without concomitant EGFR alterations – 30.2% (19/63). Overall, cases from groups A and B (69.8%) are potentially eligible for anti-EGFR therapy. Moreover, from the experience with colorectal cancer it is expectable that cases with oncogenic mutations affecting MAPK and PI3K signalling pathways GC patients will be non-responders for anti-EGFR therapies.

The advantage to identify deletion at the EGFR A13 repeat region is to select a novel category of patients that potentially benefit from EGFR inhibitors as therapeutic approach. Probably other genetic mechanisms, to date unknown, can also activate the EGFR pathway even in cases that belong, in our series, to the wild-type EGFR group.

We verified that oncogenic mutations mostly occur in MSI GC patient with older age at diagnosis and of the intestinal subtype, supporting that within MSI GC different molecular pathways are activated in order to generate specific phenotypes.

Palli and colleagues showed in another study performed in the same geographical region (Tuscany region) an association between MSI, positive family history of GC and high consumption of red meat and nitrates, suggesting that environmental factors, such as nutritional habits may play a key

role in inducing genomic instability and an increased risk for GC. Our data demonstrated that oncogenic mutations related significantly with this high incidence area and this probably is associated with the presence of MSI phenotype.^{27,48}

In conclusion, our results show that alterations at multiple molecules within or targeted by the EGFR pathway are frequent in MSI GC and, that within members of the pathway, deletions of the A13 repeat of EGFR were the most common genetic event followed by KRAS and PIK3CA mutations. Furthermore, in over one third of the cases, concomitant mutations occur in distinct genes, always involving EGFR. More importantly, our results open new avenues regarding the stratification of MSI GC patients for anti-EGFR therapies and pinpoint a non-neglectable group of cases that may benefit from this therapeutic approach.

Conflict of interest statement

None declared.

Acknowledgements

This work was supported by Istituto Toscano Tumori ('Gene expression profile and therapeutic implication in gastric cancer. From the clinical overview to the translational research'; Grant ITT- 2007) and by Fundação para a Ciência e a Tecnologia (PTDC/SAU-OB/68310/2006 and PIC/IC/82923/2007) and PhD Grant Ref SFRH/BD/40090/2007).

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PAPER VIII

Corso, G., *et al.*, **Etiopathogenesis of gastric cancer**. Surgery in Multimodal Management of Gastric Cancer 2011, cap. 2, (eds) Springer-Verlag Italia 2011, in press.

Abstract

The introduction of molecular biology into cancer genetics has resulted in the clarification of several crucial aspects in the etiopathogenesis and tumorigenesis of human cancer. However, gastric cancer continues to represent a clinical burden because of its unfavorable prognosis and our poor knowledge of the molecular mechanisms responsible for the early steps in the initiation of gastric tumor development. Progress has been made through the elucidation of different molecular signaling cascades, such as the mitogen-activated protein kinase cascade, in which the mutator phenotype has been ascribed to a deficiency of the mismatch repair system. While this and similar recent discoveries are still being discussed with respect to their scientific implications, it may nonetheless be appropriate to consider potential clinical applications in the management of patients with gastric cancer.

Keywords

Gastric cancer • Tobacco consumption • *Helicobacter pylori* • E-cadherin • Microsatellite instability • KRAS • PIK3CA • EGFR • MAPK cascade • Prognostic factors • Therapy

2.1 Introduction

Gastric cancer (GC) is the second most common form of cancer in Europe [1]. In 2000, there were 192,000 new cases with 158,000 deaths. In Southern Europe, the highest incidence of GC is found in the Tuscany region of Italy and in Portugal [2]. Despite advances in the prevention and diagnosis of GC; the outcome of these patients has remained poor, with an overall 5-year

survival of around 23%.

Several genetic, epigenetic, and environmental factors interact simultaneously in the early steps of gastric carcinogenesis. Among these, human genetic polymorphisms of inflammatory-related genes are thought to be responsible for an increased risk of GC. Other factors, such as tobacco consumption, dietary habits, and *Helicobacter pylori* infection, have been demonstrated to be involved in the multifactorial process of gastric carcinogenesis. Germline inactivating mutations and deletions of the E-cadherin gene (*CDH1*) are well-documented genetic events associated with early-onset GC and with hereditary diffuse gastric cancer (HDGC) syndrome. E-cadherin somatic inactivation correlates with aggressive tumor

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behavior and a worse prognosis. Conversely, microsatellite instability (MSI) phenotype, which is the hallmark of GC in about 20–25% of cases, is a relatively good prognostic factor. Within MSI patterns, somatic mutations in the oncogenes *KRAS*, *PIK3CA*, and *EGFR* play a crucial role in the genetic instability of GC. In this chapter, we focus on GC etiopathogenesis by exploring its genetic/epigenetic mechanisms.

2.2 Tobacco Consumption and Dietary Habits

Smoking is a major risk factor for adenocarcinomas of the esophagus and gastric cardia, accounting for approximately 40% of cases. Most but not all studies report a higher risk in the cardia than in non-cardia sites in cigarette smokers vs. non-smokers; conversely, alcohol consumption may be associated with an increased risk of distal and total GC [3, 4]. The risk of gastric cardia adenocarcinomas is increased among current cigarette smokers with a reduction first observed 30 years after smoking cessation; the risk of GC rises with increasing intensity and duration of smoking. Because of the long lag time before the risk of these tumors is reduced among ex-smokers, smoking is thought to affect early-stage carcinogenesis.

In gastric carcinogenesis, diet is also a determinant; salted, smoked, pickled and preserved foods (rich in salt, nitrite, and pre-formed N-nitroso compounds) as well as meat intake are associated with an increased risk of non-cardia GC. However, rather than only a single responsible agent, dietary habits, consumption frequency, amount of exposure to etiologically important agents, traditional food-preparation techniques, and storage methods all play major roles in the etiology of GC. Excessive salt consumption is a confirmed factor whereas in Japan and other Far East countries excessive consumption of fish and other seafood plays the lead role in GC etiology. A reduced consumption of fresh fruits and vegetables is another important etiologic factor. Dietary factors and improvements in food storage, such as refrigeration, which decreases the turnover of nitrosamines to nitrites, have caused a significant decline in the worldwide prevalence of GCs. Interestingly, Palli

et al. showed an association between GC and a high consumption of red meat [5]. The etiopathogenesis of gastric cardia carcinoma is related to nitrosamine consumption but also to exposure, as these compounds are present in tobacco and tobacco smoke and their carcinogenic activities have been well established. Similarly, cured fish and meat may contain N-nitroso compounds (NOC) such that the mechanism of carcinogenesis due to tobacco consumption and to certain dietary habits may be linked by the common presence of NOC. The role of NOC in GC etiology involves alteration of the pH of gastric juice; specifically, in the presence of NOC the pH of gastric juice is low (1.0–2.5), leading to a phenomenon called “chemical gastric nitrosation,” which proceeds as an acid-catalyzed reaction in the normal acidic stomach before premalignant lesions develop. High concentrations of NOC have been identified in vivo also in the inflamed gastric mucosa infected with *H. pylori*, accompanied by a high pH (6.0–8.5). Similarly, high pH induces bacterial gastric nitrosation. This chemical modification results in an increased tolerance to DNA damage, with reduced activity of mismatch repair (MMR) genes leading to an accumulation of errors during DNA replication.

2.3 *Helicobacter pylori* Infection

Several prospective studies have reported a strong association between chronic *H. pylori* infection and GC risk; as such, the World Health Organization’s International Agency for Research on Cancer recognized *H. pylori* as a group 1 carcinogen for humans. Chronic gastric inflammation and the interaction between *H. pylori* and gastric epithelial cells have been suggested as mechanisms in gastric carcinogenesis. However, only a few individuals infected by *H. pylori* go on to develop GC. These cases probably also involve environmental factors, host-inflammatory genetic susceptibility, and variation of the bacterial strains. *H. pylori* is a gram-negative bacterium that colonizes the human gastric epithelium. The severity of *H. pylori*-related disease is correlated with the presence of the *cag* pathogenicity island (PAI), associated with the production of the *cagA*

antigen. The *cagA* gene is a strain-specific *H. pylori* gene located in the right half of the PAI. It encodes the protein CagA, which is secreted via a type IV secretion system and translocated into gastric epithelial cells, affecting host cell physiology. Infection with *cag*-positive *H. pylori* strains has been recognized as a marker for strains that confer increased risk for peptic ulcer disease, gastric mucosal atrophy, and GC. By contrast, the *vacA* gene is present in all *H. pylori* strains. It encodes the vacuolating cytotoxin VacA, which induces epithelial cell injury. *H. pylori* colonizes the atrophic stomach poorly and intestinal metaplasia hardly at all, suggesting that the bacteria creates an environment for intestinal-type gastric carcinogenesis (atrophy and hypochlorhydria) rather than causing the cancer directly. The risk of developing GC for infected persons is estimated to be 2–3 times higher than for non-infected ones. More recently, the role of individual susceptibility has been stressed. In addition, proinflammatory cytokine gene polymorphisms have been demonstrated to interact in the compound process of gastric carcinogenesis [6]. For example, the relationship between interleukin-1 gene polymorphism and GC risk has been investigated. The conclusions support the theory in which host genetic factors affecting the inflammatory and immune response to *H. pylori* infection determine a higher risk of GC development, especially in high-risk areas [7, 8].

In GC, the absence of *H. pylori* infection is related to specific clinico-pathological factors impacting the long-term survival of these patients. Negative *H. pylori* status is associated with cardia tumor location, advanced pT classification, non-curative surgery, and a lower 5-year survival rate after R0 resection. Marrelli et al. demonstrated that *H. pylori* status is a significant prognostic factor, in which negative *H. pylori* status appears to be an indicator of poor prognosis in patients with GC [9].

2.4 E-Cadherin Gene (*CDH1*) and Diffuse Gastric Cancer

The *CDH1* gene maps to chromosome 16q22.1 and consists of 16 exons that encode a 120-kDa protein called E-cad, which is a member of the transmem-

brane glycoproteins family. E-cad is expressed on epithelial tissues and is responsible for calcium-dependent cell-cell adhesion. Functionally, it is critical for establishing and maintaining polarized and differentiated epithelia through intercellular adhesion complexes. Human E-cad is considered an invasion suppressor, and under-expression of E-cad is correlated with the infiltrative and metastatic ability of the tumor. *CDH1* deregulation has been identified in early tumors of patients with germline mutations and thus may be an initial event in HDGC [10, 11]. In GC, the principal epigenetic and structural mechanisms of *CDH1* inactivation/deregulation are somatic mutations, loss of heterozygosity (LOH), and promoter hypermethylation. About 90% of GC cases appear in a sporadic setting, whereas familial clustering is observed in the remaining 10%; of these cases, only 1–3% are hereditary. Among those with familial aggregation of GC, the most common disease is the HDGC syndrome, which is related to *CDH1* germline mutations. The syndrome was first identified in 1998 by Guilford in Maori kindred from New Zealand. To date, many studies have reported different types of *CDH1* alterations in HDGC families, with 30–40% of these families harboring *CDH1* germline mutations, as reviewed by Pedrazzani and colleagues [12]. In 1999, the International Gastric Cancer Linkage Consortium (IGCLC) defined the following criteria for the identification of HDGC families: (1) two or more documented cases of DGC in first- or second-degree relatives, with at least one of these relatives diagnosed before the age of 50; (2) three or more documented cases of DGC in first- or second-degree relatives, independent of age of onset.

After demonstrating the presence of *CDH1* germline mutations in patients not fulfilling the IGCLC criteria, Brooks-Wilson and colleagues recommended several modifications: (1) two or more documented cases of DGC in first-degree relatives, with at least one diagnosed before age 50; (1A) two or more cases of GC, with at least one case of DGC diagnosed before age 50; (2) three or more documented cases of DGC in first-degree relatives, diagnosed at any age; (2A) three or more cases of GC, diagnosed at any age, with at least one documented case of DGC; (3) isolated individual diagnosed with DGC at less than 45

years of age; (4) isolated individual diagnosed with both DGC and lobular breast cancer (no other criteria met); (5) one family member diagnosed with DGC and another with lobular breast cancer (no other criteria met); (6) one family member diagnosed with DGC and another with signet-ring carcinoma of the colon (no other criteria met) [13]. These novel criteria introduced a new clinical entity called early-onset DGC, in which E-cadherin mutations occur with a frequency of about 6%. The cut-off age for *CDH1* genetic screening is 35 years old at DGC diagnosis [14]. *CDH1* down-regulation plays a specific role in gastric carcinogenesis, and DGC with signet-ring cells (SRCs) is the predominant histological type in carriers of *CDH1* germline mutations. In its early stage, HDGC is characterized by the presence of multiple foci of diffuse-type SRC carcinoma confined to the superficial gastric mucosa [15]. The majority of neoplastic foci observed in the resected specimen after prophylactic gastrectomy are < 1 mm in diameter, and all lie under normal appearing surface epithelium [11]. Multifocal microscopic disease is very common, with a preferential localization along the epithelial wall of the gastric body/fundus. Carneiro and colleagues proposed a histological model for GC development in carriers of E-cad mutation: initially, histopathological analysis shows a pattern of in situ SRC carcinoma with early pagetoid spread and pagetoid proliferation of SRCs followed by invasive SRC carcinoma. *H. pylori* infection is not detected in gastric mucosa obtained from prophylactic gastrectomies [16]. Carriers of E-cad truncating germline mutations have a fairly high lifetime risk of developing GC; the estimated cumulative risk by age 80 years is 67% for men (95% CI, 39–99) and 83% for women (95% CI, 58–99), with a mean age at diagnosis of 40 years (range, 14–85) [17]. Prophylactic total gastrectomy remains the only method to abolish the inherited risk for gastric cancer in carriers of *CDH1* truncating mutation; however, the role of prophylactic gastrectomy in missense mutation carriers remains to be clarified, because to date prophylactic surgery has yet to be performed in these patients [12]. The strategy for clinical management includes a review of the family history, genetic testing chromoendoscopic surveillance, and prophylactic surgery.

2.5 Microsatellite Instability and Oncogenic Mutations

The mutator or MSI phenotype is caused by a defect in the MMR system, which is responsible for the correction of mismatches that occur during DNA replication. In gastric carcinoma, this phenotype is found in about 15–25% of the cases [18]. To date, no germline mutations involving MMR genes have been described in familial GC; on the other hand, somatic mutations in MMR genes are rare in sporadic GC [19]. Cancers with genomic instability show distinct clinico-pathological features. Most MSI tumors are of intestinal histotype and located in the distal part of the stomach, with limited lymph node involvement; they occur more frequently in older women. Survival analysis of patients with MSI status demonstrated a good prognosis in those with advanced tumors and instability at all markers [18]. In the high-level MSI phenotype, the mitogen-activated protein kinase (*MAPK*) cascade and phosphatidylinositol 3-kinase (*PI3K*) pathways are frequently activated in the progression of gastrointestinal malignancies. The oncogenes implicated in the MSI subset of GC are *EGFR*, *KRAS*, and *PIK3CA*. While data on *EGFR* alterations as well as mutations (hotspot region) in the gene's downstream targets, namely those belonging to the *MAPK* and *PI3K* pathways, are very limited, alterations of the polyA tract at the 3'-UTR of *EGFR* have been shown to occur with a high frequency (about 50% of cases) [20]. The presence of mutations at the 3'-UTR of *EGFR* is related to a high level of *EGFR* expression [21]. *KRAS* gene mutations occur with a frequency of 3–8% and typically cluster in the MSI subset (~17% of MSI cases). In contrast, several groups, including our own, have found that *BRAF* mutations rarely occur in this type of epithelial cancer. The frequency of *PIK3CA* oncogenic mutations is about 14%; another oncogene albeit one rarely involved (3%) is *MLK3*. Alterations within multiple molecules included in or targeted by the *MAPK* pathway are frequent in MSI GC, with deletions of the A13 repeat of *EGFR* being the most common genetic event followed by *KRAS* and *PIK3CA* mutations. More importantly, this molecular model opens new

avenues regarding the stratification of MSI GC patients for anti-*EGFR* therapies and for identifying those patients who are most likely to benefit from a targeted therapeutic approach.

2.6 Conclusion

The etiopathogenesis of GC is complex and multifactorial. This review focused on the principal mechanisms involved in gastric carcinogenesis. Of particular importance are “exogenous” factors, such as tobacco, dietary habits, and *H. pylori* infection, and “endogenous,” factors, such as E-cadherin germline mutations and genetic/epigenetic events. However, endogenous and exogenous factors may not be mutually exclusive; for exam-

ple, the presence of chronic inflammation resulting from *H. pylori* infection is probably related to the MSI phenotype and thus to alterations in the *MAPK* cascade. For clinical practice, we propose a flow-chart (Fig. 2.1) in which the molecular pathway of GC etiopathogenesis is divided in terms of prognostic and therapeutic biomarkers. Group A includes those patients expressing the prognostic biomarkers MSI and E-cadherin, indicating, respectively, a good vs. a worse prognosis. Moreover, recently we described the *CDH1* structural alteration as a novel biomarker of worse prognosis in GC patients [22]. Group B consists of patients who are candidates for a new therapeutic approach, such as *EGFR*-inhibitors, whereas it remains to be determined whether *KRAS/PIK3CA* mutations can also be included in this group.

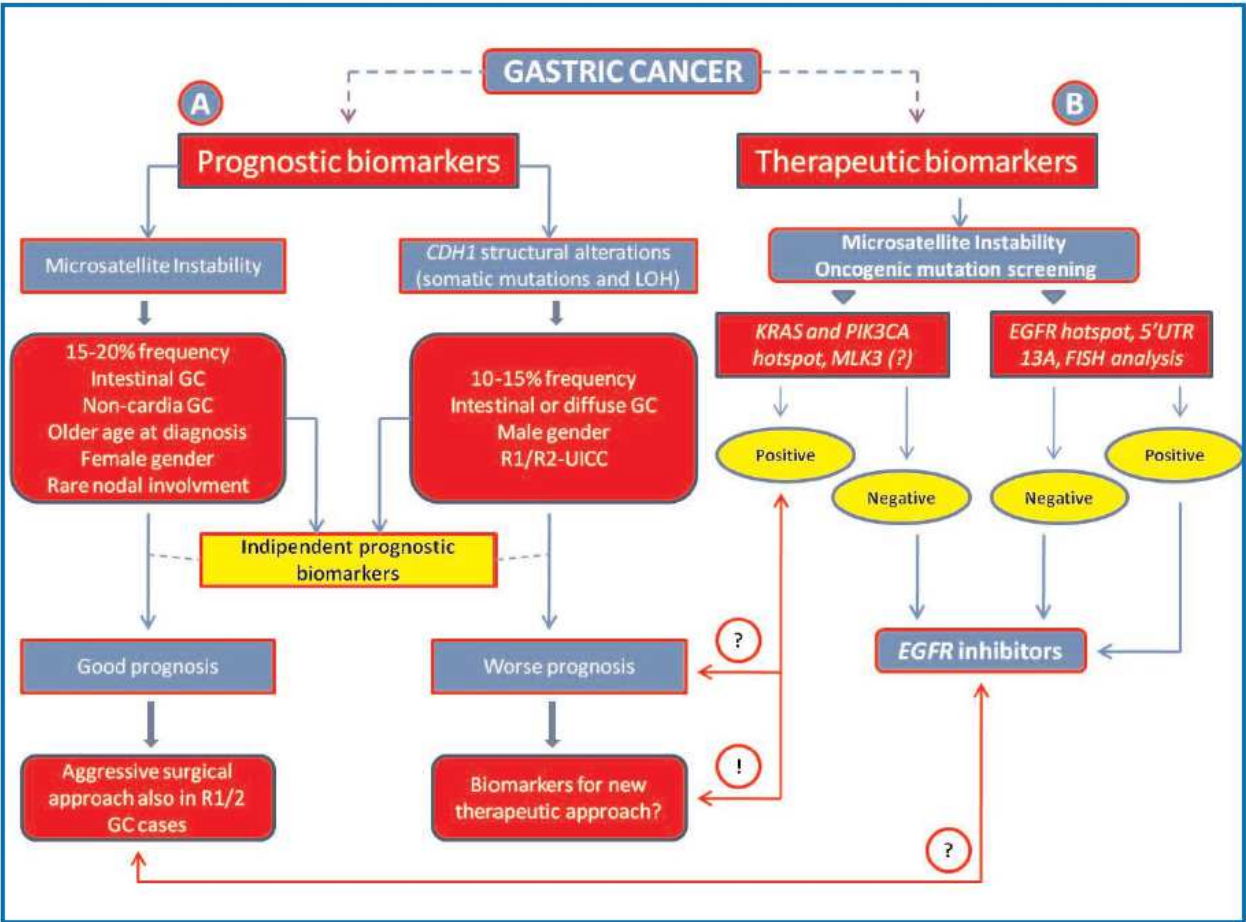


Fig. 2.1 The flow-chart proposes the clinical management of gastric carcinoma with respect to several molecular features of its etiopathogenesis. Prognostic biomarkers include MSI status and *CDH1* structural alterations. Predictive therapeutic biomarkers consider the *MAPK/PI3K* pathway and oncogenic mutations

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PAPER IX

Velho, S., *et al.*, **KRAS signalling pathway alterations in microsatellite unstable gastrointestinal tumors.** Adv Cancer Res 2010;109:123-143

KRAS Signaling Pathway Alterations in Microsatellite Unstable Gastrointestinal Cancers

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- I. Mismatch Repair, MSI, and Related Target Genes
- II. MSI-H Phenotype-Related Molecular and Clinicopathologic Features
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Microsatellite instability (MSI) occurs in about 15% of gastrointestinal cancers and it is associated with specific clinic, pathologic, and molecular features of the tumors. MSI-high (MSI-H) carcinomas also follow specific tumor development pathways. This review is focused on the molecular profile of alterations in members of the KRAS signaling pathway (*EGFR*, *KRAS*, *BRAF*, *PIK3CA*, *RASSF1A*, and *MLK3* genes) in MSI gastrointestinal carcinomas. Alterations in these genes characterize more than half of gastrointestinal cancers and frequently occur simultaneously in the same tumor, pinpointing the KRAS signaling pathway as one of the most frequently altered pathways in this subset of cancers. Nowadays, many and novel inhibitors targeting molecules of this signaling pathway are being described; therefore, it is worthwhile to test their efficacy in MSI gastrointestinal cancers in order to develop new and more directed targeted therapies for patients affected by this disease. © 2010 Elsevier Inc.

I. MISMATCH REPAIR, MSI, AND RELATED TARGET GENES

About 15% of gastrointestinal carcinomas are characterized by the accumulation of numerous mutations across the genome, mainly occurring in repetitive sequences (microsatellites), due to a defective mismatch repair (MMR) system and creating a molecular phenotype designated as microsatellite instability-high (MSI-H) (Ionov et al., 1993).

The MMR system is composed of at least seven proteins, h-MLH1, h-MLH3, h-MSH2, h-MSH3, h-MSH6, h-PMS1, and h-PMS2, which associate with specific partners to form functional heterodimers that recognizes base-pair mismatches and small nucleotide insertion/deletions (1–4 base pairs) that occur during DNA replication (Jiricny, 2006; Rustgi, 2007; Thomas and Boland, 2006). h-MLH1 and h-MSH2 are essential components of the human MMR machinery and form five functional heterodimeric complexes: h-MSH2/h-MSH3, h-MSH2/h-MSH6, h-MLH1/h-PMS1, h-MLH1/h-PMS2, and h-MLH1/h-MLH3 (Jiricny, 2006). Genetic and epigenetic alterations occurring at these MMR system effectors, namely in h-MLH1 and h-MSH2, and less frequently in h-MSH6 and h-PMS2, are the main mechanisms by which MMR system failure occurs in MSI-H gastrointestinal cancers (Peltomaki, 2001).

In cells with a defective MMR system, mutations accumulate not only in noncoding repetitive sequences but also in coding sequences. In the case of instability affecting coding repeat sequences, immediate functional consequences are expected to occur in such genes. Target genes of MMR deficiency include among many others, *TGFBR2*, *IGF1R*, *BAX*, *TCF-4*, *h-MSH3*, *h-MSH6*, *CASPASE-5*, *GRB-14*, *RHAMM*, *RAD50*, *RIZ*, *E2F4*, *PTEN*, *MBD4*, *CHK1*, *FAS*, *APAF-1*, *BCL-10*, and *EPHB2* (Davalos et al., 2006; Duval and Hamelin, 2002). *TGFBR2*, among all described MMR target genes, is the one harboring the highest mutation frequency (about 80%) and these alterations are known to associate with enhanced cell proliferation rate and higher angiogenic potential (Jakowlew, 2006).

II. MSI-H PHENOTYPE-RELATED MOLECULAR AND CLINICOPATHOLOGIC FEATURES

A. Gastric Cancer

The MSI-H phenotype generally characterizes 15–25% of GC cases and it comprises a group of stomach tumors displaying specific clinicopathologic features (Beghelli et al., 2006; Corso et al., 2009; Ottini et al., 2006).

The mechanism leading to MMR deficiency in 75% of MSI-H GC cases is *b-MLH1* promoter hypermethylation (Gu *et al.*, 2009; Pinto *et al.*, 2000). Moreover, *Helicobacter pylori* infection was recently described to be able to downregulate major DNA repair pathways (Machado *et al.*, 2009, 2010). Although somatic mutations in MMR genes have also been described in MSI-H GC, they were proved to constitute a consequence rather than a cause of the mutator phenotype (Pinto *et al.*, 2008).

In terms of clinicopathological features, most MSI-H GCs are of intestinal histotype, are located in the distal part of the stomach, and occur more frequently in older women (Corso *et al.*, 2009; dos Santos *et al.*, 1996; Oliveira *et al.*, 1998; Seruca *et al.*, 1995). Further, this phenotype delineates a subset of tumors with lower ability to invade serosal layers that preferentially spread to the periphery of the stomach via the lymphatic stream to the nodes, therefore generating an overall favorable long-term prognosis even in patients with advanced disease (Corso *et al.*, 2009; dos Santos *et al.*, 1996; Oliveira *et al.*, 1998; Seruca *et al.*, 1995). Data on long-term survival demonstrated that MSI-H advanced GC patients show higher survival rates in comparison to patients with other types of GC even if they are in the same disease stage (Beghelli *et al.*, 2006). Overall, patients with GC displaying MSI-H phenotype have better survival than those with a microsatellite stable (MSS) phenotype. Altogether, this data most probably identify a particular subset of MSI-H tumors biologically less aggressive and with a favorable prognosis (Corso *et al.*, 2009).

MSI-H GC from patients with familial history of GC were recently found to display similar clinicopathologic characteristics to those of MSI-H sporadic GC cases (Leite *et al.*, 2010, in press; Pedrazzani *et al.*, 2009).

B. Colorectal Cancer

MSI-H colorectal cancers (CRCs) arise either in a sporadic or in a hereditary context (Lynch syndrome) (Lynch *et al.*, 1991).

In MSI-H sporadic CRCs, methylation of the *b-MLH1* promoter region is the main mechanism responsible for MMR system failure and occurs in about 75% of the cases (Deng *et al.*, 2004; Lubomierski *et al.*, 2005). Another epigenetic mechanism involved in the somatic deregulation of MMR proteins expression was recently described (Valeri *et al.*, 2010). In this work, the authors reported that overexpression of miR-155 significantly downregulates the core MMR proteins, h-MSH2, h-MSH6, and h-MLH1, inducing a mutator phenotype and MSI. Furthermore, they have demonstrated an inverse correlation between the expression of miR-155 and the expression of h-MLH1 or h-MSH2 proteins in a number of MSI tumors with a previous unknown cause of somatic MMR inactivation (Valeri *et al.*, 2010).

In Lynch syndrome, the most common causal genetic events underlying MMR deficiency are germline mutations at MMR genes, namely, *h-MLH1*, *h-MSH2* (*h-MLH1* and *h-MSH2* accounts for almost 90% of all identified mutations), *h-MSH6*, and *h-PMS2* (Castells *et al.*, 2009; Lynch and de la Chapelle, 2003; Peltom ki and Vasen, 2004).

Recently, deletions of the terminal end of the *EPCAM* gene have been identified in a small number of families with Lynch syndrome whose tumors demonstrate loss of *h-MSH2* (Ligtenberg *et al.*, 2009). In these cases, a failure in transcriptional termination of *EPCAM* results in the generation of fusion transcripts with the adjacent *h-MSH2* gene downstream, giving rise to methylation of the *h-MSH2* promoter, particularly in epithelial tissues where *EPCAM* is expressed at high levels (Ligtenberg *et al.*, 2009). Constitutional epimutations of the *h-MLH1* gene have also been identified in mutation-negative individuals with a clinical diagnosis of Lynch syndrome (Gazzoli *et al.*, 2002; Goel *et al.*, 2010; Hitchins *et al.*, 2005; Miyakura *et al.*, 2004; Niessen *et al.*, 2009; Suter *et al.*, 2004). This defect is characterized by soma-wide promoter methylation and transcriptional silencing of a single allele of the *h-MLH1* gene (Hitchins *et al.*, 2005; Miyakura *et al.*, 2004; Suter *et al.*, 2004). Despite the importance of these findings, the frequencies of germline epimutations of *h-MLH1* and *h-MSH2* seem to be rather infrequent in a cohort of Lynch syndrome-suspected patients (0.6% and 0.9%, respectively) although quite high in the genetically proven Lynch syndrome cases (about 16% of all mutations) (Niessen *et al.*, 2009).

Due to the presence of the mutator phenotype, MSI-H CRCs arising either in a sporadic or in a hereditary context are associated with unique clinical and pathological features namely, proximal location, poor differentiation, and frequent presence of a mucinous component (Cai *et al.*, 2008). The presence of tumor-infiltrating lymphocytes is prevalent in MSI-H tumors and it has been suggested as a useful marker in the identification of the MSI-H phenotype (Banerjee *et al.*, 2009; Chang *et al.*, 2009; Drescher *et al.*, 2009; Greenson *et al.*, 2009). MSI-H CRCs have been found to harbor a lower tumor stage at diagnosis and rarely metastasize to distant lymph nodes or distant organs (Gryfe *et al.*, 2000; Malesci *et al.*, 2007), showing a good prognosis and a better overall and relapse-free survival (Clark *et al.*, 2004; Gryfe *et al.*, 2000). Additionally, several clinical studies have demonstrated that patients with MSI-H CRCs do not benefit from the treatment with 5-fluorouracil (5-FU)-based adjuvant chemotherapies (Arnold *et al.*, 2003; Carethers *et al.*, 1999, 2004; Des Guetz *et al.*, 2009a,b,c; Ribic *et al.*, 2003; Sinicrope and Sargent, 2009).

Nevertheless, and besides the abovementioned shared clinical and pathologic characteristics between hereditary and sporadic MSI-H CRCs,

differences also exist, mainly the sporadic setting of MSI-H tumors arising in the proximal or distal colon.

In the sporadic setting of MSI-H CRCs, cases localized in the distal colon were shown to form a distinct subgroup of tumors with particular clinico-pathological and molecular features that differ from those localized in the proximal MSI-H CRCs. Patients with MSI-H CRCs in distal location were significantly younger and of the male gender, and tumors displayed preferentially differentiated histology, smaller tumor size, distant metastasis, stability at BAT25 and BAT26, and showed h-MLH1 protein expression by immunohistochemistry as compared with proximal MSI-H CRCs. In addition, distal MSI-H CRCs demonstrated significantly worse 3-year overall and disease-free survival rates when compared to proximal MSI-H CRCs (87.0% vs. 97.4%; 81.6% vs. 95.9%). For stage III–IV CRCs, distal MSI-H CRCs also showed significantly worse 3-year overall and disease-free survival rates than proximal MSI-H CRCs (72.2% vs. 90.5%; 58.3% vs. 94.4%) (Kim *et al.*, 2010; Tanaka *et al.*, 2007).

Sporadic MSI-H CRCs are more frequent in older patients and are preferentially associated with the female gender. On the contrary, the cancers from the hereditary setting are associated with a younger age at onset and are more frequently found in males. Furthermore, lymphocytic infiltration, tumor budding (dedifferentiation), and the presence of coexisting adenomas are more evident in HNPCC. Furthermore, the cytological features of Lynch syndrome cancers recapitulate the basophilia and nuclear characteristics of conventional adenomas (Jass, 2004). MSI-H sporadic CRCs are mucinous, show poor differentiation, tumor heterogeneity, and glandular serration, and exhibit coexisting serrated polyps are more evident in sporadic MSI-H CRCs (Jass, 2004). Moreover, sporadic MSI-H CRCs are also characterized by cytoplasmic eosinophilia and nuclei that are large, round, vesicular, and contain a prominent nucleolus (Jass, 2004). Therefore, the crucial distinction between MSI-H hereditary and MSI-H sporadic CRCs should be achieved by integrating all available data including family history, age at onset of malignancy, clinicopathological, and molecular features (Jass, 2004).

III. MSI-H-RELATED EPIGENETIC FEATURES: CPG ISLAND METHYLATOR PHENOTYPE

The CpG island methylator phenotype (CIMP) was firstly described in CRC by Toyota *et al.* (1999) as being a mechanism whereby some CRCs were found to accumulate high rates of aberrant promoter methylation.

In this pioneer work, the authors analyzed the methylation status of several CpG islands in a series of CRCs, colorectal adenomas, and normal colorectal mucosa. They found that the majority of the CpG islands analyzed were methylated in both normal mucosa and in cancers, which is suggestive of an age-related phenomenon. Although, some of the CpG islands studied showed a cancer-specific methylation pattern and characterized a particular group of tumors. These findings raised the hypothesis that CIMP could contribute to CRC. Furthermore, the demonstration that the mismatch-repair gene *h-MLH1* was frequently inactivated due to promoter methylation in the CIMP positive group of CRCs was a strong argument favoring the involvement of an epigenetic mechanism working in CRC development (Toyota *et al.*, 1999). In fact, many other genes involved in cancer have a CpG island within their promoter region and so they are potential targets for transcriptional repression through methylation in a CIMP context (Toyota *et al.*, 1999).

CIMP was shown to be associated with several clinicopathologic and molecular features of CRCs. It is more frequently found in tumors located in the proximal colon (Toyota *et al.*, 1999). It is associated with female gender (Ogino *et al.*, 2006), older age, poor differentiation, and mucinous histology (Whitehall *et al.*, 2002). At the molecular level, CIMP was shown to be more frequent in sporadic cancers characterized by the presence of oncogenic BRAF and MSI-H (Ahuja *et al.*, 1997; Weisenberger *et al.*, 2006). Moreover, CIMP has been observed to occur in nonmalignant serrated lesions and implicated in the development of sporadic MSI-H CRCs in close association with the presence of oncogenic BRAF (O'Brien *et al.*, 2006).

On the other hand, Lynch syndrome cancers seldom display CIMP, but whenever present, this methylator phenotype affects genes different from those that are aberrantly methylated in the sporadic counterpart (Herman *et al.*, 1998; Kuusmanen *et al.*, 2000; Wheeler *et al.*, 2000; Yamamoto *et al.*, 2002). For example, the frequency of DNA methylation at *hMLH1*, *CDKN2a*, and *THBS1* genes was found to be significantly lower in MSI-H cancers from Lynch patients than in MSI-H sporadic cancers (Herman *et al.*, 1998; Kuusmanen *et al.*, 2000; Wheeler *et al.*, 2000; Yamamoto *et al.*, 2002).

CIMP phenotype was also reported to occur in about 31% of MSI-H gastric carcinomas (An *et al.*, 2005). Genes such as *hMLH1*, *MGMT*, *CDH1*, and *COX2* were shown to be frequently methylated (Carvalho *et al.*, 2003). Similar to CRC, in gastric carcinomas, MSI-H status is also closely associated with *hMLH1* promoter hypermethylation and CIMP status (An *et al.*, 2005; Carvalho *et al.*, 2003). Furthermore, hypermethylation at promoter regions might be a particularly important epigenetic determinant in gastric cancer development, since it has been reported to increase along progression from chronic gastritis, intestinal metaplasia, and adenomas to carcinomas of the stomach (Fleisher *et al.*, 2001).

IV. MSI-H-RELATED GENETIC FEATURES: THE KRAS SIGNALING PATHWAY

MSI-H gastrointestinal cancers display particular epigenetic and genetic backgrounds associated with specific molecular pathways, therefore reflecting their different clinic and pathologic features. A disturbed KRAS signaling pathway represents an important event in the initiation and progression of GC and CRCs, as well as, an important therapeutic biomarker.

A. Molecular Alterations in the KRAS Signaling Pathway

Activation of the KRAS signaling pathway is a frequent event in the carcinogenesis process of gastrointestinal malignancies. The KRAS signaling pathway is a very broad pathway that is involved in the control of several other signaling pathways by interacting with a wide spectrum of direct downstream effector molecules (Fig. 1). The mutational activation of *KRAS* and other molecules belonging to the pathway, such as epidermal

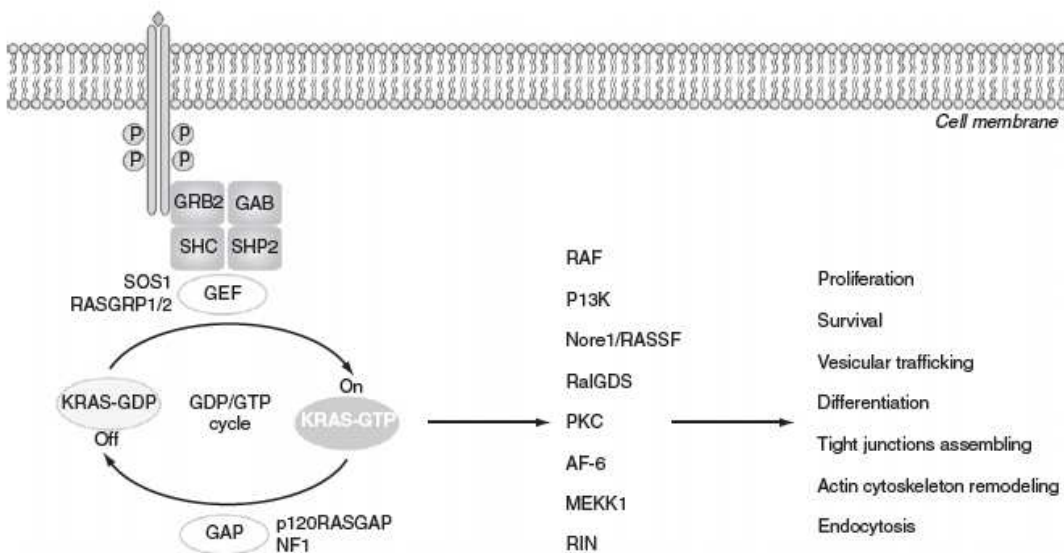


Fig. 1 KRAS activation, downstream targets, and cellular effects. At the plasma membrane, cell surface receptors are activated by extracellular stimuli. Activated cell surface receptors interact with a variety of adaptor proteins (GRB2, GAB, SHC, and SHP2) that mediate the subsequent activation of GEFs (SOS1 and RASGRP1/2). Active GEFs catalyze the transition from a GDP to an active GTP-bound state. Active KRAS interacts with several downstream effectors (RAF kinases, PI3K, Nore1/RASSF, RalGDS, PKC, AF-6, MEKK1, and RIN) and by this way, it is involved in the control of a variety of cellular functions.

growth factor receptor (*EGFR*), phosphatidylinositol 3-kinase (*PI3K*) and mixed-lineage kinase (*MLK3*), and RAS-association domain family isoform 1 A (*RASSF1A*), constitute important events in the activation of this signaling pathway in these types of cancer (Fig. 1).

In CRCs, *KRAS*, a small guanosine triphosphatase (GTPase) and *BRAF*, a serine/threonine protein kinase, which are key players of the mitogen-activated protein kinase (MAPK) signaling pathway, are often mutated (Rajagopalan *et al.*, 2002). Oncogenic mutations in these genes are preferentially found as alternative molecular alterations (Rajagopalan *et al.*, 2002) and due to this fact, they were initially thought to signal through the same pathway. However, their mutational frequency is not homogenous among the different subsets of CRC, in particular, in the MSI-H subset of carcinomas (Domingo *et al.*, 2004a; Rajagopalan *et al.*, 2002), suggesting that rather than leading to similar functional roles, *KRAS* and *BRAF* oncogenes may play different and independent functions in the development and progression of MSI-H CRCs.

KRAS mutations characterize both sporadic and hereditary MSI-H tumors but they are more frequent in the later (40%) rather than in the former (20%) (Deng *et al.*, 2004; Domingo *et al.*, 2004a; Lubomierski *et al.*, 2005; Oliveira *et al.*, 2004). Moreover, the *KRAS* mutation frequency and pattern differ among the different subsets of MSI-H CRCs (hereditary and sporadic). In CRC, the most frequent amino acid changes observed were G12D, G12V, and G13D (Oliveira *et al.*, 2004). *KRAS* mutations in codon 12 were more common in sporadic cases, whereas mutations in codon 13 were predominant in the MSI hereditary setting (Oliveira *et al.*, 2004). Although, the reasons underlying this observation are not yet elucidated, it is likely that different *KRAS* mutations are differentially selected for tumor development/progression depending on distinct tumor contexts.

BRAF mutations occur preferentially in the sporadic setting of MSI-H CRCs (40%) (Domingo *et al.*, 2004a; Oliveira *et al.*, 2003) where they strongly correlate with the presence of a genome-wide CIMP phenotype and *h-MLH1* promoter hypermethylation (Deng *et al.*, 2004; Lubomierski *et al.*, 2005; Nosho *et al.*, 2008; Ogino *et al.*, 2009; Shen *et al.*, 2007; Weisenberger *et al.*, 2006). In addition, the presence of mutant *BRAF* was demonstrated to be a poor prognostic indicator, being found associated with advanced phases of the disease (Zlobec *et al.*, 2009) and leading to abrogation of the good prognosis associated to MSI-H CRCs (Ogino *et al.*, 2009). On the other hand, *BRAF* mutations were very rarely found in Lynch syndrome associated carcinomas (Deng *et al.*, 2004; Domingo *et al.*, 2004a; Lubomierski *et al.*, 2005; Oliveira *et al.*, 2004), and therefore the detection of a positive *BRAF* mutation in a CRC is indicative of its sporadic origin and suggests the use of *BRAF* mutation testing as an exclusion criteria when selecting hereditary MSI cancers (Domingo *et al.*, 2004b, 2005).

In the case of cancers arising in the stomach, *KRAS* mutations were described in about 4% of the cases, while *BRAF* mutations were extremely rare (Oliveira *et al.*, 2003). In GC, *KRAS* mutations were only observed in the MSI subset, in about 30% of the cases (Brennetot *et al.*, 2003).

In addition to *KRAS* and *BRAF* mutations, alterations in other *KRAS* downstream effectors, such as PI3K activation through *PI3K* catalytic alpha subunit (*PIK3CA*) mutations and *RASSF1A* inactivation through promoter hypermethylation, are also found in CRCs (Samuels *et al.*, 2004; van Engeland *et al.*, 2002) and represent another example of a disturbed *KRAS* signaling pathway.

PIK3CA oncogenic mutations were described to characterize both sporadic and hereditary MSI CRCs, occurring at similar frequencies in both tumor settings. In the sporadic setting, *PIK3CA* mutations were described to occur in about 16% of the cases, whereas they occur preferentially associated with the presence of *KRAS* or *BRAF* oncogenic mutations (Campbell *et al.*, 2004; Samuels and Velculescu, 2004; Velho *et al.*, 2005). *PIK3CA* gene mutations were also described to occur in about 15% of hereditary MSI colorectal carcinomas (Ekstrand *et al.*, 2009; Miyaki *et al.*, 2007), and were also found to occur in cases harboring *KRAS* oncogenic mutations (Ekstrand *et al.*, 2009). Both in sporadic and hereditary tumors, *PIK3CA* oncogenic alterations were associated with an invasive phenotype (Miyaki *et al.*, 2007).

PIK3CA mutations were also described in about 4–11% of GC cases (Lee *et al.*, 2005; Li *et al.*, 2005; Velho *et al.*, 2005). A higher frequency was initially described by Samuels *et al.* (2004) (25%), but this study was performed in a small series of tumors ($n = 12$) (Samuels *et al.*, 2004). A deeper analysis, performed later, has demonstrated that *PIK3CA* alterations occur specifically in the MSI subset of GC (Li *et al.*, 2005; Velho *et al.*, 2005). Moreover, *PIK3CA* gene mutations and *KRAS* oncogenic alterations were described as alternative genetic events in this subgroup of GC (Velho *et al.*, 2005).

Hypermethylation of the promoter region of *RASSF1A* gene was reported to occur in 52% of sporadic MSI CRCs and in 30% of MSI hereditary carcinomas (Oliveira *et al.*, 2005). Interestingly, and despite similar high frequencies of *RASSF1A* promoter hypermethylation were found in both CRC settings, sporadic MSI cancers accumulate overall significantly more not only epigenetic but also genetic alterations in genes belonging to the *KRAS* signaling pathway (Oliveira *et al.*, 2005). *RASSF1A* promoter hypermethylation was also reported as a frequent epigenetic alteration in MSI gastric malignancies and it was found to occur mainly in tumors lacking *KRAS* mutations (Oliveira *et al.*, 2005).

Nevertheless, alterations in molecules functioning upstream of *KRAS*, such as the receptor tyrosine kinase EGFR, as well as in other molecules involved in mediating *KRAS* signal, independently of being its direct targets,

such as the MLK3, are also important players in the deregulation of this signaling pathway in MSI carcinomas.

EGFR overexpression was reported to occur in 30–85% of human CRCs (Jorissen *et al.*, 2003; Shia *et al.*, 2005), while mutations in the kinase domain are rare in this type of cancer (Barber *et al.*, 2004; Ogino *et al.*, 2005). In GC, although the frequency of EGFR overexpression is high, the mechanisms responsible to this overexpression remain undiscovered in most cases (Becker *et al.*, 2006; Takehana *et al.*, 2003). *EGFR* increased copy number was reported in about 13% of GC, which was mainly attributed to polysomy of chromosome 7 rather than to *EGFR* gene amplification (Moutinho *et al.*, 2008). Furthermore, mutations at the *EGFR* kinase domain were found very rarely in GC (Mammano *et al.*, 2006; Moutinho *et al.*, 2008).

Recently, a novel genetic mechanism underlying EGFR overexpression was described in MSI-H CRCs (Yuan *et al.*, 2009). High levels of EGFR expression were detected in MSI CRC cell lines due to the presence of a novel deletion in a polyA(13) repeat element within the 3'-untranslated region (3'-UTR) of the *EGFR* gene. Approximately 64% of MSI-H CRC cell lines and 69% of MSI-H CRC cases presented deletions at this repeat sequence (Yuan *et al.*, 2009). This alteration was shown to enhance *EGFR* mRNA stability, thus leading to EGFR protein overexpression (Yuan *et al.*, 2009). Our group has recently investigated the frequency of this alteration in a cohort of 63 MSI-H GCs and respective normal mucosa. We verified that about 32% of the GCs studied displayed tumor-specific deletions at the *EGFR* polyA(13) repeat element (unpublished data). In addition, this type of *EGFR* deletions was found to occur either as isolated genetic alterations or in concomitance with mutations of *KRAS* and/or *PIK3CA* genes, suggesting a cumulative effect of both oncogenic events in MSI-H GCs.

The high frequency of deletions in *EGFR* polyA(13) repeat element found in MSI-H gastrointestinal cancers, and its association with EGFR protein overexpression pinpoints EGFR as a good candidate for target therapy in MSI-H gastrointestinal cancers. Further studies are needed in order to determine the relationship between the presence of this *EGFR* mutation and the potential use of anti-EGFR monoclonal antibodies in the treatment of MSI-H cancers, perhaps by adapting the least successful conventional chemotherapy regimens. Nevertheless, a cautionary note should be added since deletions at the *EGFR* polyA(13) repeat may also occur in association with *KRAS* mutations (unpublished data) which is by itself a negative predictor of response to anti-EGFR monoclonal targeted therapies (Lièvre *et al.*, 2006, 2008).

MLK3 is a serine/threonine protein kinase that regulates the MAPKinase pathway activating ERK, p38, and JNK, in response to extracellular signals (Chadee and Kyriakis, 2004; Gallo and Johnson, 2002). In a recent paper by our group (Velho *et al.*, 2010), we described for the first time MLK3

mutations in a series of gastrointestinal cancers (Velho *et al.*, 2010). *MLK3* gene mutations were significantly associated with MSI phenotype, occurring in 21% of the MSI tumors (Velho *et al.*, 2010). Interestingly, cells expressing mutant forms of this gene showed transforming capacity *in vitro*, and were able to develop locally invasive tumors when subcutaneously injected in mice. Therefore, *MLK3* was described as a new candidate biomarker and/or an attractive potential target for future therapy in MSI gastrointestinal tumors (Velho *et al.*, 2010).

B. Functional Effects of KRAS and BRAF Oncogenes in MSI-H Colorectal Cancer

Due to the specific *KRAS* and *BRAF* mutational profile observed either in MSI-H CRCs or in nonmalignant lesions, it is possible to hypothesize that oncogenic signals from these mutant genes may not rely on the same signaling pathway and thus they may harbor distinct functional roles in MSI-H CRC development and progression. Furthermore, and as mentioned earlier, MSI-H CRCs are described as being originated through the serrated carcinogenesis pathway in which *BRAF*, in association with *CIMP*, seems to be a key player (O'Brien *et al.*, 2006). Data from *in vitro* studies support the assumption of a different functional role of these oncogenes. By inhibiting *BRAF* protein expression by RNA interference, our group showed that *BRAF* provides proliferation signals through ERK activation, by increasing the levels of Cyclin D1 and decreasing the levels of p27Kip1 (Preto *et al.*, 2008). Further, we demonstrated that *BRAF* mutation provides survival signals through Bcl-2 in MSI-H CRC cell lines (Preto *et al.*, 2008). In addition, we also demonstrated that *BRAF* inhibition was not sufficient to decrease proliferation and induce apoptosis in *KRAS* mutant MSI-H CRC cell lines (Preto *et al.*, 2008). Overall, these results suggest that *KRAS*-mediated oncogenic signaling do not entirely follow through the *KRAS*/*BRAF*/MAPkinase signaling cascade and strengthen the idea that *KRAS* and *BRAF* activation, in MSI-H CRCs, most likely activate distinct signaling pathways by interfering with different cellular functions.

V. A SERRATED CARCINOGENESIS PATHWAY IN THE ORIGIN OF SPORADIC MSI CRC

In 1988, Fearon and Vogelstein proposed an explaining mechanism for CRC development known as the adenoma–carcinoma model (Fearon and Vogelstein, 1990). This was a linear, multistep sequence of accumulation of

genetic alterations, where inactivating mutations of *APC* gene generated the adenoma formation. Acquisition of further genetic alterations, namely *KRAS* oncogenic activation and *p53* inactivating mutations, would then function as driving forces toward progression from adenoma to carcinoma (Fearon and Vogelstein, 1990) (Fig. 2).

Currently, it is widely accepted that the abovementioned adenoma–carcinoma concept oversimplifies the heterogeneity of the molecular, clinical, and pathological basis of CRC, and therefore cannot be applied to all types of CRCs. For instance, *APC*, *KRAS*, and *p53* mutations, the typical molecular alterations of this Vogelstein pathway, were found to occur in the same tumor only in 7.7% of CRCs (Imai and Yamamoto, 2008; Samowitz *et al.*, 2007; Smith *et al.*, 2002). A much higher percentage of concomitant mutations would be expected if the adenoma–carcinoma pathway was the main sequence leading to CRC initiation and development. Furthermore, the

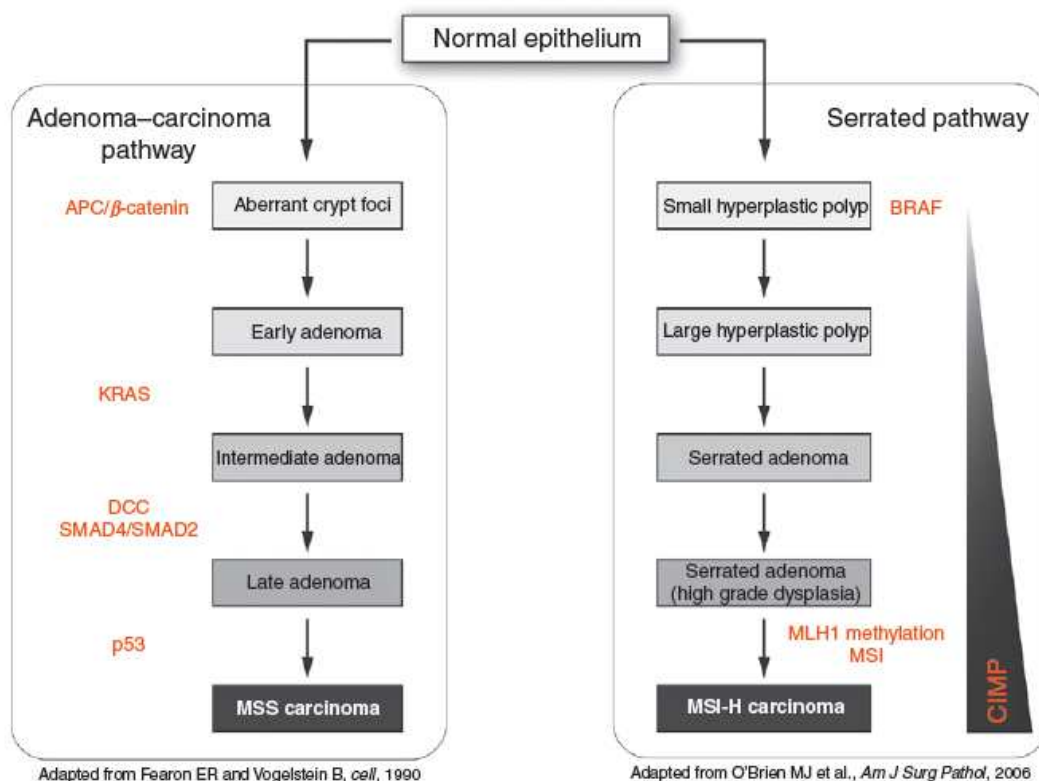


Fig. 2 Pathways for colorectal cancer development. (A) In the adenoma–carcinoma sequence, inactivating mutations of *APC* gene are the onset of adenoma formation. Acquisition of further genetic alterations, namely *KRAS* oncogenic activation and *p53* inactivating mutations, would then function as driving forces toward progression from adenoma to a MSS carcinoma. (B) *BRAF* mutation is a specific marker for the serrated polyp pathway that has its origin in a hyperplastic polyps and a potential end point as a MSI carcinoma. CIMP develops early in this sequence and MSI develops late due to *h-MLH1* silencing by promoter methylation.

molecular features of sporadic MSI-H/*BRAF* mutant/CIMP-positive CRCs do not fit within the adenoma–carcinoma pathway, since the frequency of *KRAS* mutations is low in this tumor setting and the inactivation of *APC* and *p53* genes is very rare (Jass, 2007; Samowitz *et al.*, 2007). These evidences demonstrate that CRCs are not homogenous subsets with respect to their molecular, clinical, and pathological features, and so alternative genetic pathways to CRC initiation and development have been proposed (Jass, 2006) in order to improve our knowledge about the biology of CRC and to better explain its diversity.

In this regard, a serrated pathway for CRC origin has emerged (Jass, 2005). The progressive stages of the serrated neoplasia pathway are separate and distinct from those of the traditional adenoma–carcinoma sequence (Jass, 2005; O'Brien *et al.*, 2006). In this pathway, hyperplastic polyps (HPs), traditional serrated adenomas (TSAs), and sessile serrated adenomas (SSAs) are assumed to be the precursor lesions of tumor development (O'Brien *et al.*, 2006). For a long time, these serrated polyps were seen as inoffensive lesions of the colon with no potential to become malignant. However, molecular studies performed on these type polyps demonstrated that they already harbor alterations that are commonly found in CRCs, such as *BRAF* and *KRAS* mutations, CIMP positivity, *b-MLH1* hypermethylation, and MSI-H phenotype (Kambara *et al.*, 2004; O'Brien, 2007; O'Brien *et al.*, 2006; Yang *et al.*, 2004), and thus the possibility of progression to cancer was strengthened. In fact, mutant *BRAF* is a specific marker for the serrated polyp pathway that has its origin in a HP with the potential to originate a MSI-H carcinoma (Kim *et al.*, 2008; O'Brien *et al.*, 2006). Further, *BRAF* mutations are frequently associated with the presence of CIMP-H (Kambara *et al.*, 2004; Kim *et al.*, 2008; Velho *et al.*, 2008), and both events arise early in the serrated sequence (Wynter *et al.*, 2004; Yang *et al.*, 2004). Aberrant, widespread methylation of CpG islands increases with the histological progression of serrated adenomas (Dong *et al.*, 2004). Loss of microsatellite stability due to *b-MLH1* promoter hypermethylation occurs at late stages and is the key event inducing MSI and malignant transformation (Kambara *et al.*, 2004; Velho *et al.*, 2008) (Fig. 2).

KRAS mutations might also be involved in the development of sporadic MSI-H tumors through the serrated pathway, since mutations in this gene are also found in colonic serrated lesions (O'Brien *et al.*, 2006; Velho *et al.*, 2008). However, this may represent an alternative serrated pathway that diverges from the one in which *BRAF* gene is involved. First, the type of serrated lesions in which *KRAS* mutations occur is different from the ones that harbor *BRAF* mutations. *BRAF* mutations are more frequently found in sessile-serrated adenomas while *KRAS* mutations occur more frequently in the other types of serrated lesions, such as TSAs and mixed hyperplastic and adenomatous polyps and goblet cell-serrated polyps (O'Brien *et al.*, 2006;

Velho et al., 2008; Yang et al., 2004). Second, the presence of *KRAS* mutations is not associated with the presence of CIMP-H and *b-MLH1* hypermethylation (O'Brien et al., 2006; Velho et al., 2008), which implies a different molecular environment involved in the progression from nonmalignant to a malignant state. Both evidences suggest that sporadic CRCs originated in a *KRAS* mutant context or in a *BRAF* mutant background can represent distinct molecular, clinical, and pathologic entities.

VI. CONCLUSIONS

An altered *KRAS* signaling pathway represents an important factor in the development and progression of gastrointestinal carcinomas. Mutations of *KRAS*, *PIK3CA*, and *MLK3* genes, as well as *RASSF1A* promoter hypermethylation are commonly found in MSI sporadic gastrointestinal carcinomas and in hereditary MSI CRCs. The pattern of *BRAF* oncogenic mutations show a more restricted pattern being only found in MSI sporadic CRC. Moreover, the results obtained from the *KRAS*-related genes mutation detection in premalignant lesions as well as those obtained from the functional analysis of *KRAS* and *BRAF* mutant CRC cell lines support that *KRAS*- and *BRAF*-dependent signaling may lead to different carcinogenic pathways. Moreover, concomitant alterations in genes from the *KRAS* signaling pathway are frequently observed, suggesting a strengthening of the signaling or a possible dependency of gastrointestinal cancers on this molecular pathway. This knowledge has potential clinical implications and may be translated into the development of novel and more directed targeted therapeutic strategies in a near future.

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5. GENERAL CONCLUSIONS AND FUTURE PERSPECTIVE

Molecular biomarkers will help improving the staging system of gastrointestinal cancer and their use in clinical practice is also important to find novel ways to handle patients in an efficient medical approach. In gastric carcinoma, in particular, the identification of molecular markers that can help to predict disease progression will have an important impact in the surveillance of gastric carcinoma patients and may represent a tool to impair the general worse prognosis of this cancer.

In this study we identified novel predictive molecular biomarkers that can be classified as prognostic and therapeutic biomarkers, specific for gastric carcinoma. We verified that MSI status, specifically when all markers are unstable, associates with a very good prognosis. Particularly interesting is the fact that MSI is associated to good prognosis even in advanced GC, namely in cases with pT4. The identification of good predictive prognostic biomarkers open new surgical approaches in surgical treatment of advanced GC and of tumor recurrence. To date, physicians observe GC recurrence as a limitation of surgical treatment, offering only palliative treatment as conventional chemotherapies. We demonstrated that patients with GC relapses show a long term survival when carcinomas show a MSI phenotype. This finding justifies a more radical surgery also in GC with metastatization. In fact we suggest that GC patients with MSI advanced gastric carcinoma ($\geq$ pT2) can perform an aggressive surgical treatment (R0 surgery).

Among the prognostic biomarkers, we verified that structural alterations of *CDH1* gene, as somatic mutation and intragenic deletion, are related with a worse GC prognosis. Interestingly, among *CDH1* structural alterations, the LOH status is not related with a specific histotype. In cases with somatic alterations of E-cadherin the clinical approach to these patients should be reconsidered. Moreover, somatic epigenetic change as the E-cad promoter hypermethylation, can be considered further for medical treatment with demethylating agents, as the 5'-aza-cytidine.

Exploring the role of *CDH1* germline mutations in sporadic EODGC, we proposed a revision of the clinical criteria suggesting the cut-off age of ≤ 35 years for the E-cad genetic screening; this discovery was supported by the presence of pathogenic mutations only in very young GC patients (≤ 35 years).

Our study opened a new window in the scenario for GC treatment, namely the use of tyrosine kinase inhibitors as *EGFR* inhibitor drugs, as Cetuximab and Trastuzumab. To date, clinical trials demonstrated consistently the benefit in long term survival of these molecular antibodies, in particular when associating with conventional chemotherapies (plus protocol). However it remained to determine which are the predictive biomarkers that can be used for eligibility of GC patients for anti-*EGFR* therapies. We identified a novel mechanism of *EGFR* activation in GC, as mutation/deletion at the polyA site in the 3'-UTR region. The presence of *EGFR* A-13 repeat mutations and the mutation screening of the *MAPK/PI3K* pathway represents a setting of molecular markers that should be analysed in order to elicit GC patients for *EGFR* inhibitors therapy. These results were presented at the 2011 Gastrointestinal Cancers Symposium in San Francisco (CA) and the report was selected by the ASCO Cancer Foundation as merit award winner. *Steven A. Curley, MD, chair of the GI Symposium Steering Committee comments these discoveries: "These intelligent young researchers have demonstrated passion for improving the quality of life for people living with gastrointestinal cancers. We are proud to recognize this year's recipients and hope these awards will strengthen their commitment to advancing clinical cancer diagnosis, treatment and research."* (Citation reported by the following journals: ASCO official journal, Medical News Today, World Book and News and Liquida: worldwide news and opinion).

For clinical approach we proposed a flow-chart (figure 19) in which biomarkers are divided in two groups: A) prognostic and B) therapeutic biomarkers. The group A includes the MSI status and structural *CDH1* alterations as prognostic biomarkers, respectively with a good and worse prognosis. The group B considers therapeutic biomarkers in patients that can benefit of *EGFR* inhibitors.

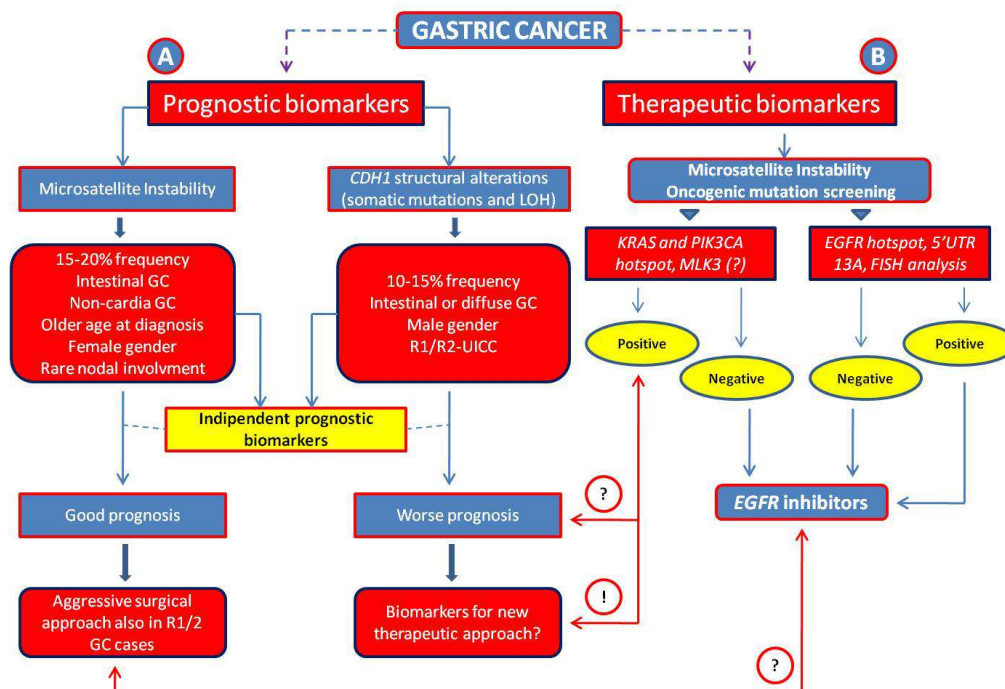


Figure 19

The discovery of different molecular biomarkers predicting the efficacy of novel therapeutic agents in advanced gastric carcinoma treatment represents the way to GC control.

We need to determine which markers will be best for each type of cancer, and studies like this are important steps. The application of novel chemotherapy drugs in gastric carcinoma treatment requires the acknowledgment of selected molecular targets. In particular, we will study other specific molecular markers predictives of response to inhibitors targeting trastuzumab that inactivates the *HER-2* over-expression, everolimus and mTOR inhibitors that block at *PIK3CA* hotspot mutations and PHA-665752 or JNJ38877605 also namely as the c-MET inhibitors (figure 12). All these cancer inhibitors are currently in clinical trial evaluation and will represent the future of advanced gastric carcinoma treatment.

7. SUMMARY

The general goal of this working plan was to identify molecular parameters that can predict prognosis, response to treatment and thus improving the management of GC. Specifically, we aimed in this project to study the role of *EGFR* signalling cascade in GC, to identify novel therapeutic and prognostic biomarkers.

Our goal was achieved by following a bottom-up approach that addressed the role of alterations of selected genes (namely E-cadherin) in GC progression.

In order to accomplish our goal data information on 500 GC patients were collected and observed with clinical, familial, diagnostic, surgical, pathological, and follow-up data. The biological material of 500 GCs included tumor and normal gastric tissue flash frozen, directly collected in the operating room. All materials were obtained from the Department of Human Pathology and Oncology, section of General Surgery and Surgery Oncology, University of Siena, Italy.

During the working plan we:

1. *Address the role of MSI as molecular biomarkers.* We studied the frequency of MSI in GC, and verified the clinico-pathologic associations in sporadic and in cases with familial clustering of GC.
2. *Analyse of the -77T/C XRCC1 polymorphism in GC risk development.* We considered the potential tumor risk in patient carriers of -77T/C variation.
3. *Address the role of E-cadherin deregulation in GC progression.* We searched for the presence of somatic E-cadherin mutations as well as E-cadherin promoter hypermethylation and loss of heterozygosity in a large series of GC patients and selected families which fulfil the HDGC criteria as well as EOGC cases searching for the presence of E-cadherin germline mutations.
4. *Address the role of EGFR activation in GC progression.* We searched for the presence of *EGFR* somatic mutations within the kinase domain and the repeated region of the gene. The mutation screening was extended also at the other oncogenes in the *MAPK/PI3K* pathway, namely *KRAS*, *BRAF*, *MLK3*, *PIK3CA*.

With this working plan we verified that:

1. MSI tumors showed peculiar clinico-pathologic features and better prognosis when compared to stable tumors. Considering these results, a peculiar subset of tumors with instability at all five loci and a very good prognosis has been observed. Moreover, we verified that in our gastric carcinoma MSI do not represents a standard biomarker of hereditary or familial setting, opposing to what is observed in colon cancer where MSI phenotype is the hallmark of the HNPCC syndrome,.
2. We found that the -77C/C variaton was associated with increased risk of GC located in the upper third region, specifically the cardia zone. Moreover, this polymorphism is associated to smoking habits and with familial aggregation for lung cancer. These results suggested us that part of GC can be underlied by the same genetic factor of lung cancer.
3. Structural alterations, in particular LOH and somatic mutations, of *CDH1* gene were assessed as predictive biomarkers of worse prognosis. Hypermethylation results from this study could be used to define the basis for the use of demethylating agents as possible novel anticancer therapy. Considering the very low frequency of E-cad germline mutations (2.3%) in juvenile GC patients and the high incidence of non-pathogenic mutations, we revised the clinical criteria for *CDH1* genetic screening and recommend this screening in GC cases with age at onset ≤ 35 years and with diffuse histotype.
4. We identified a novel mechanism of *EGFR* activation in GC, located at the polyadenylation site in the 3'-UTR region. The presence of *EGFR* A-13 repeat mutations and the information of the mutation status of the *MAPK/PI3K* pathway could be used as novel biomarkers to include GC patients for target therapies using *EGFR* inhibitors in advanced GC patients, in particular with cetuximab-plus protocol.

8. SUMÁRIO

Este projeto de doutoramento teve como principal objetivo a identificação de parâmetros moleculares com poder preditivo de prognóstico ou resposta à terapia, contribuindo assim para um melhoramento na gestão e aconselhamento dos doentes com cancro gástrico. Em particular, no decorrer deste projecto foram estudadas várias moléculas envolvidas na via de sinalização do *EGFR* com o intuito de identificar novos biomarcadores de prognóstico e terapia.

Este objectivo foi alcançado seguindo uma abordagem retrospectiva que incidiu no estudo do papel desempenhado por alterações em genes (nomeadamente Caderina-E) previamente seleccionados na progressão do cancro gástrico.

Para completar os nossos objectivos; informação relativa às caraterísticas clínicas, familiares, de diagnóstico, cirúrgicas, patológicas e dados de seguimento clínico de 500 casos de cancro gástrico foram recolhidas e analisadas. O material biológico recolhido incluía tecido tumoral e normal congelado, colhido diretamente na sala de operações. Todo o material foi obtido no Departamento de Patologia Humana e Oncologia, secção de Cirurgia Geral e Cirurgia Oncológica, da Universidade de Siena, Itália.

Durante o plano de trabalho foram estabelecidos os seguintes objectivos:

1. *Determinação do uso do fenótipo “MSI” como potencial biomarcador molecular.* Procedeu-se ao estudo da frequência de “MSI” em cancro gástrico e da sua correlação com as caraterísticas clinic-patológicas e agregação familiar dos carcinomas gástricos.
2. *Análise do polimorfismo -77T/C XRCC1 no desenvolvimento de cancro gástrico.* Neste objectivo, foi avaliado o risco de desenvolvimento tumoral em portadores da variação -77T/C.
3. *Determinação do papel da desregulação da Caderina-E na progressão de cancro gástrico.* Este objectivo foi alcançado através da pesquisa de mutações do gene da Caderina-E, da determinação do estado hipermetilação do seu promotor e do estudo de perda de heterozigotia numa série alargada de cancro gástrico. Adicionalmente, procedeu-se à análise de mutações germinativas no gene da

Caderina-E em casos de cancro gástrico que preenchiam os critérios de HDGC e EOGC.

4. *Estudo do papel da activação do EGFR na progressão de cancro gástrico.* Este objectivo foi alcançado através da determinação da presença de mutações somáticas no domínio de cinase do *EGFR*; a pesquisa mutacional foi alargada a outros oncogenes envolvidos na via de sinalização das *MAPK/PI3K*, tais como, *KRAS*, *BRAF*, *MLK3* e *PIK3CA*.

Com este plano de trabalho nós verificámos que:

1. Os tumores MSI analisados apresentam características clinico-patológicas específicas e um melhor prognóstico quando comparados com tumores estáveis. Tendo em conta estes resultados, verifica-se uma correlação positiva entre tumores com instabilidade de microssatélites em todos os locus analisados e um melhor prognóstico. Adicionalmente, observa-se que nos carcinomas gástricos a presença de MSI não representa um biomarcador de hereditabilidade ou agregação familiar, ao contrário do que se verifica nos tumores colorectais, nos quais a presença do fenótipo MSI é uma das características principais da síndrome de “HNPCC”.

2. A presença da variação -77C/C está associada a um risco aumentado de cancro gástrico no terço superior do estômago, nomeadamente na região da cárdia. Adicionalmente, a presença do polimorfismo está também associada a fumadores activos e a casos com agregação familiar de cancro do pulmão. Estes resultados sugerem que uma parte dos cancro gástrico podem ter um factor etiológico comum aos tumores do pulmão.

3. As alterações estruturais, em particular perda de heterozigotia e mutações somáticas, do gene *CDH1* são determinadas como preditivas de um pior prognóstico. Os resultados de hipermetilação obtidos neste estudo permitem definir uma possível base para uso de agentes que revertem a metilação como uma nova terapia anti-cancro. Tendo em conta a baixa frequência de mutações germinativas da Caderina-E (2.3%) em doentes jovens com cancro gástrico e a alta incidência de mutações não patogénicas, propusemos uma revisão dos critérios clínicos para pesquisa de alterações da Caderina-E e recomendamos

que esta pesquisa seja feita em pacientes com cancro gástrico difuso e com idade ≤ 35 anos.

4. Um novo mecanismo de activação do *EGFR*, localizado na região de poliadenilação do 3'-UTR, em cancro gástrico. A determinação da presença de mutações na repetição A-13 do *EGFR* em associação com informação acerca do estado mutacional da via *MAPK/PI3K* poderão ser factores usados como marcadores de resposta a terapias alvo em doentes com CG avançado, como no caso do protocolo plus-Cetuximab.

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10. Appendices

The following papers were used only in the Introduction section.

PAPER 10.1

Marrelli, D., et. al., Negative Helicobacter pylori status is associated with poor prognosis in patients with gastric cancer. Cancer 2009;115:2071-2080.

Negative *Helicobacter Pylori* Status Is Associated With Poor Prognosis in Patients With Gastric Cancer

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BACKGROUND: Recent studies have suggested that *Helicobacter pylori* (*H. pylori*) infection may be related to better prognosis in patients with gastric cancer, but to the authors' knowledge, this finding has not yet been validated. In the current study, the association between *H. pylori* status and clinical outcome was investigated in a large cohort of patients. **METHODS:** Frozen non-neoplastic gastric mucosa and serum samples obtained from 297 patients who underwent surgery for primary gastric cancer between 1988 and 2004 were retrieved from the serum and tissue bank of the study department. *H. pylori* status was defined by means of polymerase chain reaction (PCR) analysis for the *vacA* gene in gastric mucosa and by serologic assay of *H. pylori* and CagA antibodies. Univariate and multivariate analyses were used for the association between clinicopathologic variables and long-term outcome. **RESULTS:** Positivity for *H. pylori* infection was observed in 256 of 297 patients (86%), whereas in 41 patients (14%), PCR for *vacA* and both serologic tests were negative. Negative *H. pylori* status was found to be significantly associated with cardia location, advanced pT classification, noncurative surgery, and a lower 5-year survival rate after R0 resection (24% vs 57%; $P < .001$). Multivariate survival analysis confirmed *H. pylori* status as a significant prognostic factor (hazards ratio, 2.47; 95% confidence interval, 1.40-4.35 [$P = .002$]). The influence of *H. pylori* status on long-term survival was observed in patients with early as well as advanced pT classifications. **CONCLUSIONS:** Negative *H. pylori* status appears to be an indicator of poor prognosis in patients with gastric cancer, and is independent of other well-known clinical and pathologic prognostic variables. **Cancer** 2009;115:2071-80. © 2009 American Cancer Society.

KEY WORDS: gastric cancer, *Helicobacter pylori*, *vacA*, CagA, prognosis.

Gastric cancer is 1 of the leading causes of death from cancer worldwide. At the current time, surgical resection without residual tumor represents the only chance for cure, and it may be performed in approximately two-thirds of patients who undergo surgery.¹ However, even after potentially curative surgery, tumor recurrence occurs in greater than half of patients. Lymph node involvement, depth of invasion, age, and tumor location have been identified as the most significant clinicopathologic prognostic factors.^{2,3}

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Received: August 25, 2008; **Revised:** October 17, 2008; **Accepted:** November 11, 2008

Published online: March 11, 2009 © 2009 American Cancer Society

DOI: 10.1002/cncr.24253, www.interscience.wiley.com

Several prospective studies reported a strong association between *Helicobacter pylori* (*H. pylori*) infection and gastric cancer; as such, *H. pylori* is now recognized as a Group 1 carcinogen for humans.^{4,5} Chronic gastric inflammation and the interaction between *H. pylori* and gastric epithelial cells have been suggested as potential mechanisms in gastric carcinogenesis.⁶ However, only a few individuals infected by *H. pylori* eventually develop gastric cancer. The clinical outcome of *H. pylori* infection may be because of several factors, such as age at the time of first infection, environmental factors, immune response, and genetic characteristics.⁷ The *vacA* gene is present in all *H. pylori* strains and encodes the vacuolating cytotoxin VacA, which induces epithelial cell injury. The gene comprises 2 variable regions: m (middle region) and s (signal peptide).⁸ The *cagA* gene is located in the right half of the pathogenicity island, and encodes the protein CagA, which is secreted via a type IV secretion system and translocated into gastric epithelial cells affecting host cell physiology.⁹

In a variable proportion of gastric cancer patients, *H. pylori* infection cannot be detected, and distinct clinical and pathologic features have been described in this subgroup.^{10,11} Furthermore, a recent German study provided evidence of a better prognosis in patients with *H. pylori* infection compared with negative cases.¹² However, to our knowledge, these clinically relevant findings have been not yet validated in large experiences from other centers.

In the current study, the potential impact of *H. pylori* status on the clinical and pathologic characteristics of gastric cancer was investigated in a large cohort of patients who were surgically treated in a single tertiary center and followed for a significant period of time.

MATERIALS AND METHODS

Beginning in 1988, tumor tissue and non-neoplastic mucosa from patients with primary gastric cancer who underwent surgery at the study institution were collected from the resected specimen in the operating room within 30 minutes after the removal of the stomach. Non-neoplastic mucosa was removed from the gastric antrum at a distance of at least 5 cm from the tumor. When neoplasm involved the entire antrum, non-neoplastic mucosa was removed from the middle or upper third of the stomach. Specimens were immediately frozen in liquid nitrogen, processed,

coded, and stored at -80°C in a tissue bank located in the laboratory of the study department.

In the same period, blood samples (20 mL each) were taken from patients at the time of admission to the hospital and then processed, aliquoted, and immediately frozen; serum samples were coded and stored at -80°C in a serum bank, as previously described.¹³ Patient consent for storing tissue and blood samples and their use in scientific studies was always obtained.

For the current study, 297 patients who underwent surgery for primary gastric cancer between 1988 and 2004 were considered; patients who underwent nonresective surgery, those who underwent previous resection for gastric cancer, and those with Siewert type I cardia adenocarcinoma were excluded from the study, as well as patients for whom frozen tissue or serum samples were unavailable. The patient population included 172 males (58%) and 125 females (42%), with a mean age (standard deviation [SD]) of 68 (11) years (range, 30-92 years). Surgeries performed included 192 partial gastrectomies (65%) and 105 total gastrectomies (35%). A standardized technique was used for surgical resection and lymphadenectomy, as described elsewhere²; the overall mean (SD) number of lymph nodes removed was 32 (19). In 220 patients (74%), surgical resection was classified as potentially curative (R0) according to the International Union Against Cancer (UICC) criteria.¹⁴ None of the patients included in the current study received preoperative chemotherapy or radio-chemotherapy. No patient who underwent R0 resection received adjuvant chemotherapy.

Tumor location, depth of invasion, lymph node involvement, and Lauren histotype were classified according to a standard protocol by an expert pathologist (C.V.). pTNM classification followed the criteria of the 6th edition of the UICC.¹⁴ Clinical and pathologic data from the patients were prospectively recorded in the database.

DNA Extraction and PCR for *vacA*

Non-neoplastic mucosa that was obtained from all patients included in the study was retrieved from the tissue bank. Genomic analysis was performed by a biologist (A.B) who was unaware of the clinical and pathologic data of the patients.

Briefly, total DNA was extracted from 50 mg of solid tissue according to the Puregene DNA Purification Kit

Table 1. Oligonucleotide Sequences of PCR Primers for the *vacA* Gene of *Helicobacter Pylori*

Allele	Direction	Sequence	Product, bp
<i>vacA1</i>	Sense	5'-GAAATACAACAAACACACCGC-3'	201
	Antisense	5'-GGCTTGTTTGAGCCCCAG-3'	
<i>vacA3</i>	Sense	5'-GGTCAAATGCGGTCATGG-3'	388
	Antisense	5'-CATCAGTATTTGACACCACA-3'	
<i>vacA4</i>	Sense	5'-CCAGGAAACATTGCCGGCAA-3'	346
	Antisense	5'-CATAACTAGCGCCTTGAC-3'	

PCR indicates polymerase chain reaction; bp, base pairs.

protocol (Gentra Systems, Minneapolis, Minn). The DNA concentration was calculated by spectrophotometry.

Polymerase chain reaction (PCR) for 3 *vacA* alleles was performed to increase sensitivity. Oligonucleotide sequences of PCR primers were designed as reported in Table 1. As described in previous studies from our group, the *vacA* s region was genotyped by a *vacA1* primer set, whereas for the *vacA* m region, *vacA3* and *vacA4* primer sets were used.¹⁵ Cases were considered positive for *vacA* when 1 of these 3 alleles was present on PCR product amplification. Each PCR was performed in a final volume of 25 μ L containing a buffer of 10×100 ng/ μ L of genomic DNA, 0.5 μ M of each primer, 200 μ M of each deoxynucleotide, 1.5 mM of MgCl₂, and 2U of Taq polymerase (Euroclone Life Sciences Division, Pavia, Italy). Each reaction mixture was amplified as follows: denaturation at 95°C for 10 minutes followed by 35 cycles of denaturation at 95°C for 40 seconds; annealing at 58°C (*vacA1*), 53°C (*vacA3*), and 56°C (*vacA4*) for 40 seconds; and extension at 72°C for 40 seconds. Final extension was continued for 10 minutes at 72°C. Amplifications were performed with a MJ-PTC-200 DNA engine (MJ Research, Inc, Waltham, Mass). PCR products were run on 2% agarose gels, stained with ethidium bromide, and visualized by means of photography under ultraviolet transillumination.

Serology

Preoperative frozen serum samples obtained from all patients under study were retrieved from the serum bank and processed for serologic examination. Antibodies against both *H. pylori* and CagA were determined to increase sensitivity.¹⁶ Analysis of serologic infection status was performed using an enzyme-linked immunoadsorb-

ent assay (ELISA) method using commercially available products: *Helicobacter* immunoglobulin (Ig) G (Diesse Diagnostica Senese, Siena, Italy), with a sensitivity and specificity of 96%; and CagA-IgG (Genesis Diagnostics Ltd, Cambridgeshire, UK), with a sensitivity of 95% and a specificity of 90%. As provided by the manufacturer, for both serologic tests a cutoff level of 7.0 U/mL was established for classifying cases as "positive."

Follow-up

All patients, after discharge from the hospital, entered a follow-up program according to a standard protocol.¹³ The follow-up was closed in December 2007. For survival analysis, only patients who underwent potentially curative surgery (R0) were considered. None of these patients was lost to follow-up. The mean (SD) follow-up period was 62 (54) months (range, 1-220 months) for the entire R0 group and was 89 (56) months (range, 1-220 months) for the subgroup of patients who were still alive at the end of follow-up or who had died of causes other than tumor recurrence.

Statistical Analysis

Two groups of patients were considered. Group 1 was comprised of patients who were positive for *H. pylori* infection; this group included patients who had positive test results for *vacA*, thus confirming the presence of *H. pylori* in the gastric mucosa adjacent to the tumor, and patients who tested negative for *vacA* but were serologically positive for *H. pylori* or CagA antibodies, thus indicating a previous infection with *H. pylori*. Group 2 was comprised of patients who had negative results for all tests.¹²

Potential differences in clinical and pathologic characteristics between the 2 groups of patients were investigated. Categorical variables were compared using the Fisher exact test or the Pearson chi-square test (2-tailed tests in each). For continuous variables, the Kolmogorov-Smirnov test was used to investigate the assumption of the normality of distribution. When continuous variables resulted as normally distributed, the independent-sample Student *t* test was used for statistical comparisons between the 2 groups.

Long-term survival was evaluated using the Kaplan-Meier method, considering death from cancer as the endpoint; patients who died of causes other than tumor recurrence were considered as censored at the time of death. The log-rank test was used for statistical comparisons between factors. Variables independently affecting prognosis were investigated by means of multivariate analysis using the Cox proportional hazards model. A stepwise forward procedure was performed, and the score statistic was used to select variables for entry into the model. A statistical level of $P < .05$ was used for the inclusion of prognostic variables, and a $P > .06$ was used for removing variables. Results were expressed as the hazards ratio (95% confidence interval [95% CI]). The assumption of the proportionality of the risk (ie, that the ratio of hazard function in each group of patients does not depend on time, which is necessary for the inclusion of the variables in the Cox model) was verified by plotting the graph of the $\ln(-\ln[\text{survival}])$ curves for each category of covariates.¹⁷ Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) software (version 15.0; SPSS Inc, Chicago, Ill).

RESULTS

H. pylori Status as Determined by PCR and Serology

Of the 297 patients examined for the presence of *vacA*, 199 (67%) tested positive for 1 of the 3 primers used. A total of 210 patients (71%) demonstrated positive *H. pylori* serology (HPs), and 157 patients (53%) demonstrated positive CagA serology; the combined sensitivity of the 2 serologic tests was 77% (229 patients).

The majority of patients found to be positive on PCR were also found to be positive for HPs or CagA (172

Table 2. Combination of PCR for *vacA*, *Helicobacter Pylori* Serology, and CagA Serology in 297 Patients Included in the Study

<i>vacA</i> (PCR)	<i>H. pylori</i> Serology	CagA Serology	
		No. of Negative Cases	No. of Positive Cases
Negative	Negative	41	2
	Positive	22	33
Positive	Negative	27	17
	Positive	50	105

PCR indicates polymerase chain reaction; *H. pylori*, *Helicobacter pylori*.

of 199 patients); however, both serologic tests were negative in 27 of the 199 PCR-positive cases (Table 2). In 57 patients (19% of the total number), the PCR for *vacA* was negative, but a serologic positivity was observed. On the whole, 256 patients (86%) were positive for *H. pylori* infection (Group 1), whereas in 41 patients (14%), PCR for *vacA* alleles and serologic tests (HPs and CagA) were negative (Group 2).

H. pylori Status and Clinicopathologic Factors

In Table 3, a comparison of the clinical and pathologic characteristics between the patients in Group 1 and Group 2 is reported. In patients in Group 2, neoplasms were more frequently located in the cardia (27% vs 13%), a more advanced pT classification was observed (above all for tumors involving the serosa [66% vs 48%]), and the percentage of curative surgery (R0) performed was significantly lower (59% vs 77%). No significant differences with regard to sex, age, tumor size, Lauren histotype, lymph node involvement, distant metastasis, and the number of removed lymph nodes were observed between the 2 groups.

H. pylori Status and Survival

The potential impact of *H. pylori* status on the long-term survival of patients was investigated by means of univariate and multivariate analysis. Survival analysis was performed in 220 patients who underwent R0 resection. At the end of follow-up, 91 patients were still alive, 97 patients had died of tumor recurrence, and 32 patients had died of other causes; the cancer-related 5-year survival

Table 3. Association Between Clinicopathologic Variables and *H. pylori* Status

Variable	No. of Patients	Group 1 (Positive) n=256 (%)	Group 2 (Negative) n=41 (%)	P value
Gender				
Male	172	147 (57)	25 (61)	.735*
Female	125	109 (43)	16 (39)	
Age (mean $\pm$ SD), y		68 $\pm$ 11	69 $\pm$ 11	.486†
Tumor location				
Cardia	45	34 (13)	11 (27)	.034*
Noncardia	252	222 (87)	30 (73)	
Tumor size (mean $\pm$ SD), cm		5.4 $\pm$ 2.8	5.8 $\pm$ 2.7	.451†
Lauren histotype				
Intestinal	208	180 (70)	28 (68)	.855*
Diffuse or mixed	89	76 (30)	13 (32)	
Depth of invasion				
pT1	39	33 (13)	6 (15)	.049‡
pT2	108	100 (39)	8 (19)	
pT3-pT4	150	123 (48)	27 (66)	
Lymph node involvement				
pN0	98	88 (34)	10 (24)	.316‡
pN1	96	79 (31)	17 (42)	
pN2-pN3	103	89 (35)	14 (34)	
Distant metastases				
Absent	276	240 (94)	36 (88)	.186*
Present	21	16 (6)	5 (12)	
No. of lymph nodes removed (mean $\pm$ SD)		32 $\pm$ 19	33 $\pm$ 17	.834†
Curative surgery				
R0	220	196 (77)	24 (59)	.021*
R1/R2	77	60 (23)	17 (41)	

H. pylori indicates *Helicobacter pylori*; SD, standard deviation.

*Determined using the 2-tailed Fisher exact test.

†Determined using the 2-tailed independent-sample Student *t* test.

‡Determined using the 2-tailed Pearson chi-square test.

rate in the entire series was 54% (standard error [SE], 3.6). The 5-year survival rate was 57% (SE, 3.8) in 196 *H. pylori*-positive patients versus 24% (SE, 9.7) in 24 *H. pylori*-negative patients (log-rank test, $P < .001$) (Fig. 1).

Multivariate analysis was performed using the Cox proportional hazards model to investigate the prognostic value of *H. pylori* status when adjusting for the effect of other clinicopathologic variables. Age, sex, tumor location, tumor size, Lauren histotype, depth of invasion, lymph node involvement, number of removed lymph nodes, and *H. pylori* status were included in the analysis as covariates. The statistical model identified depth of invasion, lymph node involvement, tumor location, and *H. pylori* infection as significant and independent prognostic factors (Table 4); the hazards ratio in patients who were negative for *H. pylori* infection compared with positive patients was 2.47 (95% CI, 1.40-4.35).

Prognostic Value of *H. pylori* Status in Different Tumor Stages and Histotypes

Kaplan-Meier survival curves of patients with different *H. pylori* status were stratified according to the most significant prognostic variables, such as depth of invasion and lymph node involvement. This analysis confirmed the observation that the absence of *H. pylori* infection negatively affected long-term survival. Statistically significant differences between Group 1 and Group 2 were observed in the early (pT1-pT2) as well as in the more advanced pT classification groups (pT3-pT4), and in patients with lymph node involvement (pN+) (Fig. 2). Survival differences were also noted when patients were stratified according to Lauren histotype. In patients with the intestinal type, the 5-year survival rate was 63% in Group 1 versus 34% in Group 2 (log-rank test, $P = .023$) and in patients with the diffuse-mixed type, the 5-year survival rate was 44%

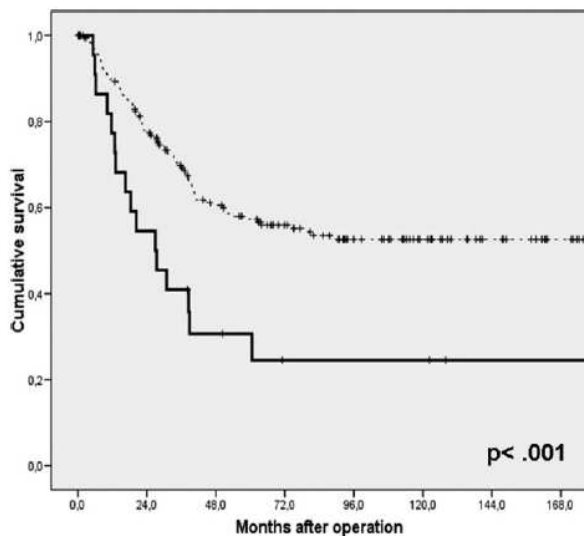


FIGURE 1. Tumor-related survival according to *Helicobacter pylori* (*H. pylori*) status in patients who underwent potentially curative resection (RO). The difference between positive cases (indicated by the broken line, $n=196$) and negative cases (solid line, $n=24$) was statistically significant (log-rank test, $P<.001$).

in Group 1, whereas none of the patients in Group 2 were still alive at 5 years ($P=.001$).

Survival According to the Timing of Infection

An additional analysis was conducted categorizing the patients into 3 groups: current infection (*vacA* positive), prior infection (*vacA* negative but with positive serology), and never infected. This analysis confirmed a significant difference between patients with current infection versus those who were never infected ($P=.001$) and those with a prior infection versus those who were never infected ($P=.016$). A trend toward higher long-term survival in patients with current infection compared with patients with a prior infection was observed, but the difference was not statistically significant ($P=.708$) (Fig. 3).

DISCUSSION

The influence of *H. pylori* infection on the development, progression, and clinical outcome of gastric cancer remains unclear. Of the several epidemiologic studies to

Table 4. Significant Prognostic Factors in 220 Patients Treated With Potentially Curative Surgery (RO), as Evaluated Using the Cox Proportional Hazards Model

Variable	Coefficient	Hazards Ratio (95% CI)	P Value for Global Test*
Depth of invasion			
pT1	Reference	—	<.001
pT2	0.18	1.20 (0.39-3.72)	
pT3-T4	1.67	5.32 (1.77-15.95)	
Lymph node status			
pN0	Reference	—	<.001
pN1	1.24	3.47 (1.74-6.90)	
pN2/pN3	2.09	8.05 (3.93-16.47)	
<i>H. pylori</i> status			
Positive	Reference	—	.002
Negative	0.90	2.47 (1.40-4.35)	
Tumor location			
Noncardia	Reference	—	.050
Cardia	0.52	1.69 (1.00-2.85)	

95% CI indicates 95% confidence interval; *H. pylori*, *Helicobacter pylori*.

*Global chi-square of the model: 150.656 ($P<.001$). Residual chi-square: 5.231 ($P>.05$).

have investigated potential correlations between *H. pylori* infection and gastric cancer risk, to our knowledge only a few focused on the association with long-term survival. In the current study, we had the opportunity to investigate such a correlation in a large cohort of patients because several years ago we initiated the collection and preservation of gastric mucosa, tumor tissue, and serum samples from patients who underwent surgery at the study department. From these same patients, follow-up data were prospectively recorded, and a long period of follow-up was obtained.

The main findings of the current study were: 1) in 67% of patients, *H. pylori* infection was detected by PCR analysis in non-neoplastic mucosa adjacent to the tumor, and in a further 19% of patients, positive serology indicated a previous infection; 2) patients without evidence of *H. pylori* infection demonstrated tumors that were more frequently located in the cardia and involved the serosal layer; and 3) in patients with negative *H. pylori* status, a higher rate of residual tumor after surgical resection and a worse prognosis after curative surgery were observed.

Bacterial DNA was extracted from frozen non-neoplastic mucosa previously collected from the resected stomach in the operating room. Because the *vacA* gene is

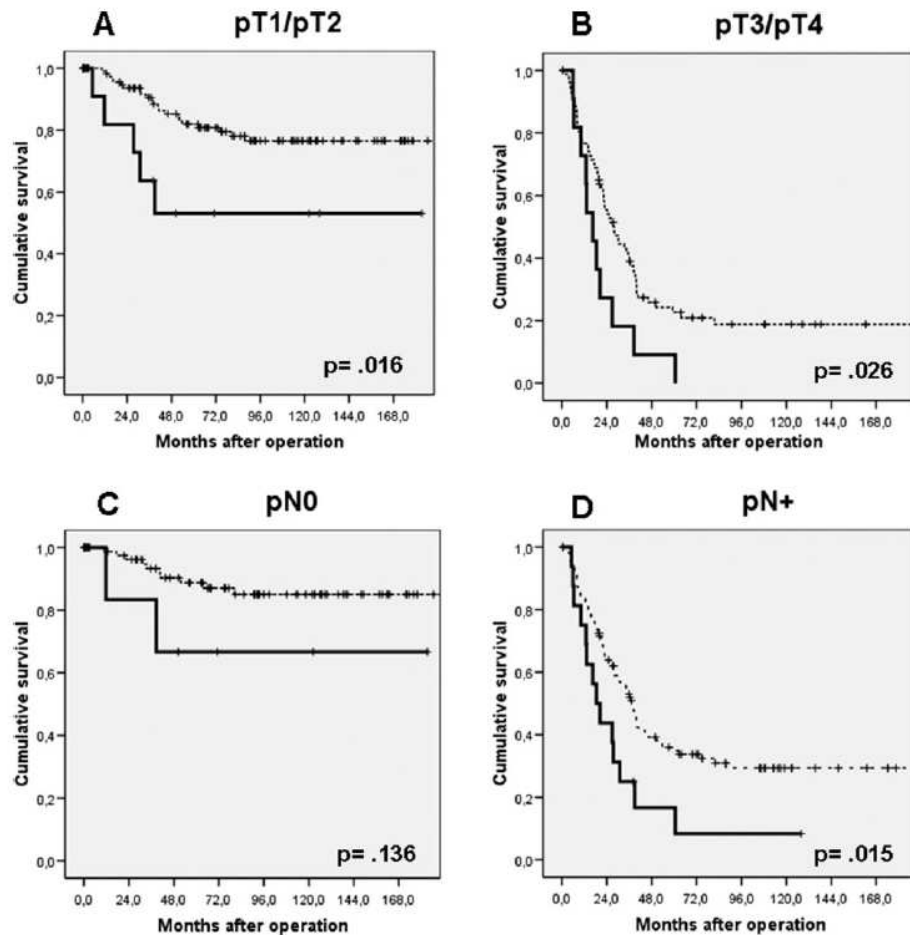


FIGURE 2. Influence of *Helicobacter pylori* (*H. pylori*) status on long-term survival in patients who underwent potentially curative resection (RO) stratified according to (A and B) pT classification and (C and D) lymph node status. A statistically significant difference between positive (indicated by the broken line) and negative cases (solid line) was observed in Panels A, B, and D.

present in all *H. pylori* strains, we used this marker to identify patients with *H. pylori* infection.^{7,8} We emphasize that PCR analysis has been reported to be associated with a higher sensitivity when compared with other detection methods such as bacterial culture or histologic analysis.¹⁸ Serologic positivity in our series (77%) was similar to the overall rate reported in a recent meta-analysis including a large number of gastric cancer patients.¹⁹ In 19 patients, serology was negative for *H. pylori* antibodies but positive for CagA; this finding has been reported in the literature with similar rates, because CagA antibodies can remain positive for a longer period of time compared with *H. pylori* surface antibodies.^{16,19}

In 27 patients (9% of the entire series) PCR for *vacA* was positive but no serologic positivity was found. One

study reported that advanced age and infiltrative tumor growth are factors associated with negative serology in the presence of *H. pylori* infection.¹¹ In the current series, no significant clinical or pathologic differences were found in PCR-positive patients who demonstrated positive or negative serology (data not shown).

Data from the current study indicate that the combination of PCR for *vacA* and serologic analysis for *H. pylori* and CagA antibodies is able to increase the accuracy of the definition of *H. pylori* status; an overall positivity rate of 86% was observed in the patients in the current study. This value is higher with respect to rates reported in other studies.^{11,12} However, very high infection rates also have been reported from Japan, and negative *H. pylori* status in Japanese gastric cancer patients has been recently

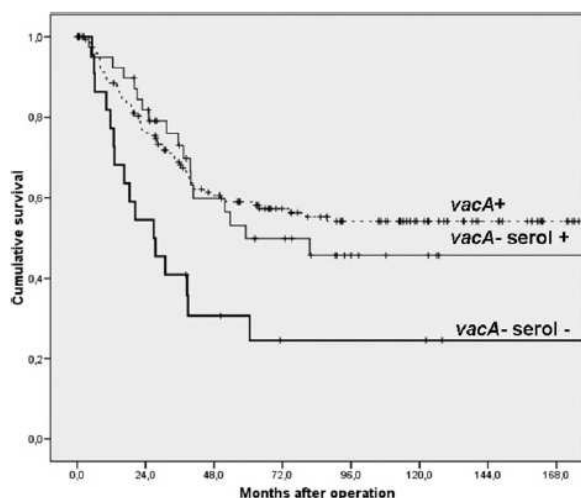


FIGURE 3. Survival according to the timing of infection: current infection (*vacA* positive [*vacA*+]), prior infection (*vacA* negative [*vacA*-], serology positive [serol+]), and never infected (*vacA*-, serol-). A significant difference between current infection versus never infected ($P = .001$) and prior infection versus never infected ($P = .016$) was observed. No statistically significant difference was found between current infection versus prior infection ($P = .708$).

estimated to range from between 2% and 11%.²⁰ It is well known that the prevalence of *H. pylori* infection in the general population is higher in countries with a higher incidence of gastric cancer²¹; in Tuscany, the incidence of gastric cancer is very high compared with other regions of Italy.²² Epidemiologic factors and the sensitivity of the methods used could explain the high rates of *H. pylori* positivity reported in the current series.

The characteristics of *H. pylori*-positive patients were compared with those of the small group of patients with negative *H. pylori* status. Statistical analysis revealed that cardia location and serosal involvement were more frequent in *H. pylori*-negative patients. The association with proximal tumor location also has been observed in the study by Meimarakis et al.¹² We also found a higher rate of noncurative surgery in *H. pylori*-negative patients. This may be indicative of the potential predictive value of this factor in noncurative surgery, which should be confirmed in a specifically designed prospective study. Because the majority of noncurative cases also had tumors involving the serosa, this may explain the correlation between this parameter and *H. pylori* status.

From a clinical point of view, the most significant finding of the current study is the association between

negative *H. pylori* status and poor prognosis. Several years ago, a study from Taiwan to our knowledge was the first to report better survival in gastric cancer patients demonstrating seropositivity for *H. pylori*.¹⁰ Multivariate analysis demonstrated that only TNM stage was an independent prognostic factor; however, the definition of *H. pylori* status was performed only by serology, the number of patients was small, and follow-up was rather short. Other authors found better survival in seropositive patients with stage I disease compared with seronegative patients with the same stage of disease.²³

A recent prospective study identified *H. pylori* status as an independent prognostic factor.¹² Culture examination, histologic analysis, and serologic assay were performed to define *H. pylori* status. The better prognosis noted in patients with *H. pylori* infection was explained on the basis of an improved immune response against the tumor.¹² It also has been assumed that because *H. pylori* components mimic specific receptors or surface molecules on gastric epithelial cells, autoantibodies could induce a cross-reaction against gastric cancer cells.²⁴ However, several authors have raised doubts regarding the real prognostic value of *H. pylori* status, suggesting that *H. pylori* negativity may be simply related to more advanced tumor progression.^{25,26} Currently, the definition of *H. pylori* status is far from being introduced into clinical practice for gastric cancer patients.

To our knowledge, the current study is the first to confirm *H. pylori* status as an independent prognostic factor in a large number of patients with gastric cancer. The influence of this factor on clinical outcome was observed in patients with early as well as advanced stages of disease. Multivariate analysis confirmed the prognostic value, even when adjusting for other well-known prognostic factors such as pT classification, lymph node involvement, and tumor location. Furthermore, *H. pylori* status was found to significantly influence survival in patients with intestinal as well as diffuse-mixed histotypes. It is interesting to note that all *H. pylori*-negative patients but 1 with tumors involving the serosa or with positive lymph nodes died of tumor recurrence during follow-up. The adverse effect of *H. pylori* status on prognosis was observed in all the subgroups examined. The difference was not found to be statistically significant in patients with pN0 disease, most likely because of the low number of *H. pylori*-negative cases (10 patients) in that subgroup (type beta error). In the current study, statistical analysis was conducted on

2 groups with different numbers of patients, and this may be considered a limitation; however, this is similar to other experiences in this field, because of the low proportion of *H. pylori*-negative patients in several reported series.¹⁰⁻¹² When considering the low percentage of patients with negative *H. pylori* status, very large number of patients should be analyzed in the future to perform stratified analyses on subgroups.

Both groups of patients with current infection and prior infection demonstrated better survival than never-infected patients. A trend toward a higher survival rate was observed in patients with a current infection compared with patients with a prior infection, but the difference was not found to be statistically significant. This trend may be because of a higher positive effect of actual infection on long-term survival, or the higher specificity of the PCR test for *H. pylori* with respect to serology. Potential associations between the timing of infection, tumor characteristics, and long-term survival should be investigated in further studies to clarify whether *H. pylori* eradication could be associated with a worse prognosis in patients with gastric cancer.

In addition to the improved immune response against the tumor noted in patients with *H. pylori* infection, other potential explanations may be suggested. Although similar molecular characteristics of gastric cancer based on *H. pylori* status have been reported in some studies,^{11,27} other authors have observed differences in the incidence of microsatellite instability, which may be involved in long-term outcome.²⁸ However, *H. pylori* status may simply represent an epiphenomenon of other factors primarily involved in the prognosis of gastric cancer. Specific studies in a large number of patients should be designed to investigate potential correlations between biologic characteristics, *H. pylori* status, and long-term outcome in patients with gastric cancer.

Data from the current study also give rise to some intriguing speculations. Because *H. pylori* status is associated with gastric cancer prognosis, could the different prevalences of *H. pylori* infection explain the geographic variability in survival rates noted after surgery? *H. pylori* positivity in the general population as well in gastric cancer patients is very high in Japan and in countries with a high incidence of gastric cancer.^{19-21,29} It is interesting to note that in gastric cancer patients, very low positivity rates of *H. pylori* infection have been reported from the

US,³⁰⁻³³ and clinical studies demonstrated clearly lower survival rates when compared with Japanese patients at the same stages of disease.³⁴ Differences in prognosis between Eastern and Western series have been explained on the basis of different surgical approaches, less advanced stage of disease, and more careful tumor staging³⁵; the association with *H. pylori* status may shed more light on geographic variability in the prognosis of patients with gastric cancer. These speculations should be investigated in multicenter prospective studies.

We strongly believe that the determination of *H. pylori* status may be clinically relevant because it could be obtained in a pretreatment phase; more aggressive surgical treatment or the use of neoadjuvant therapies in high-risk patients may be investigated in clinical trials. While awaiting potential explanations of the mechanisms that affect the clinical outcome of patients with gastric cancer, we confirm that, in addition to other well-known clinicopathologic parameters, *H. pylori* status is a prognostic factor in gastric cancer. Preoperative assessment of *H. pylori* status should be obtained, and in the case of a negative finding, a higher risk of tumor recurrence after curative surgery should be attributed to those patients.

Conflict of Interest Disclosures

Supported by Ministero dell'Università e della Ricerca (MIUR) under grant Progetti di Ricerca di Interesse Nazionale (PRIN), number 2004065502-004.

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PAPER 10.2

Oliveira, C., et al., **Germline CDH1 deletions in hereditary diffuse gastric cancer families.** Hum Mol Genet 2009;18:1545-1555.

Germline *CDH1* deletions in hereditary diffuse gastric cancer families

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Received December 23, 2008; Revised and Accepted January 22, 2009

Germline *CDH1* point or small frameshift mutations can be identified in 30–50% of hereditary diffuse gastric cancer (HDGC) families. We hypothesized that *CDH1* genomic rearrangements would be found in HDGC and identified 160 families with either two gastric cancers in first-degree relatives and with at least one diffuse gastric cancer (DGC) diagnosed before age 50, or three or more DGC in close relatives diagnosed at any age. Sixty-seven carried germline *CDH1* point or small frameshift mutations. We screened germline DNA from the 93 mutation negative probands for large genomic rearrangements by Multiplex Ligation-Dependent Probe Amplification. Potential deletions were validated by RT-PCR and breakpoints cloned using a combination of oligo-CGH-arrays and long-range-PCR. *In-silico* analysis of the *CDH1* locus was used to determine a potential mechanism for these rearrangements. Six of 93 (6.5%) previously described mutation negative HDGC probands, from low GC incidence populations (UK and North America), carried genomic deletions (UK and North America). Two families carried an identical deletion spanning 193 593 bp, encompassing the full *CDH3* sequence and *CDH1* exons 1 and 2. Other deletions affecting exons 1, 2, 15 and/or 16 were identified. The statistically significant over-representation of Alus around breakpoints indicates it as a likely mechanism for these deletions. When all mutations and deletions are considered, the overall frequency of *CDH1* alterations in HDGC is ~46% (73/160). *CDH1* large deletions occur in 4% of HDGC families by mechanisms involving mainly non-allelic homologous recombination in Alu repeat sequences. As the finding of pathogenic *CDH1* mutations is useful for management of HDGC families, screening for deletions should be offered to at-risk families.

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INTRODUCTION

Hereditary diffuse gastric cancer (HDGC) is an autosomal dominant disorder that accounts for <1% of all cases of gastric cancer (GC). Although uncommon, this disease constitutes an important health issue due to its severity, its high penetrance, early age at presentation and the unavailability of effective screening tools. Diffuse gastric cancer (DGC) is the most important cause of cancer lethality in mutation positive HDGC families with lobular breast cancer being a secondary medical concern (1).

Heterozygous germline point or small frameshift mutations in E-cadherin gene (*CDH1*) [OMIM +192090], a calcium-dependent cell-to-cell adhesion molecule and a tumor suppressor protein, are the only germline molecular defect associated with HDGC (2–4). Overall, carriers of *CDH1* germline mutations have a cumulative GC risk, before age 75, of 40–67% for men and 63–83% for women and a risk for lobular breast cancer of 39–52% (5,6). Clinical expression of the disease often occurs before the third decade of life (1), but pre-clinical multifocal tumors, which may have the potential for metastatic spread, are already present in the stomachs of young asymptomatic *CDH1* mutation carriers (7,8).

Clinical criteria have been established, in 1999, by the International Gastric Cancer Linkage Consortium (IGCLC) to select families for mutation screening in the *CDH1* gene, based mainly on early age of disease presentation and diffuse histology of the tumors (9). These have been later adapted in an attempt to enlarge the population to which this screening could apply, in countries with low incidence of GC (10). Overall, 30–40% of HDGC families harbor *CDH1* germline mutations, but this frequency is highly variable between countries with different incidences of GC, with low incidence countries like UK, North America and Canada, displaying nearly 50% of HDGC mutation positive families, while high incidence countries like Portugal and Italy, displaying around 10% of HDGC mutation positive families (4,6,11).

Approximately two-thirds of families, screened worldwide, remain genetically unexplained and the concern related with the management of *CDH1* mutation negative gastric cancer families persists. Since the required samples for full genome linkage, in any GC family, are difficult to obtain, a candidate gene approach to identify novel susceptibility genes has been more frequently used. Putative tumor suppressor genes, which are commonly inactivated in sporadic GC and/or associated with GC development, could also represent good candidate susceptibility genes to familial GC. Previous studies including ours, however, have lead us to rule out RUNX3, Caspase-10, SMAD4, HPP1, and Desmoglein 2 as major GC predisposition genes in families with aggregation of gastric carcinoma (12–14). Virtually all diffuse gastric carcinomas, both hereditary and sporadic, independently of whether they carry a *CDH1* mutation, display identical morphological features as well as aberrant patterns of expression of E-cadherin (unpublished data). As no other gene has been so far shown to play a major genetic role in the disease, we hypothesize that other genetic events at the *CDH1* locus, potentially missed by standard screening techniques, such as large genomic rearrangements, deletions or insertions could be found in families with multiple cases of DGC. Examples of this type of

molecular alterations have been recently reported for other cancer associated syndromes. Large genomic rearrangements have been described in *hMLH1* and *hMSH2* in 10–20% of HNPCC mutation negative families, in *APC* in 15% of FAP mutation negative families and in lower frequencies in *BRCA1* and *BRCA2* in breast cancer families initially identified as negative, as well as in *CDKN2A* in melanoma negative families (15–21).

Based on these observations, we aimed at characterizing *CDH1* genomic rearrangements in a large population of HDGC patients, negative for *CDH1* point mutations. Moreover, by using a larger series of families screened for *CDH1* point mutations and rearrangements, in five reference centers world-wide and arising in countries with different GC incidence rates, we aimed at clarifying the frequency of *CDH1* germline alterations and its relationship with GC incidence as well as with clinical criteria for selection of patients at risk.

RESULTS

Multiplex ligation-dependent probe amplification analysis for the *CDH1* gene

We collected, from five reference centers, 93 DNA samples from families with aggregation of DGC and negative for *CDH1* germline point mutations. These families were selected based on their risk to carry a germline alteration in *CDH1* and the clinical criteria described in Materials and Methods section.

We found that six out of the 93 (6.5%) probands showed abnormal multiplex ligation-dependent probe amplification (MLPA) features when compared with the controls, displaying an ~70% signal reduction in one or more exons of the *CDH1* gene and, suggesting the presence of large deletions affecting the *CDH1* locus (Fig. 1, Table 1). No abnormalities were observed in the remaining 87 probands.

After reproducing these results in an independent MLPA reaction, we verified that four probands (families 1 to 4) presented deletions at the 5'-end of the gene: in three probands deletions encompassed at least exon 1, intron 1 and exon 2 and, in one proband a smaller deletion that encompassed one of the three probes designed for *CDH1* exon 1 was found. The remaining two probands (families 5 and 6) displayed deletions at the further 3'-end of the gene: in one proband a deletion of the genomic sequence encompassing exons 14 and 16 was observed, in the other a deletion of exon 16 only was observed (Fig. 1).

Fine mapping of the deletions breakpoints

A combination of array CGH and PCR was utilized to map the putative breakpoints in five of the six probands (Fig. 2). The array CGH indicated the regions of chromosome 16 at which the breakpoints were located. PCR primers were then designed to span the breakpoints and the resulting PCR products sequenced (Table 2). Family 1 and family 2 showed identical MLPA patterns as well as virtually identical array CGH results. Sequencing of the breakpoint revealed that these two families harbored an identical 193 593 bp deletion encompassing the full sequence of *CDH3* and extending to

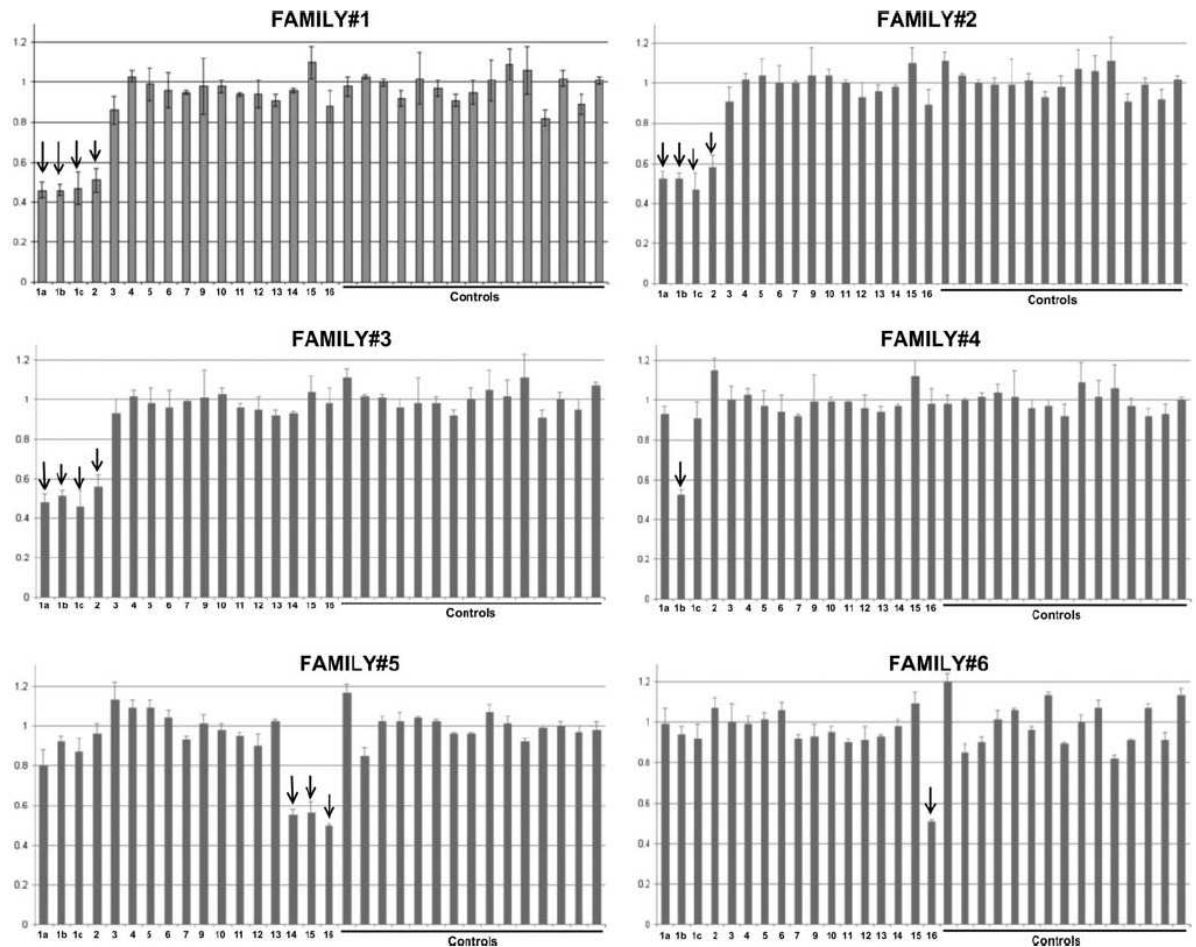


Figure 1. MLPA output from the six HDGC families carrying germline deletions of the *CDH1* locus. The first 17 bars represent signal obtained with MLPA probes within the *CDH1* gene. Please note that for exon 1, three probes were used (1a, 1b and 1c). *CDH1* deletions are represented, in the graph, by smaller bars marked with arrows. Bars on the right hand-side represent results obtained with control probes for MLPA ($n = 15$).

position IVS2+57 595 in *CDH1*. Family 3 was found to carry a 5671 bp deletion with breakpoints at position -3809 upstream of the *CDH1* transcription start site (TSS) and at position IVS2+742 in *CDH1*. Family 4 harbored a 150 bp deletion encompassing the TSS of *CDH1*, with breakpoints located 125 bp upstream and 25 bp downstream of the TSS. Array CGH was not done for this proband since the PCR encompassing *CDH1* exon 1 produced both a normal sized and a shorter band that was visible by dHPLC and further used to clone the breakpoints for the deletion by direct sequencing. Family 5 carried an 8078 bp deletion ranging from IVS13-2738 to 20 bp downstream of the stop codon of *CDH1*, as confirmed by long-range PCR using specific primers flanking the breakpoints (Fig. 3A). Family 6 was found to carry an 828 bp deletion ranging from IVS15+3097 to 223 bp downstream of the stop codon of *CDH1* (Fig. 3B).

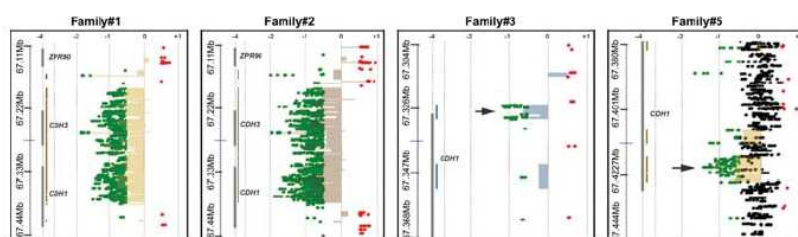
Bioinformatics analysis of breakpoints and deletion containing sequences

Assuming that deletions are more likely to occur due to recombination events, we analyzed the genomic sequences flanking the boundaries of these five deletions, which are predicted to constitute target regions for breaks and recombinations to occur. We performed a search for Alu elements which have been reported to mediate non-allelic recombination events in several other malignancies. Evidence for non-homologous end joining (NHEJ) and non-allelic homologous recombination (NAHR) has been described in atypical NF1 microdeletions. These deletions were traced back to short sequences with similarities to Alu elements near the deletion junction (22). Moreover, deletions in the PMP22 gene have been described to be a product of homologous recombination between AluS elements present in intronic regions of the

Table 1. Table summarizing HDGC families analyzed for large *CDH1* rearrangements (gray columns) and compilation of frequency of *CDH1* alterations indicating geographic origin grouped by GC incidence rates

Incidence	Origin (n)	Rearrangement analysis	Deletion (%)	<i>CDH1</i> positive (%)	<i>CDH1</i> negative (%)
Low	North America (92)	49	4 (8.2)	47 (51.1)	45 (48.9)
	UK (33)	18	2 (11.1)	17 (51.5)	16 (48.5)
	Holland (1)	0	0	1 (100.0)	0
	Total (126)	67	6 (9.0)	65 (51.6)	61 (48.4)
Moderate	Germany (16)	12	0	4 (25.0)	12 (75.0)
	Total (16)	12	0	(25.0)	2 (75.0)
High	Portugal (15)	12	0	3 (20.0)	12 (80.0)
	Italy (3)	2	0	1 (33.3)	2 (66.6)
	Total (18)	14	(22.2)	14 (77.8)	
	Total (160)	93	6 (6.5) ^a	73 (45.6)	87 (54.4)

^aThis frequency represents 6/93 *CDH1* deletions found in negative families. The overall frequency of large *CDH1* deletions is 3.8% (6/160).

**Figure 2.** Array CGH profiles obtained for HDGC families carrying large *CDH1* deletions. Examples are shown for four of the six families. See Tables 2 and 3 for details on the deletion found in each family. Deletions are represented by dense areas of green dots on the left side of each panel. Vertical thick gray bars on the left represent genes in the genomic area surrounding the *CDH1* locus and corresponding gene symbols are also shown. Smaller deletions in families#3 and #5 are pointed with black arrows. Please note the scale in Mb on the right, for chromosome position.**Table 2.** Summary of HDGC families displaying *CDH1* germline deletions

ID	Ancestry	GC incidence	Other cancers (age)	Genomic rearrangement
Family 1	Northern European	Low	LBC (61)	Del exon 1–2 (193 593 bp)
Family 2	Canadian	Low		Del exon 1–2 (193 593 bp)
Family 3	Eastern European	Low	PCa (43)	Del exon 1–2 (5671 bp)
Family 4	Southern European	Low	LCa (nd), PCa (42)	Del 5'-UTR-exon 1 (150 bp)
Family 5	Central European	Low	Unknown	Del exon 14–16 (8078 bp)
Family 6	Central European	Low	Unknown	Del exon 16 (828 bp)

LBC, lobular breast cancer; PCa, pancreatic cancer; LCa, liver cancer.

gene (23). We also performed a search for the presence of cryptic sequences recognizable by the RAG1/2 recombinase near the breakpoints and the insertion of P and/or N nucleotides in the rejoining sites. Such sequence features, reminiscent of V(D)J recombination sites, are expected to indicate whether cleavage by the RAG1/2 recombinase may occur near these joining sites (24,25).

In three families carrying two different deletions we found that deletions overlap Alu repeats (Table 3). In families 1 and 2, bearing the same deletion at chr16:67193822–67387415, both deletion boundaries fall within annotated Alu repeats, AluSp near the start and AluSg near the end. The sequences of the two repeats are highly similar (percentage identity >75%) (Table 3, Fig. 4A). In family 3, carrying a deletion at chr16:67324886–67330557, the start of the deletion maps inside an AluSx repeat while the end map into a repeat AluSg and, the sequences of these two repeats display an identity of ~80% (Table 3, Fig. 4B).

For the deletion at chr16:67328695–67328844, present in family 4, an AluJo repeat is present 304 bp upstream from the start of the deletion, and another AluJo is present 1497 bp downstream the end of the deletion. The closest repeat external to the deletion is a MIR named MIRb, 1235 bp downstream of the end-point but no sequence identity can be seen in the –150/+50 and –50/+150 bp fragments centered, respectively, on the start and the end of the deletion. An interesting feature of this 150 bp deletion is the presence of microsatellite/microhomology around the breakpoints due to the presence of five nucleotides sequence (CTCTC) between the upstream and downstream breakpoints.

Family 5 displays a deletion at chr16:67416845–67424923 that does not overlap Alu repeats, although it occurs in the proximity of this type of repetitive regions (Table 3). The closest repeats both upstream and downstream are AT rich sequences, ~300 bp distant from the deletion (Fig. 4C). Moreover, 344 bp upstream of the deletion start a FLAM_C repeat

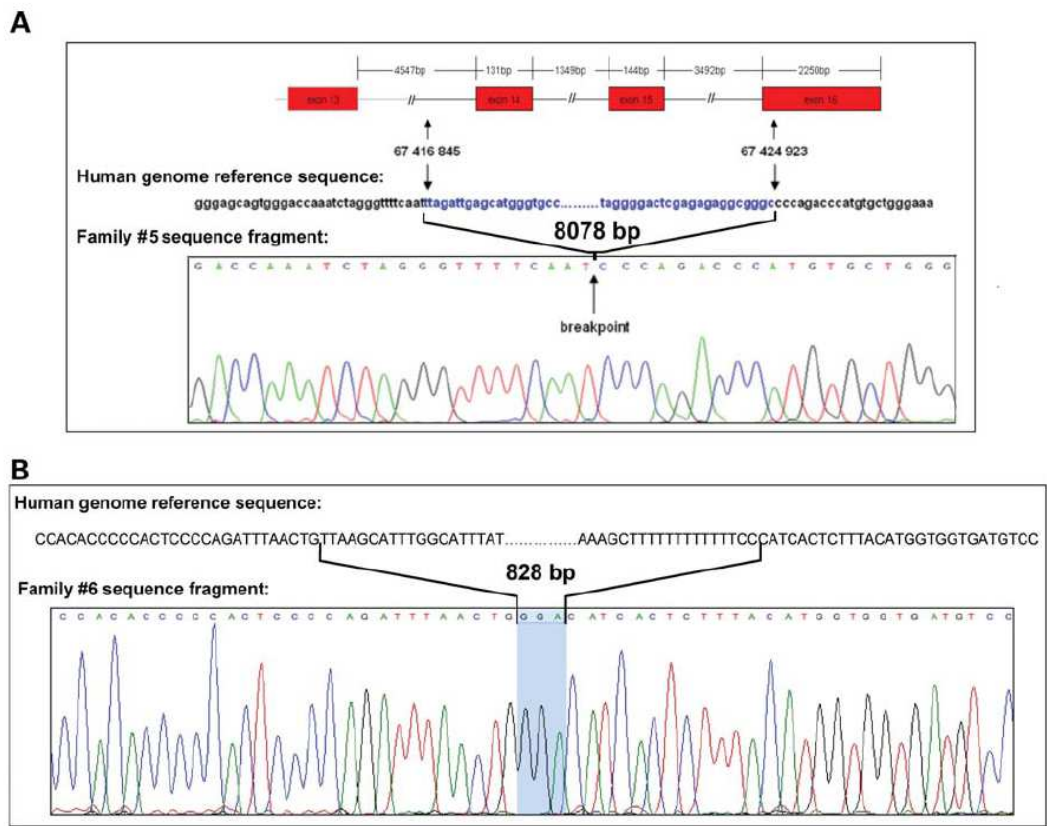


Figure 3. Sequencing chromatogram of the mapped breakpoints in family 5 (A) and family 6 (B). Comparison with the human genome reference sequence. (A) Deletion of 8078 bp without insertion of P or N nucleotides. (B) Deletion of 828 bp and insertion of a triplet at the breakpoint.

Table 3. Deletion coordinates and distance from deletion ends to the closest Alu containing sequences

Family ID	Deletion coordinates	Closest start Alu	Closest start Alu distance	Closest end Alu	Closest end Alu distance
Family 1	chr16:67193822–67387415	AluSp	overlap	AluSg	overlap
Family 2	chr16:67193822–67387415	AluSp	overlap	AluSg	overlap
Family 3	chr16:67324886–67330557	AluSx	overlap	AluSg	overlap
Family 4	chr16:67328695–67328844	AluJo	–304	AluJo	1497
Family 5	chr16:67416845–67424923	FLAM_C	–344	AluJb	442
Family 6	chr16:67424298–67425126	AluSq	–50	AluJb	239

exists and 442 bp downstream of the endpoint there is an AluJb sequence (Table 3). The alignment of the –150/+150 bp surrounding the deletion ends did not show any identity.

The deletion in family 6, at chr16:67424298–67425126, is unlikely to have been produced by homology-dependent mechanisms, since a small (3 bp) sequence insertion at the junction between the deletion breakpoints was identified (Fig. 3B). For this deletion, the closest Alu repeats found on both sides of the deletion are an AluSq 50 bp upstream and an AluJb 239 bp downstream of the deletion's start and end, which share a sequence identity >77% (Table 3).

In a range of +/-5 Mb flanking the five deletions reported in this study, we did not find evidence for the presence of

RAG1/2 consensus sequences accompanied or not by the insertion of P and/or N nucleotides in the rejoining sites. Nevertheless, the presence of Alus overlapping 6 out of the 12 points analyzed (the ends of the six deletion sequences) is statistically significant (z-score 3.3, *P*-value 0.0005) as tested by performing a randomization analysis with 1000 random sampling of 12 random locations (see Materials and Methods).

Clinical features of deletion-carrying HDGC families

The proband in family 1 was a 61-year-old female of European Irish/German/Scottish/Danish ancestry presenting with lobular

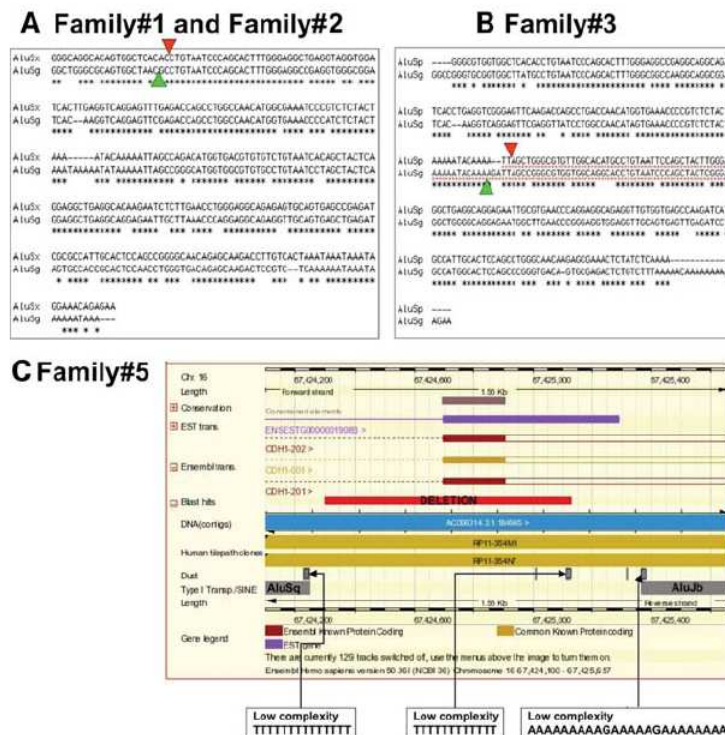


Figure 4. Analysis of sequences flanking the deletion breakpoints. (A and B) Homology analysis of AluS type elements flanking deletions in families 1, 2 and 3. (C) Ensembl based scheme displaying the repetitive elements flanking the deletion in family 5.

breast cancer. Her daughter and son, as well as a granddaughter developed DGC at the age of 40, 37 and 28, respectively, and are already diseased. The deletion was only looked for in the proband that is currently alive.

In family 2, the proband, of unknown origin but currently living in Canada, was a female who developed DGC at 38 and later at 43 years old and the only patient tested in this family. Her two sisters also developed DGC at the ages of 30 and 35, respectively, but were not tested for the presence of the same deletion.

The presence of the same deletion in two different families could be explained two ways. Either of these families had a common ancestor or the deletions arose independently. To clarify this issue, we performed haplotype analysis with seven microsatellite markers surrounding the *CDHI* gene and verified that an allele for each of the microsatellite markers was common between the two families, demonstrating the existence of a common ancestor carrying this deletion (Fig. 5).

The proband in family 3, was a 34-year-old female of European Lithuanian origin, with DGC. Her mother was also affected by the same disease at uncertain age, and her grandfather developed pancreatic cancer at the age of 43 and none of them was tested for the deletion. The proband in family 4, was a 40-year-old male of Hispanic origin who developed DGC at the age of 40. His 24-year-old brother died from exactly the same disease and his father died at 42 with a pan-

creatic cancer, neither has been tested for the deletion. Carrier tests have been performed in ten other family members and one of which was positive.

In family 5, the proband was a patient of European ancestry, who developed DGC at 33 years. His two sisters developed DGC at 33 and 35 years. At least one second degree relative developed GC, but no more information was available. So far, only the proband was tested, but five other family members are currently being tested for the presence of the deletion. As for family 6, the proband was a 51-year-old patient of European ancestry who developed DGC. His mother died of GC of unconfirmed histology at age of 52. Two sisters were recently diagnosed with DGC by surveillance endoscopy, although only one of them was tested for the deletion and proved to be a carrier. This sister's offspring, constituted of two asymptomatic individuals, also tested positive for the deletion. Four other second degree relatives died of cancer, one with DGC, two of GC of unknown histology and one of lobular breast cancer.

Clinical criteria, frequency of CDH1 alterations and GC incidence

In the present study, we analyzed 93 families based on two clinical criteria: (i) three or more DGC in first degree relatives diagnosed at any age or; (ii) two or more GC in first degree relatives with at least one DGC diagnosed before age 50;

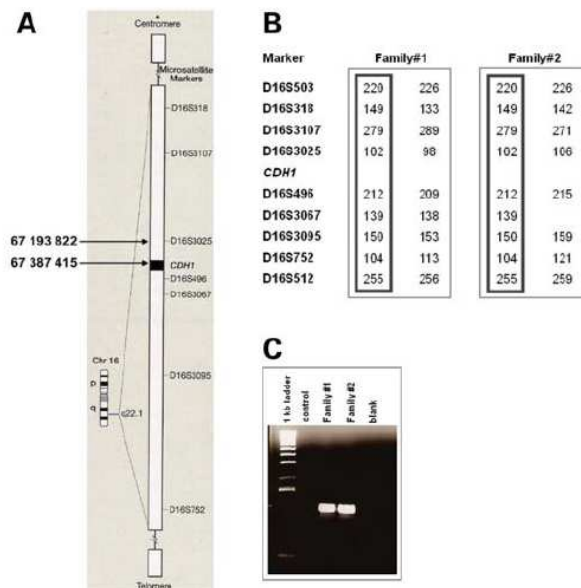


Figure 5. Haplotype analysis of family 1 and family 2 probands. (A) Scheme of chromosome 16 with reference to microsatellite markers used in the haplotype analysis and to the breakpoints of the deletion. (B) Common haplotypes displayed by probands of family 1 and family 2 and proving a common ancestry. (C) Agarose gel of the PCR that allowed the mapping of the deletion, showing similar band sizes for probands in both families.

and found that 6.5% of these families carry large *CDH1* germline deletions.

With the purpose of clarifying the relative contribution of germline *CDH1* point mutations and deletions in a large series of families fulfilling the aforementioned criteria, we collected data not only on the 93 negative families, herein analyzed for genomic rearrangements, but also on 67 HDGC families carrying *CDH1* point mutations, from five different reference centers, and originated from countries with low, moderate and high GC incidence rates.

From the series of 160 probands, 73 (45.6%) were proved to carry germline *CDH1* alterations: 41.9% point mutations and 3.8% large deletions (Fig. 6).

In low incidence countries namely in the North America, Canada, UK and Holland, the frequency of germline *CDH1* alterations was 51.6%, while in moderate (Germany) and high (Portugal and Italy) incidence countries the frequency of alterations was 25 and 22.2%, respectively (Fig. 6). Large deletions were only identified in countries with low incidence of GC and account for 9% of all *CDH1* alterations in HDGC families from such countries (Table 1).

DISCUSSION

In this report, we demonstrate that germline large deletions affecting the *CDH1* locus are one of the mechanisms underlying HDGC. Our data supports the addition of MLPA based

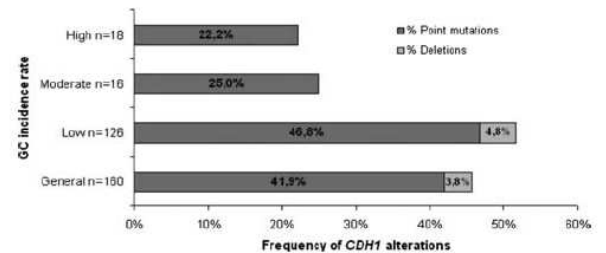


Figure 6. Frequency of *CDH1* alterations in a series of 160 HDGC families, subdivided by GC incidence rates as well as by type of gene alteration (point or small frameshift mutations and large deletions).

screening for such deletions to clinical screening for *CDH1* mutations.

The *CDH1* locus, like the *hMLH1*, *hMSH2*, *BRCA1*, *APC* and *CDKN2A* loci, is a region susceptible to genomic rearrangements leading to increased cancer susceptibility (18,19,21,26).

The analysis of deletions found in our HDGC patients, revealed that two out of five deletions showed extensive sequence identity between the two breakpoints, strongly suggesting that NAHR was the mechanism of their formation (Families 1, 2 and 3). The deletion breakpoints of these two deletions fall within Alu repeats similar to what has been previously reported for *hMLH1*, *hMSH2*, *BRCA1* and infrequently for *APC* (18,19,26). Alu repetitive sequences have long been believed to give rise to genomic deletions promoting recombinational instability (27). *PMP22* deletions have been described to be a product of homologous recombination between intronic AluS elements in hereditary neuropathies (23), but the type of Alu sequences involved in deleterious deletions underlying cancer-associated syndromes have not been systematically explored in the literature. Nevertheless, at least one of the Alu sequences located at large deletion breakpoints in *hMLH1* gene are described to be of the AluSx type (28). In our study, we analyzed the type of Alu sequences associated with *CDH1* large deletions in HDGC, and found that six breakpoints overlap AluS elements: three overlap AluSg, two overlap AluSp and one overlaps an AluSx (Table 3).

One of the deletions herein identified occurs in a context of microhomology and could have arisen by microhomology-mediated recombination, a mechanism of NHEJ. This mechanism is associated with very short stretches of sequence identity (a few bp) between the two ends of the breakpoint junctions (29,30), rather than with long stretches of sequence identity at these locations (31,32). Moreover, it involves the double strand breakage of DNA followed by end joining in the absence of extensive sequence homology. A similar microhomology-mediated intra-strand repair mechanism has been previously described to occur either in pathological conditions such as trisomy in a family displaying an inverted duplication/deletion of 2p25.1–25.3 (33) or in recombination events underlying genomic variation during evolution (34). The same putative mechanism underlying the type of deletion and end re-joining, found in this family, has been described in a recent report where the treatment of mice,

with specific chemicals or radiation during late spermatogenic stages, generates very short deletions, duplications, or inversions with microhomology around breakpoints. The ligation by NHEJ mediated by microhomology, is raised in the aforementioned report, as the major path of repair (35), and provides insight on how this specific *CDH1* deletion may have initially arisen.

Another of our deletions is unlikely to have been produced by homology-dependent mechanisms, since a small sequence insertion (3 bp) at the junction between the deletion breakpoints was identified. Since no extensive homology exists in the vicinity of breakpoints, it is likely that this deletion have been mediated by NHEJ. Identical deletions involving small insertions (3, 4, 6 bp), of unknown origin at the site of the breakpoints, have been previously identified within copy number variants (CNVs) breakpoint-region sequences, in normal population (32,34).

In contrast to all other *CDH1* deletions described so far in this report, one does not seem to be caused by NAHR, microhomology-mediated recombination or small sequence insertions at the junction site. This deletion is flanked by upstream and downstream low complexity AT rich sequences, as well as FLAM_C repeat and an AluJb sequence. Similar features surrounding deletions have been previously described to occur in CNVs described in apparently normal individuals (34), but the mechanisms involved in their generation is not known. The AT rich sequences could mediate a microhomology-mediated deletion mechanism based on their base composition bias, though further evidence would be needed to prove this.

Deletions found in HDGC families mainly fall into three separate classes: products of NAHR, in which recombination between Alu containing sequences leads to deletion of the sequence between them; products of microhomology-mediated recombination, a mechanism of NHEJ; and results of small insertions of unknown origin at the site of the breakpoint junction. These deleterious deletions in *CDH1* appear as the pathogenic counterpart of apparently normal evolution generated CNVs (32,34).

The probands studied ($n = 160$) fulfilled clinically used criteria for HDGC (see Materials and Methods). We found that 45.6% (73/160) of them displayed *CDH1* alterations (Fig. 6). In comparison, only 7/123 (5.7%) of the probands of families who did not meet the above testing criteria, had point mutations or small frameshift mutations, and none had deletions detectable through MLPA (data not shown). Based on this analysis, we have demonstrated that the clinical criteria herein used (three or more DGC in first degree relatives diagnosed at any age or two or more GC in first degree relatives with at least one DGC diagnosed before age 50) will identify most families with HDGC associated germline *CDH1* alterations, with a mutation pick up rate of ~46%. Based on these criteria, ~4% of HDGC patients display large *CDH1* germline deletions (Fig. 6).

Two of the families shared the same large deletion that included the whole of the *CDH3* gene and the first two exons of *CDH1*. Individuals with homozygous germline truncating mutations in the *CDH3* gene present with hypotrichosis with juvenile macular dystrophy (HJMD) syndrome (36,37). Heterozygous carriers of such mutations

are phenotypically normal and have not been reported to have increased GC risk. Haplotype analysis supports a shared ancestor as the source of the (chr16:67193822–67387415) in these two families. We have previously used this approach to demonstrate that recurrent HDGC associated *CDH1* germline point mutations can arise from a common ancestor (6).

In conclusion, 6.5% of probands from strictly defined HDGC families who previously tested negative for *CDH1* mutations carried heterozygous germline deletions. As these can be readily identified using MLPA, an assay frequently used in clinical cancer genetics laboratories, we suggest that MLPA be added to the testing strategy for all families who meet clinical criteria for HDGC.

MATERIALS AND METHODS

Patients and families

The collection of clinical data and peripheral blood samples was approved by the appropriate Ethics Committee from each of the centers participating in this work: University of British Columbia, Vancouver, Canada; Institute of Molecular Pathology and Immunology, University of Porto, Porto, Portugal; Department of Human Pathology and Oncology, Section of Surgical Oncology, Translational Research Laboratory, University of Siena, Italy; Institute of Pathology, Technische Universität München, Munich, Germany; Department of Oncology, University of Cambridge, Cambridge, UK. A series of 160 probands from families with aggregation of DGC was analyzed in this study, based on the following clinical criteria: (i) three or more DGC in first degree relatives diagnosed at any age or; (ii) two or more GC in first degree relatives with at least one DGC diagnosed before age 50 (see Table 1). From these 160 probands, 67 showed point mutations in the *CDH1* gene and were used only for analysis of general frequency of *CDH1* mutations within HDGC families. The majority of these families have been published elsewhere (3,6,10–13,38–46) except for 15 families from University of British Columbia, Vancouver, Canada (unpublished data DGH), six families from Department of Oncology, University of Cambridge, Cambridge (unpublished data CC) and two families from Technische Universität München, Munich, Germany (unpublished data GK). The remaining 93 probands belonged to families that tested negative for *CDH1* germline point mutations. These were analyzed for *CDH1* genomic rearrangements.

Peripheral blood leukocytes from the 93 negative probands were referred for analysis at the different centers. High molecular weight genomic DNA was isolated from all patients using standard methodologies.

Multiplex ligation-dependent probe amplification

A series of 93 probands, negative for *CDH1* germline point mutations, were tested for the presence of large genomic alterations at the *CDH1* locus using MLPA. MLPA is a PCR-based technique for identifying gene dosage alterations, which has been previously described in detail (47). All reagents were provided by the manufacturer in a kit (SALSA P085 *CDH1* MLPA

kit; MRC-Holland, Amsterdam, Holland), and testing was performed according to the manufacturer's recommendations. The assay contains probes for all but one of the *CDHI* exons. For technical reasons exon 8 has been excluded and exon 1 has three different probes. The assay was done according to manufacturer's instructions. Briefly, the probes were hybridized overnight at 60°C with 100 ng of germline DNA and subsequently ligated. Ten microliters of the ligation reaction was used in the PCR which contained FAM labeled primers. The PCR products were analyzed on an ABI 3130×L Genetic Analyzer. The output for each patient displayed a peak that corresponded to the amount of amplified ligated probe present for each exon of the *CDHI* gene, along with several peaks representative of extragenic regions used for ligation and PCR monitoring controls. Peak heights for fragments corresponding to specific exons and control regions were binned, appended to a table, and then saved as a text file, all within the Applied Biosystems Genotyper software. The resulting data were analyzed using the Coffalyser software downloaded from MRC-Holland. Any exon with a decrease or increase in peak height of 70% was scored as a deletion or duplication, respectively. MLPA assays which showed a variation between peak ratios were repeated.

Comparative genomic hybridization microarray

A CGH array strategy was done to confirm the deletions identified by MLPA and to identify the location of deletion breakpoints. Custom CGH arrays were designed using Agilent's eArray program. An 8×15K array format was generated with Agilent HD CGH Database Catalog Probes for coverage of chromosome 16: 066880834–067879823. Genomic DNA (1.5 µg) was labeled using the Agilent Genomic DNA Labeling Kit PLUS (Agilent Technologies, Santa Clara, CA). Samples labeled with Cy3 were co-hybridized with Cy5 labeled gender-matched normal reference gDNA (Promega, Madison WI) to custom 8×15K Agilent human CGH microarrays. Microarrays were washed with Agilent CGH wash buffers and scanned on an Agilent DNA Microarray Scanner. Images were analyzed using Agilent Feature Extraction (version 8.5) and data were analyzed using Agilent CGH Analytics (version 3.4.27).

Breakpoint identification

Based on the information obtained from the CGH array, primers were designed spanning the putative breakpoints for each case and used in long-range PCR. A table with primer sequences is supplied as supplementary material (Supplementary Material, Table S1).

Haplotype analysis

Analysis was performed using the following microsatellite markers surrounding the *CDHI* gene: D16S318, D16S3107, D16S3025, D16S496, D16S3067, D16S3095 and D16S752. Each marker was amplified and then analyzed on an ABI 3130×L Genetic Analyzer followed by analysis using GeneMapper V7.0 software. Primer sequences for the microsatellite markers D16S3025, D16S496, D16S3095 and D16S752 have been previously reported (11). Sequences for the remaining

markers, D16S318, D16S3107 and D16S3067 are listed in the genome database (<http://www.gdb.org>).

Analysis of breakpoints sequence context

Breakpoints were defined as a set of coordinates on the genome spanning the genomic sequence of the deletions. The coordinates have been loaded on the UCSC genome browser as a BED file accessible on <http://biodev.cbm.fvg.it/remo/DEL.txt>. Both automatic and manual bioinformatics analysis have been carried out in order to analyze the genomic context of these regions.

For each deletion we analyzed the closest repeats in a range of 1500 bp flanking the start/end points. Repeats were obtained from the Ensembl *Homo sapiens* core database version 50 and UCSC genome browser Human Mar. 2006 Assembly. Sequences in a range of −150/+50 bp flanking the start-point and −50/+150 bp flanking the end-point of the deletions have been collected in order to test the similarity around the breakpoints by using both clustalw and blast2 with default parameters. An interval of 5 Mb around the deletion start/end points has been analyzed in order to look for the presence of the RAG1 binding site on both strands by using a custom perl script and known consensus sequences (CACTGTG.{11,13}GGTTTTTGT,GGTTTTTGT.{11,13}CACTGTG,ACAAAAACC.{11,13}CACAGTG,CACAGTG.{11,13}ACAAAAACC,CACTGTG.{22,24}GGTTTTTGT,GGTTTTTGT.{22,24}CACTGTG,ACAAAAACC.{22,24}CACAGTG,CACAGTG.{22,24}ACAAAAACC). In order to perform randomization analysis we collected 150 000 random points on the human genome and calculated for each of them the distance from the closest Alu. The distribution of the distances has been trimmed in order to avoid considering points in poorly annotated regions and/or in gene deserts and/or in genomic gaps, which resulted nonetheless to be very far from Alus. We trimmed out all the points retaining a distance bigger than 4519 bp (the 1.5*IQR of the distribution of all the distances) from the closest Alu.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

ACKNOWLEDGEMENTS

We gratefully acknowledge patients, families and their caregivers for their willing participation in this research project and who provided consent regarding use of the information obtained from the study.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by the following grants: The National Cancer Institute of Canada (Canadian Cancer Society); The Michael Smith Foundation for Health Research to D.H.; The Portuguese Foundation for Science and Technology—FCT (grant numbers POCTI/SAU-OBS/58111/2004,

PTDC/SAU-GMG/72168/2006, Program *Ciência 2007* to C.O.); Sixth Framework Programme from European Union FP6 (grant number LSHC-CT-2005-018754); The Deutsche krebshilfe (grant number 10-1345-HÖ2) and Istituto Toscano Tumori (ITT) (Grant 2007 Ref: *Gene expression profile and therapeutic implication in GC. From the clinical overview to the translational research*). Funding to pay the Open Access charge was provided by National Cancer Institute of Canada Grant #018381 "The effect of E-cadherin mutations on gastric and breast cancer risk: from families to populations."

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PAPER 10.3

Roviello, F., et al., **Long term results after R0 resection in the surgical treatment of gastric cancer.** Surgery in Multimodal Management of Gastric Cancer 2011, cap. 17, (eds) Springer-Verlag Italia 2011. In press

Long-Term Results after R0 Resection in the Surgical Treatment of Gastric Cancer

17

Franco Roviello, Giovanni Corso and Daniele Marrelli

Abstract

The International Union against Cancer and the American Joint Committee on Cancer system define residual tumor as R0 when the surgical procedure is concluded without any evidence of macroscopic residual tumor. This index is considered an important factor in terms of the prognosis and long-term survival of gastric carcinoma patients. Our data demonstrate that prognosis and survival differ in high- vs. low-risk areas of gastric cancer; in particular, we determined that prognosis is better in high-risk areas for gastric cancer in patients undergoing R0 surgery. These differences may also take into account the multiple etiologies of gastric tumors. In this chapter, we discuss the R classification in gastric cancer, focusing on long-term survival after radical surgery (R0) and stratifying these data with respect to high- and low-incidence areas for gastric carcinoma development.

Keywords

Gastric cancer • Radical surgery • High- and low-risk areas

17.1 Introduction

Long-term survival in patients with gastric cancer (GC) after curative (R0) resection is strongly influenced by pTNM stage classification, in which lymphatic dissemination is the main predictor of tumor recurrence. Furthermore, it has been demonstrated that extended lymphadenectomy may decrease the risk of tumor relapse after gastric resection, with several other prognostic factors indicative of long-

term outcome. Indeed, overall 5-year survival for GC after curative (R0) resection is extremely variable, and significant differences in survival have been reported from institutions throughout the world.

In R1 and R2 cases, prognosis is determined primarily by the absence or presence of distant metastases while the pTNM classification is of minor significance; by contrast, in R0 patients, pTNM is a good predictor of prognosis.

Figure 17.1 provides an overview of gastric carcinoma management with respect to the R0, R1, and R2 classification. In this chapter, we review the overall survival of GC patients who underwent radical (R0) surgery, as reported by several institutions located in different geographic areas.

F. Roviello (✉)

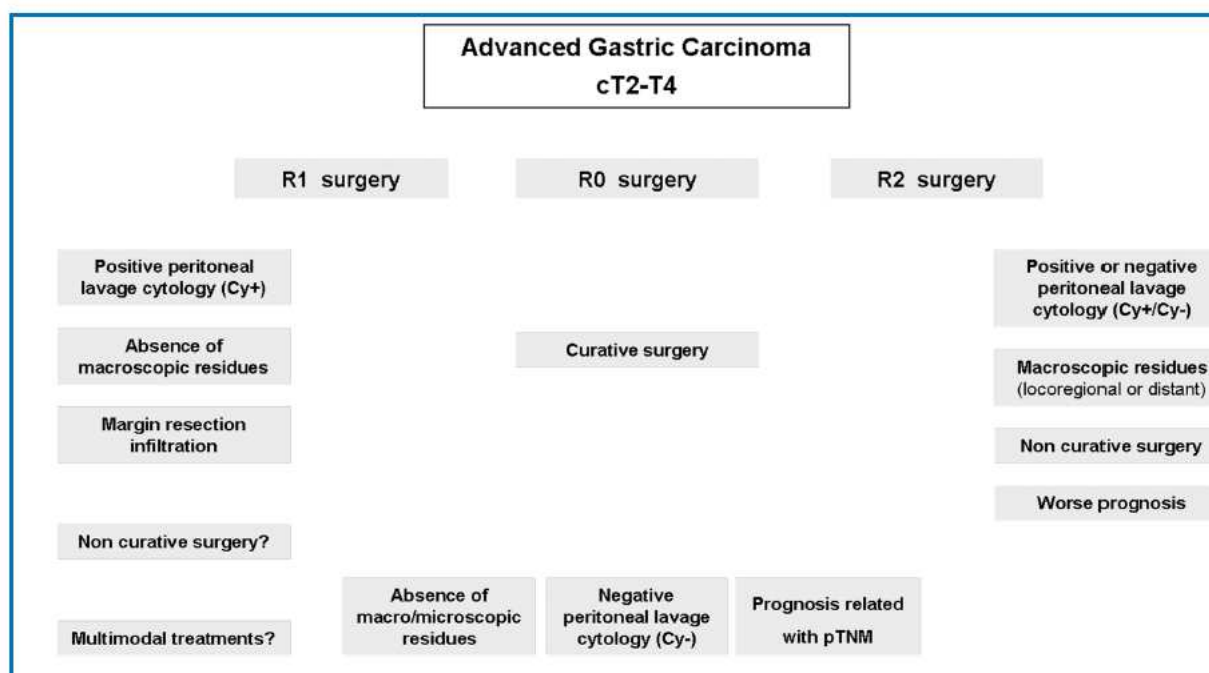


Fig. 17.1 The proposed flow-chart is a model for gastric carcinoma management with respect to the R0, R1, and R2 classification

17.2 Definition of R0 Resection

The residual tumor (R) classification system was defined by Hermanek and Colleagues in 1994 [1, 2]. Surgery is defined as “potentially curative” (R0) when the surgical procedure is concluded without any evidence of macroscopic residual tumor in the tumor bed, lymph nodes, and/or at distant sites. In addition, macroscopic and microscopic examination of resection margins is negative. R1 and R2 indicate, respectively, microscopic and macroscopic residual tumor. According to this definition, the R classification is considered as a predictor of prognosis, in which good long-term survival can be expected only in R0 patients. However, this classification system is currently discussed controversially by different authors because the definitions do not consider the presence of tumor dissemination through the blood stream, peritoneal dissemination, and/or lymphatic involvement. Consequently, the presence of microscopic and distant residual disease could be missed [3]. In this regard, clinical pre-operative staging is fundamental, including the selection of the appropriate modality to determine the extent of tumor spread-

ing: endoscopic ultrasound, computed tomography, positron emission tomography, magnetic resonance imaging, or laparoscopic exploration [4]. More recently, the International Union against Cancer (UICC)/American Joint Committee on Cancer (AJCC) system defined as R0 resection a complete resection of the primary cancer without macroscopic or microscopic residual disease. Patients with circulating intraperitoneal cancer cells, identified by cytology examination after lavage, are classified as having R1 disease. According to the new UICC/AJCC TNM, the number of positive lymph nodes is considered a strong prognostic determinant, and cases with positive peritoneal cytology are considered as M1 and included in stage IV [5, 6].

17.3 Long-Term Results after R0 Resection in High-Incidence Areas

In this and the following sections, we analyze R0 GC cases and the long-term results, as reported from areas around the world where there is either a high or a low incidence of GC. Among the areas with a high GC incidence are Japan, Korea, China, and Latvia. In Korea and Japan, extended lymph

node dissection (D2 or D3) is routinely performed.

Three studies of interest were carried out in Japan. Maruyama et al. [7] analyzed the data of 8851 patients with primary gastric carcinoma (JGCA 2006). The authors identified 4959 GC patients as having R0 disease (namely curability A); the cumulative 5-year survival was 88.5% [7]. A Japanese study based on tumor histology grade was performed on 1119 GC patients, including a subset of R0 cases. The tumors were graded as follows: grade 1, well differentiated; grade 2, moderately differentiated; and grade 3, poorly differentiated [8]. Five-year survival was 71% for patients with grade 1, 65.7% for those with grade 2, and 66.7% for those with grade 3 disease. In the third Japanese study [8], the outcome of 587 GC patients who underwent R0 gastrectomy with D2 lymphadenectomy was analyzed. Five-year survival according to the AJCC/UICC staging system was 94.6% for Ia, 88.4% for Ib, 70.6% for II, 54.1% for IIIa, 35.5% for IIIb, and 25.6% for IV.

A population of 9998 GC patients was analyzed by Ahn et al. [10] from Korea, which has the world's highest incidence of GC. Overall 5-year survival in their R0 resection patients was 66.4%. According to the TNM stage, the 5-year disease-free survival of patients with stages Ia, Ib, IIa, IIb, IIIa, IIIb, and IIIc was 95.1, 88.4, 84, 71.7, 58.4, 41.3, and 26.1%, respectively.

Among Asian countries, a paper described 2159 patients in China who underwent both en bloc resection of primary GC and D2/D3 lymphadenectomy, without microscopic or macroscopic residual disease (R0). The 5-year disease-free survival of patients with stages I, II, III, and IV was 84, 50.8, 29.1, and 12.4%, respectively [11].

The advantage of these Asian studies was that in each one screening programs led to the diagnosis of GC at an early stage of the disease. Furthermore, most patients achieved radical (R0) resection with D2 or D3 lymphadenectomy.

Good results were also reported in a Latvian study of 444 patients with GC who underwent R0 surgery [12]. Five-year survival was 52.5% but considering pTNM stage was 89.7% for stage Ia, 72.6% for stage Ib, 52.9% for stage II, 34.8% for stage IIIa, 27.4% for stage IIIb, and 18.7% for stage IV.

Considered as a whole, the overall 5-year sur-

vival of patients from high-incidence areas treated by R0 surgery is approximately 44-88%. The lowest overall survival was registered in China (44%) and the highest in Japan (88.5%).

17.4 Long-Term Results after R0 Resection in Low-Incidence Areas

Among the countries with a low GC incidence, we consider studies from the USA, Germany, and Norway.

In the USA, the 711 patients analyzed in the study of Strong et al. [13] were treated in a specialized center by R0 resection, with extended lymphadenectomy performed in most cases. The authors reported an overall 5-year survival rate of 58%. The study of Cunningham et al. [14] consisted of 436 patients; overall 5-year survival after R0 resection in this group was 33%. In a trial comparing chemoradiotherapy after surgery vs. surgery alone, Macdonald et al. [15] reported an overall 5-year survival of 44% in 281 patients treated by gastrectomy and chemoradiotherapy vs. 28% in 275 patients treated by surgery alone. Notably, in that trial only 10% of patients were treated by D2 dissection.

A study from Germany analyzed 124 GC patients who underwent curative surgery (R0) with D2 lymphadenectomy [16]; the overall survival rate over a period of 94 months was 51%. A multicentric study was conducted in Germany by Siewert et al. [17], in which 1182 consecutive patients underwent radical surgery with D1 or D2 lymphadenectomy. The 5-year survival rate with respect to stage was: Ia 82.8%, Ib 68.3%, II 42.9%, IIIa 28.2%, IIIb 16.7%, and IV 17.3%. Long-term survival was better in patients receiving R0 surgery and D2 lymphadenectomy at stage II (29% vs. 56.7%). In another Western study, from Norway [18], 97 patients underwent R0 surgery for gastric carcinoma with D1 lymphadenectomy; 5-year survival was 39%.

In low-incidence areas (USA, Norway, Germany), there are more cases of advanced stage disease and thus a lower overall 5-year survival (28-58%) than in high-incidence areas (Korea, Japan, China, Latvia), where survival is 44-67%. Specifically, the American data showed lower over-

all 5-year survival in GC patients with R0 resection (28-58%), with similar results in the population from Norway (39%). In Germany, the overall survival rate (42-51%) is within the range of that in high-incidence areas (44-88.5%). Generally, however, patients with R0 GC in countries with a higher incidence of the disease have a better prognosis than analogous patients in low-incidence countries.

17.5 The Experience of the Italian Research Group for Gastric Cancer

The Italian Research Group for Gastric Cancer (IRGGC) assessed a prognostic score predicting tumor recurrence in GC patients with curative resection (R0) [19] based on 536 patients who underwent UICC R0 resection at three surgical department in Italy (University of Siena, University of Verona, and Morgagni Hospital of Forlì). During the follow-up period, tumor relapse was identified in 50.7% of these patients. The results of this study demonstrated that the predicted and observed risk of recurrence overlapped, with a sensitivity of 83.5% and specificity of 81.1%. The statistical model identified nodal status, pT stage, tumor loca-

tion, extent of lymphadenectomy, and advanced age as independent predictors of recurrence.

Extended lymphadenectomy (D2) is considered the standard approach for the treatment of advanced GC, with benefits in long-term survival. The IRGGC demonstrated that the 5-year overall survival was 54% in patients with R0 surgery and D2 lymphadenectomy, and with a real survival benefit for patients with locoregional lymph node involvement [20]. Another study [21], conducted on 286 R0 GC patients treated with super-extended (D3) lymphadenectomy, demonstrated a potential benefit of this procedure, especially in patients with pT2 disease and positive nodes (5-year overall survival 60%).

The overall geographic Italian area is considered to be at low-risk for GC, but in some regions of the country the incidence is relatively high [22, 23]. In Tuscany, for example, the estimated incidence of GC is three-fold higher than in Southern Italy. GC patients in Tuscany have a 5-year survival probability of 63.2% compared to 48% in patients in Southern Italy.

Table 17.1 reviews the overall survival rate reported in the literature for GC patients undergoing potentially curative surgery (R0).

Table 17.1 Overall survival rate reported in the literature in patients with gastric cancer who underwent potentially curative surgery (R0)

References	Country	Risk area	Number of patients	UICC	Lymphadenectomy	Follow-up end-time (months)	Overall survival (%)
[8]	Korea	High	9.998	R0	D2-D3	60	55.6
[7]	Japan	High	4.959	R0	D2	60	88.5
[9]	Japan	High	1.119	R0	Unknown	60	67.8
[10]	Japan	High	587	R0	D2	60	61.5
[11]	China	High	2.159	R0	D2-D3	60	44.0
[12]	Latvia	High	444	R0	D1	60	52.5
[13]	USA	Low	711	R0	D2	60	58.0
[14]	USA	Low	436	R0	Unknown	60	33.0
[15]	USA	Low	556	R0	D1	60	28-44 ^a
[16]	Germany	Low	124	R0	D2	94	51.0
[17]	Germany	Low	1.182	R0	D1-D2	60	42.7
[18]	Norway	Low	97	R0	D1	60	39.0
[Present report]	Italy-Tuscany	High	545	R0	D2-D3	60	63.2
	Italy-Southern	Low	89	R0	D2-D3	60	48.0

^aSurgery alone vs. surgery + radio-/chemotherapy.

17.6 Conclusions

Radical resection (R0) for GC offers the possibility of cure although it requires complete surgical removal of the tumor. The UICC classification for GC considers curative resection (R0) as a good predictive prognostic factor; nevertheless, the limitation of this classification (R0 vs. R1) is the risk of overlooking microscopic tumor diffusion, especially in cases with hematogenous or peritoneal metastasis. Therefore, the R classification may represent a more valid prognostic indicator when associated with pTNM stage.

The overall survival of R0 patients who undergo GC resection is 30-70%, and varies notably in different institutions worldwide. The survival rate of R0 GC patients is better in high-incidence than in low-incidence areas. This difference has been explained by: (a) differences in the disease itself; (b) stage migration; and (c) differences in treatment. These explanations are probably not mutually exclusive. Moreover, environmental factors can interact with and influence molecular pathways, as can single-nucleotide polymorphisms and dietary and life-style habits.

The widespread introduction of screening programs in high-incidence countries has resulted in the detection of early stage GC, which in turn would explain differences in prognosis; in addition, those countries generally have a better diagnostic and therapeutic approach, which is also likely play a prognostic role. This points to the need for a standard surgical approach to the treatment of GC; for example, it has been demonstrated that extended (D2) and superextended (D3) lymphadenectomy are associated with better R0 outcome in advanced cases of GC, also in low-risk areas.

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PAPER 10.4

Roviello, F., et al., **Super-extended (D3) lymphadenectomy in advanced gastric cancer.** Eur J Surg Oncol 2010;36:439-446

Super-extended (D3) lymphadenectomy in advanced gastric cancer

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Accepted 22 March 2010
Available online 13 April 2010

Abstract

Purpose: To analyze our experience with D3 lymphadenectomy in the treatment of advanced GC with specific reference to post-operative morbidity and mortality, incidence of para-aortic node (PAN) metastases, and long-term prognosis.

Methods: Short- and long-term results of D3 lymphadenectomy were analyzed in 286 patients with advanced GC.

Results: PAN metastases were demonstrated in 37 patients. PAN involvement was significantly higher in upper third tumours (29%) compared to middle and lower third (7%; $P < 0.001$). Eighty patients developed post-operative complications, being pulmonary disorders (6%), abdominal abscesses (4.5%) and pancreatic fistulas (3%) the most frequently observed. In-hospital mortality was 2%. Overall 5-year survival rate for R0 pT2–4 patients was 52%. When considering survival in relation to nodal involvement, both pN3 and non-regional lymph node metastases (M1a) patients showed a chance of long-term survival: 5-year survival was 31% for pN3 and 17% for M1a cases. Furthermore, the 5-year survival rate was remarkably high (about 60%) even in pN2 and pN3 subsets when no serosal invasion (pT2) was demonstrated.

Conclusions: D3 lymphadenectomy could be further explored in specialized centers for curative surgery of advanced GC, especially for upper third tumours, providing that an acceptable morbidity and no increase in mortality can be offered.

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Keywords: Gastric cancer; Surgery; D3 Lymphadenectomy; Prognosis

Introduction

The reduction in GC mortality reported during recent years by Japanese authors has been attributed to the increased detection of early cancers as well as to the widespread use of extended lymphadenectomy.¹ To date, little evidence exists from prospective randomized trials to confirm the advantage of extended lymph node dissection compared to limited lymphadenectomy.^{2–4} Despite that, long-term results after D2–D3 lymphadenectomy reported by Japanese as well as by dedicated Western centers are definitely better compared to European and US results.^{5–7} In previous publications, we identified lymphadenectomy together with depth of tumour invasion and nodal involvement as independent predictors

of survival, demonstrating a more favourable prognosis with extensive nodal dissection.^{8,9}

The purpose of the present study was to analyze our experience with super-extended (D3) lymphadenectomy in the treatment of gastric cancer with specific reference to post-operative morbidity and mortality, incidence of PAN metastases, and long-term prognosis.

Patients and methods

Population under study

This study analyzed 286 patients who underwent a macroscopic potentially curative gastrectomy with D3 lymph node dissection for advanced primary gastric adenocarcinomas from January 1993 to June 2007 at two Italian surgical departments (Department of General Surgery and Surgical Oncology, University of Siena; First Department

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of General Surgery, University of Verona) both part of the Italian Research Group for Gastric Cancer (IRGGC).

Eligibility criteria

The inclusion criteria were as follows: 1) histologically confirmed gastric adenocarcinoma; 2) macroscopic curative (R0-1) resection; 3) advanced (pT2-4) tumours; 4) systematic and standardized D3 lymphadenectomy (JGCA guidelines). Gastric stump and linitis plastica type tumours were excluded from the analysis. The main demographic and clinical characteristics of the cohort in relation to tumour location are reported in Table 1.

The study was approved by the local ethic committees.

Extent of gastric resection

Gastric resection was performed according to the extent of the tumour in the stomach. Distal subtotal gastrectomy was preferred for tumours located in the lower and middle third of the stomach provided that the proximal resection margin stayed at least 5 cm from the tumour edge, otherwise total gastrectomy was carried out.⁹

Extent of lymphadenectomy

Lymphadenectomy was classified and standardized in accordance with the Japanese Gastric Cancer Association (JGCA) guidelines. D3 lymphadenectomy consisted of the removal of regional nodes in the first, second and third level stations as defined by the JGCA.¹⁰ This procedure has been described in detail elsewhere, and progressively introduced in our practice since 1993.¹¹ PAN dissection implied

the resection of nodes between the level of the celiac axis and the left renal vein (Station 16-a2) and nodes between the left renal vein and the inferior mesenteric artery (Station 16-b1). Dissection of the left upper lateral nodes (Station 16-a2-lat) was generally not performed and reserved to patients with enlarged nodes in this area; in particular, in all upper third tumors total gastrectomy with macroscopic evaluation of 16-a2-lat nodes was performed. Lymphadenectomy at the splenic hilum was limited to patients with upper third tumours and enlarged nodes or tumours reaching the greater curvature or the gastric fundus. This was achieved by performing a pancreas preserving splenectomy.

Staging and lymph node classification

Perigastric nodes were dissected from the excised specimen immediately after resection, assigned to the appropriate station according to the JGCA classification,¹⁰ and sent for histological examination. In order to avoid error in correct lymph node classification after en bloc resection, the second and third tier nodes were subdivided by the surgeon during lymphadenectomy, as previously described in details.¹²

Tumours were staged according to the last version of the pathologic classification (pTNM) of the International Union Against Cancer (UICC). The histological classification followed the Lauren criteria.

Follow-up

All patients were followed-up with a standardized protocol.⁹ The median follow-up period for surviving patients was 57.2 months (range 6–200) and no patients were lost to follow-up procedure.

Table 1
Main demographic and clinical characteristics according to the tumour location in the 286 patients under study.

	Upper third (n = 82)	Middle third (n = 93)	Lower third (n = 111)	Total
Age (years)				<i>P</i> = 0.722
Mean (range)	63.2 (30–83)	63.3 (32–87)	62.2 (30–87)	62.8 (30–87)
Gender				<i>P</i> = 0.023
Male	60	60	60	180
Female	22	33	51	106
Lauren classification				<i>P</i> = 0.510
Intestinal	52	55	57	164
Diffuse	23	31	44	98
Mixed	7	7	10	24
Depth of invasion (pT)				<i>P</i> = 0.004
pT2	38	41	49	128
pT3	36	38	61	135
pT4	8	14	1	23
Nodal involvement (pN)				<i>P</i> = 0.012
pN0	19	22	28	69
pN1	17	35	35	87
pN2	18	20	22	60
pN3	5	9	13	27
M1a	23	7	13	43

Statistical analysis

In 254 cases, there was pathological confirmation that a potentially curative resection (classified as R0) was achieved. Conversely, microscopic residual tumour (R1) or positive peritoneal washing were diagnosed in 32 patients (11.2%). To evaluate the impact of D3 dissection on long-term survival, we avoided to analyze patients with microscopic non-curative (R1) resection, for which the prognosis is well established to be poor and independent from the extent of lymphadenectomy. Post-operative mortality was assessed while deaths unrelated to tumour recurrence were considered censored observations at the time of death. Survival curves were estimated using the Kaplan–Meier method and compared by the Log-rank test. Chi-square test was used for categorical data and ANOVA test for continuous variables.

Results

Number of lymph nodes

The number of excised and positive nodes according to the main characteristics of the tumour for the cohort under study is reported in Table 2. Of the 286 patients, a total of 13,459 lymph nodes were dissected, with a median number of 45 (range, 15–110) dissected nodes per case. The median number of metastatic nodes was 5 (range, 0–75) in the overall series and 8 (range, 1–75) in pN + patients.

No difference in the average number of dissected nodes was observed in relation to the depth of tumour invasion (pT) ($P = 0.234$). Conversely, the mean number of metastatic nodes increased from pT2 to pT3 and pT4 tumours ($P < 0.001$) (Table 2).

Number of para-aortic lymph nodes

A total of 1508 nodes were retrieved from the PAN stations, with a median of 5 (range, 0–22). Thirty-seven patients (12.9%) showed PANs involvement for a total

number of 175 nodes. The median number of metastatic nodes in para-aortic area was 3 (range 1–22).

Frequency of para-aortic lymph node involvement and pT category

When considering PAN stations, no increase in the average number of dissected ($P = 0.972$) as well as positive nodes ($P = 0.074$) was observed in relation to the depth of tumour invasion (pT) (Table 2). Likewise, the increase in the frequency of involvement of PANs according to pT classes was not statistically significant (8.6% for pT2, 16.3% for pT3 and 17.4% for pT4; $P = 0.142$).

Frequency of para-aortic lymph node involvement and tumour location

Considering PAN metastases according to the location of the tumour, the frequency of nodal involvement was significantly higher in upper third tumours (26.8%) compared to middle (7.5%) and lower third (7.2%) tumours ($P < 0.001$).

Short-term results

Eighty of the 286 patients developed post-operative general and surgical complications (morbidity: 28%); of these, 6 patients died (post-operative mortality: 2.1%). Table 3 lists the type of complications and their frequency. As shown, pulmonary complications (5.6%), abdominal abscesses (4.5%), pancreatic fistulas (3.1%) and anastomotic leaks (2.8%) were the most frequently observed complications.

Long-term results

At the follow-up end date, 150 out of the 286 patients were still alive, and 144 were disease-free. Conversely, 122 patients died because of cancer recurrence, 6 from post-operative complications and 8 from causes other than tumour relapse.

Table 2
Overall and para-aortic number of excised and positive lymph nodes according to the main characteristics of the tumour in the 286 patients under study.^a

	Total		Para-aortic stations	
	Excised	Positive	Excised	Positive
Overall	47.1 (±16.6)	8.7 (±11.5)	5.5 (±4.1)	0.6 (±2.5)
Tumour location				
Upper third	49.4 (±17.6)	10 (±12)	6.1 (±4.2)	1 (±2.7)
Middle third	47.6 (±17)	8 (±11.6)	5.3 (±4.1)	0.4 (±2.1)
Lower third	44.8 (±15.4)	8.5 (±11.2)	5.3 (±3.9)	0.5 (±2.6)
Depth of invasion (pT)				
pT2	46.1 (±16.8)	5.1 (±7.9)	5.5 (±4.2)	0.3 (±1.5)
pT3	48.6 (±16.1)	12 (±13.3)	5.6 (±4.1)	1 (±3.3)
pT4	43 (±18)	9.9 (±11.5)	5.4 (±3.5)	0.3 (±0.8)

^a Numbers represent the mean value with ±SD in parenthesis.

Table 3
Major post-operative complications observed in the 286 patients under study.

Type of complication	No. of cases (286 patients)
Pulmonary	16
Abdominal abscess	13
Pancreatic fistula	9
Anastomotic leak	8
Delayed gastric emptying	7
Lymphorrhea	6
Paralytic ileus	3
Duodenal stump leak	3
Abdominal hemorrhage	3
Severe pancreatitis	2
Anastomotic haemorrhage	1
Biliary fistula	1
Cardiovascular	4
Others	4
Total	80

The 5-year survival rate for the 254 patients who underwent R0 resection was 52.2% with a median survival time beyond the observation period. The 5-year survival rate according to the depth of tumour invasion (pT) was 67% for pT2 tumours (median beyond the observation period), 39.2% for pT3 (median, 31.1 months) and 29.1% for pT4 (median, 10.3 months) ($P < 0.001$). Survival curves in relation to nodal involvement (pN) are illustrated in Fig. 1. It is noteworthy that pN3 cases as well as patients with non-regional lymph node metastases (M1a) had a chance of long-term survival.

Fig. 2 shows the 5-year survival rate for each pN subset according to pT stage (pT2 vs. pT3–4). In node negative

patients, the probability of survival was very high independently from pT class ($P = 0.615$). In node positive patients, the 5-year survival rate for pN1, pN2 and pN3 patients was about 60% in pT2 cases while it decreased progressively in patients with serosal invasion (52.3% in pN1, 22.2% in pN2 and 23.6% in pN3). No difference was observed in M1a patients.

Discussion

Extent of lymphadenectomy and long-term results

The extent of lymphadenectomy remains a matter of debate in gastric cancer surgery. The well-known randomized Dutch² and British³ trials that compared limited (D1) versus extended (D2) lymphadenectomy showed no significant differences in 5-year survival rates between the two groups. This topic was re-considered by a Cochrane Review¹³ which concluded that “randomized studies show no evidence of overall survival benefit (after D2 dissection), but possible benefit in T3N+ tumours. These results may be confounded by surgical learning curves and poor surgeon compliance. Non-randomized comparisons suggest a possible survival benefit for D2 in intermediate UICC stages.”

Recently, Wu et al.^{4,14} published the short- and long-term results from a prospective randomized trial comparing limited and extended lymph node dissection carried out at a single specialized institution in Taiwan. Two-arm randomization considered 221 patients and allowed to demonstrate a survival benefit in overall and disease-free survival rates after extended lymph node dissection.

Extent of lymphadenectomy and tumour recurrence

It is well-demonstrated that the great majority of tumour relapses occurs within 3 years ensuing potentially curative (R0) surgery.^{9,15} In the randomized Taiwan trial, the number of patients who relapsed beyond 3 years after extended dissection was limited, but several relapses occurred over time in the D1 group making overall and disease-free survival curves of the two groups to diverge more clearly after 36 months.⁴ This figure was also evident in the final results of the randomized Dutch trial in pN1 and pN2 subgroups¹⁶ and, similarly, in our experience. These data seem to suggest that microscopic residual tumour in surgically un-excised nodes should proliferate and grow in several years leading to overt cancer recurrence. Besides, adjuvant chemo-radiotherapy seems not to influence this aspect as shown in the Intergroup 0116 trial (SWOG 9008).¹⁷ In addition, there are some evidences that lymph node excision, although negative at microscopic examination, can improve survival¹⁸ and that lymphadenectomy is beneficial only when the disease is widely encompassed, probably because cancer cells are present in the regional nodes even in cases classified as pN0.¹⁹

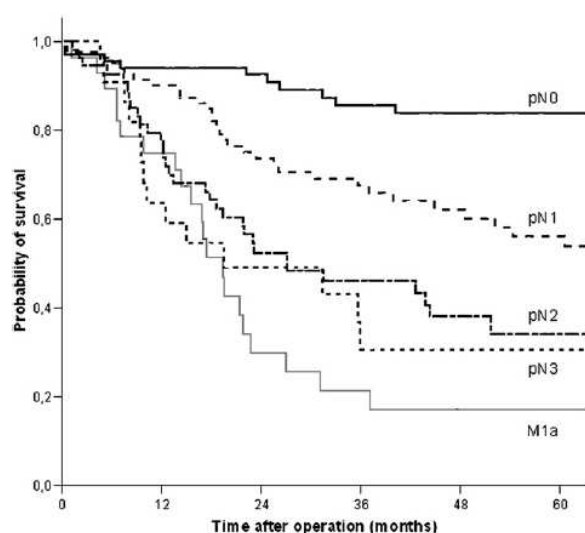


Figure 1. Kaplan–Meier estimates of survival probability according to nodal involvement (pN) in the 254 patients who underwent potentially curative gastrectomy with D3 lymphadenectomy. Median survival time was beyond the observation period for pN0 and pN1; 27.1 (95% CI 5.6–48.5) months for pN2; 19.5 (95% CI 0–44.4) months for pN3 and 19.4 (95% CI 15.6–23.2) months for M1a ($P < 0.001$).

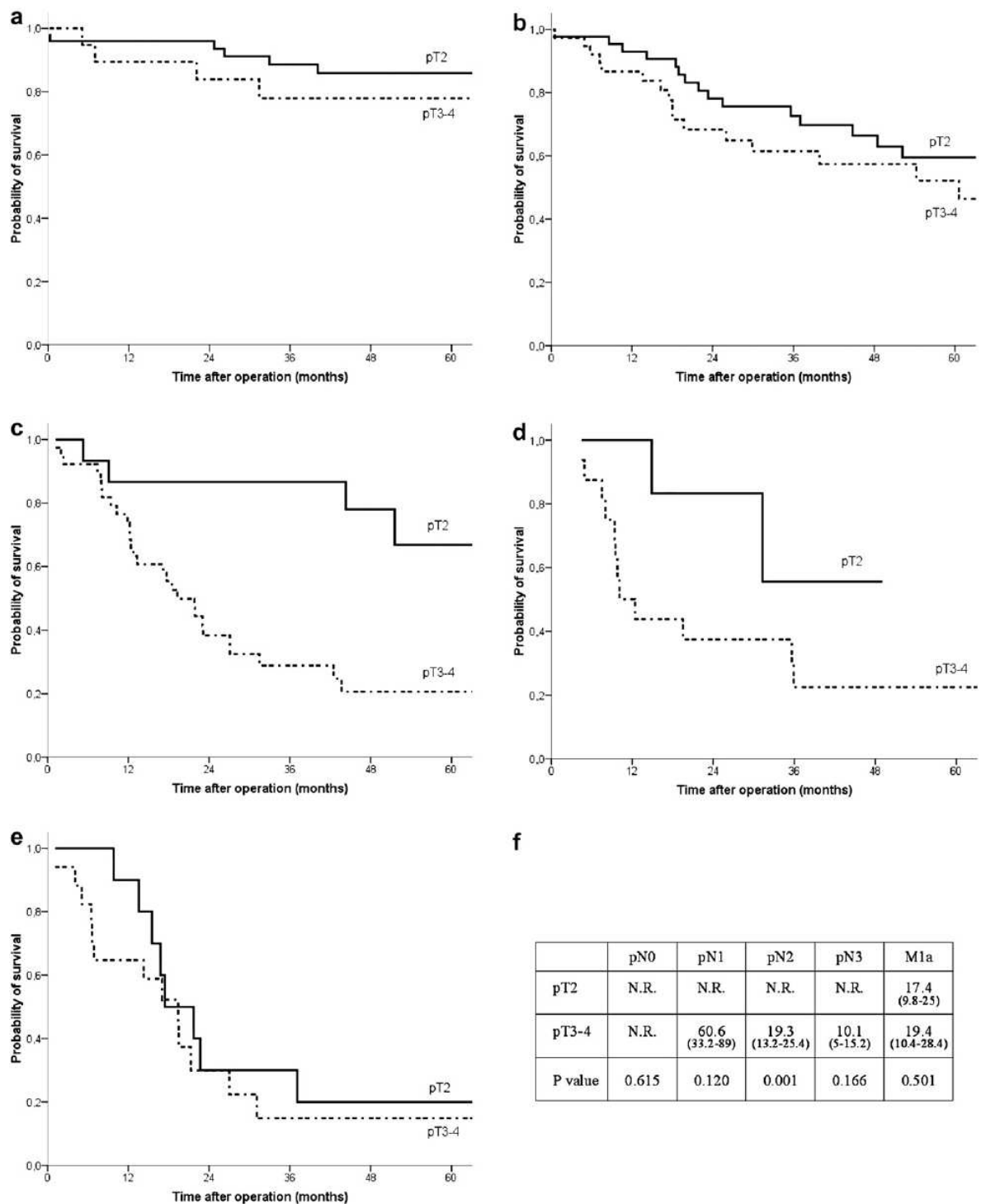


Figure 2. Kaplan–Meier estimates of survival probability according to the depth of tumour invasion for each pN subset in the 254 patients who underwent potentially curative gastrectomy with D3 lymphadenectomy. Fig. 2a–e refers to pN0, pN1, pN2, pN3, M1a subsets, respectively. Median survival times and *P* values are reported in the Table (Fig. 2f).

Pros of extended lymphadenectomy

Nodal metastases are an early event in gastric cancer and one of the key factors affecting prognosis. Extended (D2) and super-extended (D3) lymph node dissection have been suggested to lead to improved staging and better long-term results in several prospective observational series.^{7,8,20,21} Specifically, in a previous study we showed that D2 and D3 lymphadenectomy enables positive nodes clearance that would not have been achieved with limited dissection (D1) in 39% of cases.²² Similar findings were reported by a Dutch study wherein 32% of the patients had positive nodes removed in the second and third tiers.²⁰ In our study, the percentage of patients requiring extended or super-extended lymphadenectomy to remove positive nodes in the second and third levels was 9% in pT1 tumours, 42% in pT2, 56% in pT3, and 67% in pT4.²²

Para-aortic lymph node involvement

PAN involvement is regarded as distant metastasis in the TNM classification. However, the results recently reported by Japanese surgeons after super-extended lymphadenectomy (i.e. with complete dissection of the para-aortic stations) have cast doubts on this statement. These studies revealed that the incidence of PAN involvement is higher than expected, ranging from 8 to 20% in patients with advanced gastric cancer and that the 5-year survival rate for this subgroup of patients after super-extended lymph node dissection is 13–20%.^{23–25}

In our study, PAN deposits were identified in 13% of the cases with a long-term outcome consistent with that reported in the Japanese literature (Fig. 2). In keeping with previous papers,¹¹ metastatic spread to PANs was significantly more frequent in upper third compared to middle and lower third tumours (27% vs. 7%; $P < 0.001$).

Morbidity and mortality after extended lymphadenectomy

Extended and super-extended lymphadenectomy can be associated with large increases in post-operative morbidity and mortality, as reported by trials from the UK³ and The Netherlands,² where the volumes of interventions for centre and surgeon were extremely low and pancreatosplenectomy was routinely combined with extended lymph node dissection. The Taiwan trial⁴ as well as the interim analysis of a randomized trial from Italy²⁶ showed comparable short-term results after pancreas preserving D2 gastrectomy carried out in high volume centres.

The Japanese prospective randomized trial compared D2 and D2 plus para-aortic lymphadenectomy (JCOGS 9501) in 523 gastrectomies performed by experienced surgeons. While morbidity for the super-extended surgery group (28%) was slightly higher than the extended group (21%), there was no difference in the incidence of four major

complications (anastomotic leak, pancreatic fistula, abdominal abscess, pneumonia) and the reported hospital mortality was 0.8% for the two groups.²⁷ Furthermore, no association between post-operative mortality and the extension of lymphadenectomy was reported in a multicenter randomized trial from Poland²⁸ and several observational studies published by high volume European centres.^{6,8,9,29} In particular, we have recently demonstrated that it is possible to resemble Japanese results (mortality rate of 0.5%) in 203 patients aged 75 years or younger who underwent D2–D3 curative gastrectomy.¹¹ In the current experience, 28% of patients developed post-operative general or surgical complications with pulmonary complaints (6%), abdominal abscesses (4.5%) and pancreatic fistulas (3%) as the most frequently observed complaints, while the mortality rate at 2% is consistent with best data reported in the literature.

The Japanese JCOGS 9501 randomized trial

The final long-term results of the JCOGS 9501 randomized trial comparing D2 with respect to D2 plus para-aortic lymphadenectomy have been recently published and failed to show an overall survival benefit for the super-extended group.³⁰ Despite this, several considerations should be taken into account: i) the overall survival rates (5-year survival: 70%) are at least better by one third than those reported by specialized Western centres in advanced forms; ii) the adopted lymphadenectomy in D2 group included third level nodes (posterior hepatic artery nodes, posterior hepatoduodenal ligament nodes, etc.) in tumour with antral location that lead to a 'D3 lymphadenectomy minus PAN dissection'. The extent of lymphadenectomy is indeed demonstrated by the extremely high number of removed nodes (mean: 53 for D2 and 73 for D2 plus para-aortic); iii) cases in which macroscopic involvement of PANs was evident at surgery were excluded from the study. As stated by the authors themselves, the percentage of involvement was lower than expected (8%) and the potential survival benefit possibly weakened.

Certainly this study demonstrated the exceptional high standards of gastric cancer surgery in Japan giving the motivation to Western surgeons to accomplish such a high level of excellence. On the other hand it could lead us to argue that this study does not fit completely to the Western experience where the standards of surgery are not so high and gastric cancer is still diagnosed in a very advanced stage in a high percentage of cases.

Furthermore, the post hoc subgroup analysis based on pathologic N and T stages showed a better survival rate in the D2 plus para-aortic group for patients without nodal involvement and for patients with tumour invasion limited to the subserosal layer (less than pT3). In this regard, our experience seems to resemble these results. An extremely high chance of cure was observed in our patients with tumors not involving the serosa (pT2) or absence of nodal metastases (pN0), even if survival probability did not reach the results of the Japanese trial. Particularly, long-term

results were remarkable in pT2 N2 and N3 patients. One could speculate that in these particular subsets of patients, where the local control of the disease is essential, D3 lymphadenectomy may be of value in improving long-term results. On the contrary, in serosally exposed neoplasms, which are particularly prone to peritoneal dissemination, the extent of lymphadenectomy might not provide a survival advantage with respect to more limited dissection.¹⁵ However, we emphasize that this is not a randomized trial, and as a consequence we cannot advise this procedure in clinical practice. However, in our opinion the potential benefit of D3 lymphadenectomy cannot be excluded and should be investigated in subgroups of patients.

Conclusions

Our data are indicative of a potential benefit of super-extended lymphadenectomy in patients with advanced gastric cancer without serosal involvement; furthermore, a chance of cure may also be offered to patients with para-aortic lymph node involvement. Further specifically designed prospective studies should confirm our results and clearly assess which patients may benefit from such an extended procedure. In the meantime, it should only be applied in high volume specialized centers in the context of a clinical study.

Conflict of interest

None declared.

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PAPER 10.5

Roviello, F., et al., **Standard and extended lymphadenectomy: technical notes.** Surgery in Multimodal Management of Gastric Cancer 2011, cap. 33, (eds) Springer-Verlag Italia 2011, in press.

Standard and Extended Lymphadenectomy: Technical Notes

33

Franco Roviello, Giovanni Corso and Daniele Marrelli

Abstract

Surgical procedures for the dissection of the various lymph nodes stations differ depending on the location and stage of the gastric tumor. The guidelines of the Japanese Gastric Cancer Association recommend D2 (standard) lymphadenectomy for the treatment of advanced gastric carcinoma. However, an emerging surgical approach to the treatment of gastric tumors is the D3 (extended) lymphadenectomy, indicated for advanced cancers and in patients in good general health who are under 75 years of age. In this chapter, we discuss the D2 standard and D3 extended surgical techniques for the treatment of advanced gastric carcinoma.

Keywords

33.1 Introduction

The Japanese Gastric Cancer Association (JGCA) guidelines define and recommend D2 lymph node (LN) dissection for most gastric cancers. Limited lymphadenectomy, i.e., **D1+?** or **D1+?** LN dissection, is indicated for selected patients with stage T1 cancers. D1+? dissection refers to the removal of level 1 (right paracardial group) stations plus station 7 (left gastric artery), with the addition of station 8a (common hepatic artery), and **D1+?** dissection to the removal of level 1 (right paracardial group) stations plus stations 7 (left gastric artery),

8a (common hepatic artery), and 9 (celiac artery) [1, 2]. Gastrectomy with D1+? is indicated for T1N0 tumors limited to the mucosa (M) and for differentiated submucosal (SM) tumors < 1.5 cm in diameter. Submucosal tumors that do not meet this condition and T1N1 (M, SM) tumors < 2.0 cm should be treated by gastrectomy with D1+? [3].

Following publication of the 13th edition of the Japanese Classification of Gastric Carcinoma (JCGC), in June 1999, the standard for advanced carcinomas has been the D2 dissection [4]. Indeed, gastric cancer who patients underwent R0 surgery with standard D2 lymphadenectomy were found to have better long-term survival [5]. The more recent, extended lymphadenectomy, D3 dissection, is indicated also in advanced gastric cancer, as shown in a selected sub-group of these patients who had longer survival after this procedure [6]. Clearly, adequate knowledge of LN classification

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is essential in gastric cancer in order to correctly perform a D2 standard or D3 extended lymphadenectomy, as defined by the JCGC.

In the late 1980s, Keiichi Maruyama and colleagues at the National Cancer Center Hospital in Tokyo created a computer program, known as the “Maruyama program,” that analyzed a large series of gastric cancer patients treated by extended lymphadenectomy (i.e., D2 or more). The program estimates the rate of metastasis in each of the 16 main LN stations. The Maruyama program has been used to assess Japanese, German, and Italian populations and found to be highly accurate [7-9]. It provides a highly valuable tool for surgeons in pre-operative or intra-operative planning, as a convenient means of rationally determining an adequate lymphadenectomy for the treatment of gastric cancer.

In this chapter, we discuss the D2 standard and D3 extended surgical techniques for the treatment of advanced gastric cancer.

33.2 D2 Standard Lymphadenectomy and Lymph Node Stations

In patients with advanced gastric carcinomas, D2 dissection depends on the tumor's location.

Upper third. D2 classification of these tumors includes the following LN stations: right (station 1), and left (station 2) paracardial, lesser curvature (station 3), short gastric, right and left gastroepiploic vessels (stations 4sa, 4sb, 4d), supra- and infra-pyloric (stations 5, 6), left gastric artery (station 7), common hepatic artery (anterosuperior group station 8a), celiac artery (station 9), proximal (station 11p), and left hepatoduodenal (station 12a). Lymphadenectomy along the splenic hilum (station 10) and distal splenic artery (station 11) should be performed in cases of T3–T4 tumors with serosal infiltration.

Middle and middle/lower third. For gastric tumors located at this site, the D2 LN stations are: right (station 1), and left (station 2) paracardial, lesser curvature (station 3), short gastric, right and left gastroepiploic vessels (stations 4sa, 4sb, 4d), supra- and infra-pyloric (stations 5, 6), left gastric artery (station 7), common hepatic artery (anterosuperior group, station 8a), celiac artery (station 9), proximal splenic artery (station 11p), and hepato-

duodenal ligament (along the hepatic artery, station 12a). Lymphadenectomy along the splenic hilum (station 10) and distal splenic artery (station 11) should be performed in cases of T3–T4 tumor with macroscopic lymphatic metastases.

Lower third. The D2 classification for antral/pyloric gastric tumors comprises the following: right paracardial group (station 1), lesser curvature (station 3), right and left gastroepiploic vessels (stations 4sb, 4d), supra- and infra-pyloric (stations 5, 6), left gastric artery (station 7), common hepatic artery (anterosuperior group, station 8a), celiac artery (station 9), proximal splenic artery (station 11p), hepatoduodenal ligament (along the hepatic artery, station 12a), and superior mesenteric vein (station 14v).

33.3 Lymph Node Stations in D3 Extended Lymphadenectomy

Similar to the D2 classification, LNs of the D3 groups are ranked according to tumor location.

Upper third. Suprapyloric (station 5) and infrapyloric stations (station 6), common hepatic artery (posterior group, station 8p), hepatoduodenal ligament (anterior and posterior hepatic artery, stations 12a, 12p), middle para-aortic (station 16a2,b1), infradiaphragmatic (station 19), and esophageal hiatus (station 20).

Middle and middle/lower third. Left paracardial (station 2), short gastric (station 4sa), common hepatic artery (LN posterior group; station 8p), splenic hilum (station 10), hepatoduodenal ligament (posterior hepatic artery, station 12p), retropancreatic (station 13), superior mesenteric vein (station 14v), and middle para-aortic (station 16a2,b1).

Lower third. Left gastroepiploic vessels (station 4sb), common hepatic artery (posterior group, station 8p), hepatoduodenal ligament (posterior hepatic artery, station 12p), retropancreatic (station 13), and middle para-aortic (station 16a2,b1).

33.4 D2 Lymph Node Dissection: Surgical Procedure

The JGCA guidelines for gastric surgery spell out the correct approach to standard lymphadenectomy [10].

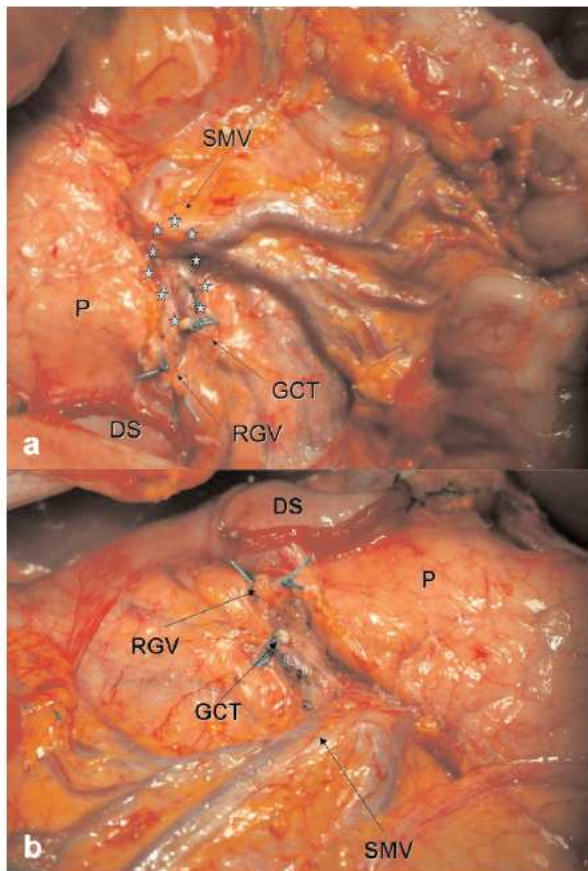


Fig. 33.1 Dissection of station 14. **a** Skeletonization of the gastroduodenal trunk (GCT, Henle's trunk), superior mesenteric vein (SMV), pancreas (P), and duodenum (D). Stars encircle lymph nodes of station 14v. **b** Duodenum section (DS), dissection of the GCT and the right gastroepiploic vein (RGV)

The D2 dissection begins with a bursectomy, with removal of the superior gastro-colic ligament and the pancreatic capsule. Mobilization of the stomach exposes LN stations 4d (right gastroepiploic) and 4sb (left gastroepiploic) through the greater gastric curvature toward the lower pole of the spleen. Particular attention should be paid to the head of the pancreas. The middle colic and superior mesenteric veins, the gastroduodenal trunk, and the right gastroepiploic vein are exposed and station 14v (superior mesenteric vein) is dissected (Fig. 33.1a). Station 6 (infrapyloric) is exposed after elevation of the stomach, allowing the exposure and subsequent ligation of the right gastroepiploic vessels. Duodenal transection aids in the dissection of station 6 (Fig. 33.1b).

The next step is the isolation of the gastroduo-

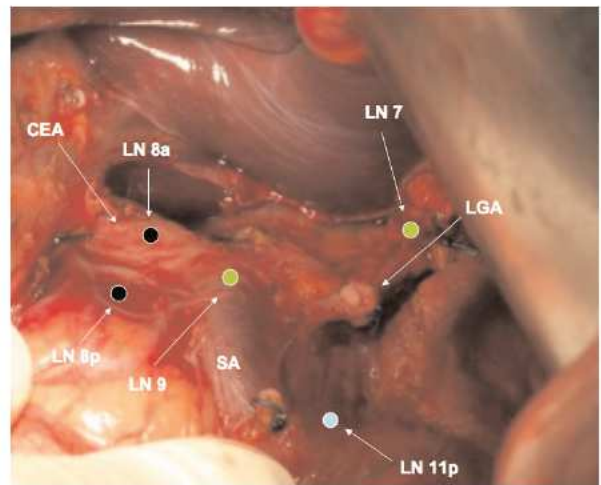


Fig. 33.2 Dissection of stations 7, 8a, 8p, 9, and 11p. Isolation and sectioning of the left gastric artery (LGA) and removal of station 7, with exposure of the celiac trunk (CT) and lymphadenectomy of station 9. This procedure exposes the branches of the CT, i.e., the common hepatic artery (CEA) and the splenic artery (SA), with removal, respectively, of stations 8a (anterior), 8p (posterior), and 11p (splenic proximal)

denal, common hepatic, and proper hepatic arteries, allowing dissection of stations 5 (suprapyloric) and 12a (left hepatoduodenal). The removal of stations 12a and 12p (hepatoduodenal ligament), 13 (retropancreatic), and 14v (superior mesenteric vein) is indicated for tumors located in the lower third of the stomach [11]. The procedure continues with skeletonization of the common hepatic artery and dissection of stations 8a and 8p, LNs around the celiac artery (station 9), and those along the proximal splenic artery (station 11p). This is followed by separation and removal of the LNs along the left gastric artery (station 7), as shown in Fig. 33.2. Station 1 (right paracardial) is dissected along with the upper-third of the lesser curvature. In a total gastrectomy, en-bloc resection of the stomach facilitates dissection of station 2 (left paracardial). This surgical step completes the standard D2 lymphadenectomy.

33.5 D3 Lymph Node Dissection: Surgical Procedure

Extended D3 lymphadenectomy requires complete mobilization of the duodenum, which is accom-

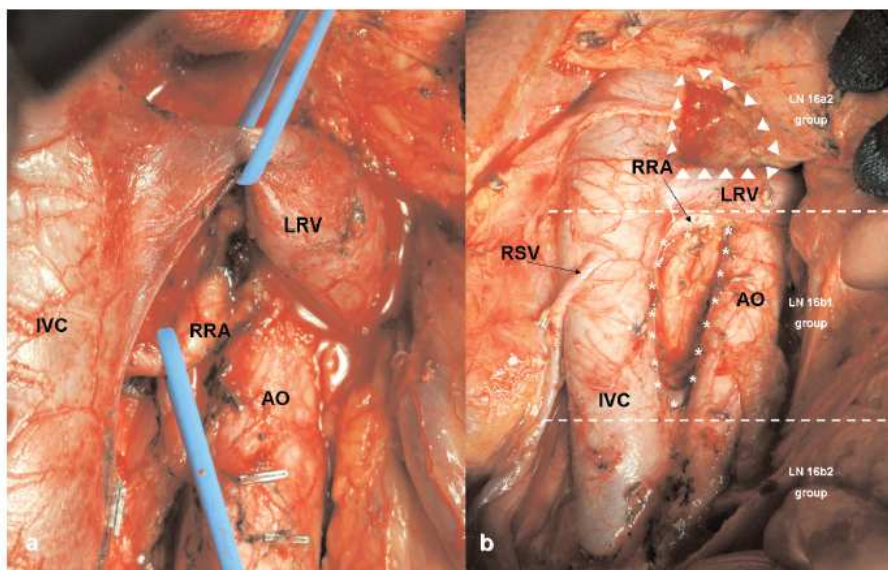


Fig. 33.3 Dissection of station 16. **a** Exposure of the aorta (AO), inferior vena cava (IVC), left renal vein (LRV), and right renal artery (RRA). **b** Arrowheads indicate station 16a2, and stars station 16b1. The arrow points to the right spermatic vein (RSV)

plished with the Kocher maneuver. This provides access to the para-aortic node (PAN) stations. PAN dissection implies the resection of LNs between the level of the celiac axis and the left renal vein (station 16a2) and of nodes between the left renal vein and the inferior mesenteric artery (station 16b1). The left upper lateral nodes (station 16a2-lat) are not generally dissected, except in cases of upper-third tumors or macroscopic LN involvement (Fig. 33.3a, b) [6]. It is important to note that the above-mentioned Kocher maneuver broadly exposes LN stations 8p and 12p; this procedure also facilitates a complete D2 lymphadenectomy.

D3 lymphadenectomy is usually indicated for advanced tumors (cT2–T4) in patients in good general health and under 75 years of age [11].

33.6 Conclusions

D2 lymphadenectomy represents the standard surgical procedure for advanced gastric carcinoma. The technique has proven to be safe when conducted in specialized centers and is associated with a low risk of post-operative complications and mortality. D3 lymphadenectomy requires removal of the PAN, stations. This procedure is an emerging technical approach especially indicated for patients

with upper-third gastric cancers. It has an acceptable morbidity rate and no increase in mortality compared to earlier approaches.

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PAPER 10.6

MacConaill, L.E., ET AL., **Profiling critical cancer gene mutations in clinical tumor samples.** PLoS One 2009;4:e7887.

Profiling Critical Cancer Gene Mutations in Clinical Tumor Samples

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Abstract

Background: Detection of critical cancer gene mutations in clinical tumor specimens may predict patient outcomes and inform treatment options; however, high-throughput mutation profiling remains underdeveloped as a diagnostic approach. We report the implementation of a genotyping and validation algorithm that enables robust tumor mutation profiling in the clinical setting.

Methodology: We developed and implemented an optimized mutation profiling platform ("OncoMap") to interrogate ~400 mutations in 33 known oncogenes and tumor suppressors, many of which are known to predict response or resistance to targeted therapies. The performance of OncoMap was analyzed using DNA derived from both frozen and FFPE clinical material in a diverse set of cancer types. A subsequent in-depth analysis was conducted on histologically and clinically annotated pediatric gliomas. The sensitivity and specificity of OncoMap were 93.8% and 100% in fresh frozen tissue; and 89.3% and 99.4% in FFPE-derived DNA. We detected known mutations at the expected frequencies in common cancers, as well as novel mutations in adult and pediatric cancers that are likely to predict heightened response or resistance to existing or developmental cancer therapies. OncoMap profiles also support a new molecular stratification of pediatric low-grade gliomas based on *BRAF* mutations that may have immediate clinical impact.

Conclusions: Our results demonstrate the clinical feasibility of high-throughput mutation profiling to query a large panel of "actionable" cancer gene mutations. In the future, this type of approach may be incorporated into both cancer epidemiologic studies and clinical decision making to specify the use of many targeted anticancer agents.

Citation: MacConaill LE, Campbell CD, Kehoe SM, Bass AJ, Hatton C, et al. (2009) Profiling Critical Cancer Gene Mutations in Clinical Tumor Samples. PLoS ONE 4(11): e7887. doi:10.1371/journal.pone.0007887

Editor: Chris Jones, Institute of Cancer Research, United Kingdom

Received: August 25, 2009; **Accepted:** October 20, 2009; **Published:** November 18, 2009

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Funding: This work was supported by the National Cancer Institute (R21CA126674) to L.A.G., (K08CA134931) to A.J.B., and the Dana-Farber Cancer Institute. A.J.B. was supported by the Harvard Clinical Investigator Training Program. Funding for analysis of gastrointestinal tumors was supplied the American Society of Clinical Oncology, Friends of the Dana-Farber Cancer Institute and the H.T. Berry Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: In terms of financial disclosures, Levi Garraway is a consultant for and/or receives sponsored research from Novartis, Inc. None of these relationships constitute a conflict of interest for this work. This does not alter his adherence to all PLoS ONE policies on data sharing and materials.

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Introduction

Many tumors contain hallmark mutations within oncogenes or tumor suppressor (TS) genes that may confer a heightened susceptibility to targeted anticancer therapies. Well-established examples include *KIT* mutations in gastrointestinal stromal tumors

(GISTs) that predict response to imatinib or nilotinib, and non-small cell lung cancers with *EGFR* mutations that are sensitive to erlotinib [1,2,3]. The presence of other mutations predicts a lack of response to targeted therapy. For example, lung and colorectal cancers that harbor mutations in the *KRAS* oncogene are unresponsive to treatment with anti-*EGFR* agents [4], and inactivating

PTEN mutations (or protein loss) in glioblastomas predict resistance to erlotinib[5]. Thus, clinical decision-making based on tumor genetic information will increasingly be informed by the mutational status of multiple cancer genes. However, generating a comprehensive profile of targetable or otherwise “actionable” tumor DNA mutations in the clinical arena remains challenging.

Recent technological advances make it feasible in principle to screen a tumor biopsy for many types of genomic changes. However, incorporation of such information into clinical decision-making requires reliable genomic profiling of frozen, paraffin-derived, and archival tumor DNA. In this context, nucleic acids are often subject to degradation and/or chemical modification, and the availability of tumor tissue may be limiting.

We previously reported the adaptation of a high-throughput genotyping approach to interrogate key mutations in a panel of 17 known oncogenes. We now demonstrate the clinical feasibility of mass-spectrometric based cancer gene mutation profiling for a large panel of oncogene and TS gene mutations. To accomplish this, we generated an approach termed OncoMap, which employs an expanded collection of 460 assays interrogating known mutations in 33 cancer genes. Using this genomic profiling approach coupled to an analytical mutation-calling algorithm and orthogonal validation step, we identified numerous mutations present in genomic DNA from both frozen and FFPE tumor tissue. Moreover, the application of systematic mutation profiling to >120 pediatric low-grade astrocytomas reveals a clinically informative molecular stratification not previously recognized in this tumor type. Such information, if it became widely available, could inform both clinical decision making and optimal clinical trial design for targeted therapeutics.

Methods

Patients and Tumor Tissue Collection

Anonymized tumor specimens were obtained from the Cooperative Human Tissue Network (CHTN), Surgical Oncology University of Siena, Italy, and Dana-Farber Cancer Institute; human glioma samples were obtained from the clinical archives of the departments of Pathology at Children’s Hospital Boston and Brigham and Women’s Hospital. (The required tumor content was >70%; necrosis <10%.) Institutional review board (IRB) exemption was obtained for all samples from the Dana-Farber/Partners

Cancer Care Office for the Protection of Research Subjects. DNA extraction was performed as described in Methods S1.

OncoMap Assay Design and Genotyping

Selection of cancer gene mutations for assay design and mass spectrometric genotyping were performed as previously described[6] with modifications indicated in Methods S1. Assay, primer and probe sequences are indicated in **Table S1**.

Sample Barcoding and Sequencing

Primers flanking *KRAS* codon 12 were used to PCR-amplify genomic DNA from 91 fresh frozen and 93 FFPE samples (primer sequences indicated in **Table S2**). PCR amplicons were analyzed by Sanger sequencing. Alternatively, *KRAS* codon 12 was PCR-amplified with barcoded primers as described in Methods S1. PCR amplicons were pooled and analyzed (Illumina Genome Analyzer II; single lane). Sequence data was analyzed as described in Methods S1.

Results

Characteristics of Clinical Tumor Samples

A total of 903 clinical tumor specimens derived from 12 different tissue sites were assessed for this study (**Table 1**). The majority of samples were adenocarcinomas ($n = 625$) from tumors of the breast, lung, prostate, and GI tract. A collection of GISTs ($n = 34$) and gliomas ($n = 155$) were also included. Of these, 643 specimens were obtained from fresh frozen tissue and 260 derived from FFPE blocks. Estimated tumor content exceeded 70% in all samples as measured by pathological review (see Methods).

Performance of the Tumor Mutation Profiling Algorithm

To facilitate cancer gene mutation profiling in clinical tumor specimens, we developed OncoMap, a panel of genotyping assays that assessed 396 unique mutations in 33 cancer genes. The complete mutation profiling algorithm (**Figure 1A**) involved mass spectrometric genotyping followed by both automated calling and manual review to generate a list of candidate mutations. These candidates were subjected to secondary genotyping validation (see Methods S1). Assuming that all primary profiling and assay validation reagents are in place, 7–10 days are required to complete the entire OncoMap sequence.

Table 1. Human tumor samples (fresh frozen and FFPE) investigated in this study.

	Total Number of Samples Tested	Number of fresh frozen vs (FFPE) samples	Total Number of Samples with Mutation	Percentage of samples with mutation
Brain	155	0 (155)	44	28.40%
Breast	53	20 (33)	21	39.60%
Colon	159	113 (46)	102	64.20%
Endometrium	23	23 (0)	18	78.30%
Esophagus	117	117 (0)	29	24.80%
Gastric	233	233 (0)	90	38.20%
Kidney	26	26 (0)	1	3.80%
Lung	26	0 (26)	15	57.70%
Ovary	9	9 (0)	1	11.10%
Prostate	95	95 (0)	10	10.50%
Thyroid	7	7 (0)	4	57.10%
Total Numbers:	903	643 (260)	335	37.10%

doi:10.1371/journal.pone.0007887.t001

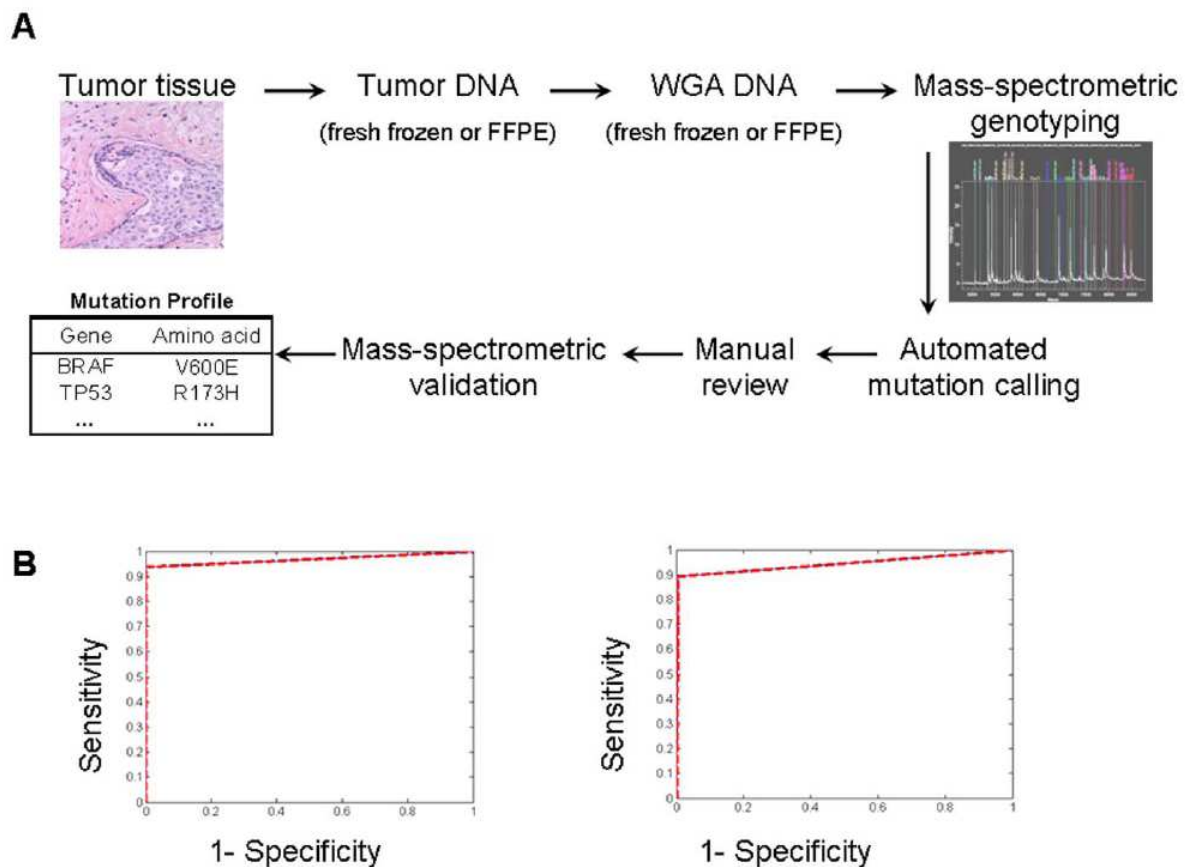


Figure 1. The OncoMap process and performance in fresh frozen and FFPE-derived DNA. A. An overview of the OncoMap process from tumor to mutation profile. See text for details. B. Receiver operator characteristic curves (ROCs) show the sensitivity and specificity for various cutoff values on the sample score of the validation samples. ROCs are plotted for fresh frozen (left) and FFPE-derived (right) DNAs, using bidirectional *KRAS* assays and Illumina data as a truth-set (see Methods S1). doi:10.1371/journal.pone.0007887.g001

OncoMap performance was assessed after determining the “ground-truth” mutational status at *KRAS* codon 12 using a DNA barcoding and massively parallel sequencing-by-synthesis strategy (see Methods S1) applied to 91 frozen and 93 FFPE-derived tumor DNAs. When these deep sequencing results were compared to genotyping assays interrogating the same *KRAS* codon, we found the OncoMap sensitivity and specificity to be 93.8% and 100%, respectively, in DNA from fresh/frozen tissue; and 89.3% and 99.4% in FFPE-derived DNA (Figure 1B). In contrast, the sensitivity of conventional Sanger sequencing was 83.3% for fresh frozen tissue and 76.0% for FFPE-derived DNA, confirming the heightened performance of genotyping-based mutation profiling [7].

OncoMap performance was evaluated more extensively by testing 215 fresh frozen tumor samples spanning multiple tumor types in which the mutational status for nucleotides interrogated by 52 OncoMap assays had been established previously. By this analysis, mutation profiling achieved a similarly high specificity of 99.8%. Assay sensitivity was optimal in tumor tissues where the tumor content exceeded 50% (data not shown). Although individual sensitivity values could not be determined for all mutations assayed, calculations using unidirectional *KRAS* assays (reflective of the majority of OncoMap assays) yielded nearly identical sensitivity and specificity values (Figure S1). These

results suggested that the genotyping-based mutation profiling platform was suitable for many clinical applications.

“Actionable” Cancer Gene Mutations across Diverse Cancer Types

Of the 903 clinical tumor specimens profiled, 37% ($n = 335$) contained one or more mutations. In total, 417 mutations were identified, with 63 samples exhibiting co-occurring mutations. Whereas 286 mutations were found by assays interrogating known or candidate oncogenes, 131 mutations were observed in TS genes. Thus, the OncoMap platform provided more extensive mutation information than earlier mutation profiling efforts which focused exclusively on oncogene mutations. As expected, the distribution of mutations reflected patterns previously observed in human tumors, although the frequency of TS mutations was lower (reflective of the reduced coverage of such mutations by OncoMap). Examples include *PIK3CA* (26%) and *TP53* (13%) mutations in breast cancer; *APC* (11%), *BRAF* (10%), *KRAS* (38%), *PIK3CA* (11%) and *TP53* (9%) mutations in colorectal cancer, and *EGFR* (12%), *KRAS* (23%) and *STK11* (8%) mutations in lung cancer. The expected mutation distributions were observed in both fresh frozen and FFPE specimens, underscoring the potential utility of OncoMap in the clinical setting.

Mutations Predicting Response to Targeted Therapies

Table 2 indicates a set of “actionable” cancer gene mutations identified herein. The OncoMap platform robustly detected mutations that constitute established markers of response to targeted therapies. For example, *EGFR* mutations predictive of response to erlotinib and gefitinib were identified at the expected frequency (12%) in non-small cell lung cancer, and *KIT* mutations linked to sensitivity to imatinib and nilotinib were detected in 76% of GISTs. Interestingly, *ERBB2* mutations were detected in four gastric adenocarcinomas, raising the possibility of testing a HER-2 inhibitor such as trastuzumab in gastric cancer patients selected based on this genetic criterion [8].

Several tumors harbored mutations that may predict response to investigational agents. For example, *BRAF*^{V600E} mutations were identified in colorectal (n = 16), ovarian (n = 1), thyroid (n = 4) and endometrial (n = 1) cancers, as well as pediatric gangliogliomas (n = 22; see below). Tumors harboring these mutations may respond to a selective *BRAF* inhibitor [6,9]. We also identified 96 samples across seven different cancer types (breast n = 14, colorectal n = 24, endometrial n = 15, esophageal n = 4, gastric n = 34, prostate n = 3, and pediatric astrocytoma n = 2) harboring mutations in either *PIK3CA* or *PTEN*. These mutations might be expected to enrich for tumors responsive to the PI3 kinase inhibitors currently in development.

Mutations Predicting Resistance to Targeted Therapies

Along with mutations that confer heightened sensitivity to targeted therapies, OncoMap robustly detected mutations associated with resistance to several agents. Established examples include *KRAS* mutations in non-small cell lung cancer (23%) and colorectal cancer (38%) that confer resistance to erlotinib, gefitinib (lung) or cetuximab (colorectal) [4,10,11]. While 94% of *KRAS* mutations identified localized to codons 12 or 13, 6% occurred elsewhere in the gene (most commonly at codon 61). Since most studies of *KRAS*-associated resistance have focused exclusively on codons 12 and 13 [3,12,13], OncoMap identified additional *KRAS* mutations that may influence sensitivity to anti-EGFR treatment. OncoMap also identified an *HRAS* mutation in a lung adenocarcinoma and an *NRAS* mutation in a colorectal adenocarcinoma. Mutations involving alternate RAS isoforms are rare in these cancer types; conceivably, these might also confer resistance to anti-EGFR therapy.

Mutation profiling also identified mutations that confer “secondary” resistance to targeted therapies (e.g., resistance alleles arising during the course of targeted therapy). In GIST tumors, where 31 *KIT* mutations were identified in 25 samples; both primary (imatinib-responsive) and secondary (imatinib-resistant) *KIT* mutations were observed, in keeping with prior mutation profiling studies [7]. In particular, several *KIT* mutations involving exon 9 (*KIT* Y503 or F504insAY; 5 cases) were detected in untreated GIST tumors. These mutations are associated with an increased drug requirement to elicit a clinical response [14,15]. A *PDGFRA* mutation (D842V) predictive of resistance to imatinib [15] was also identified in one GIST sample.

The *AKT1*^{E17K} mutation has previously been reported in breast, colorectal and ovarian cancer. The OncoMap platform identified rare *AKT1* mutations in these tumor types, as well as single *AKT1*^{E17K} instances in endometrial, esophageal squamous, gastric, and prostate cancers. This mutation may predict resistance to PI3 kinase inhibition (and conceivably receptor tyrosine kinase inhibition) in some contexts [16].

Cancers with Co-Occurring “Actionable” Mutations

The presence of co-occurring mutations involving critical cancer genes may modify the clinical response to single-agent

targeted therapy. Here, 20 adenocarcinomas (10 colorectal, two endometrial and 8 gastric) exhibited co-occurring *PIK3CA* and *KRAS* mutations. While coincident mutations in these genes have previously been reported in cancers of the large intestine [17], *PIK3CA* and *KRAS* mutations have typically exhibited a mutually exclusive pattern of occurrence in endometrial cancer [18,19]. An endometrial adenocarcinoma with co-occurring *FGFR2* and *PTEN* mutations was also identified. Two tumors harbored coincident *BRAF* and *PTEN* mutations (one endometrial, one colorectal), and an additional colorectal sample contained both a *PIK3CA* and a *BRAF* mutation. These tumors might be expected to exhibit resistance to receptor tyrosine kinase inhibition.

Molecular Classification of Pediatric Brain Tumors by Cancer Gene Mutation Profiling

The ability to perform robust mutation profiling of clinical and archival tumor tissue may promote systematic molecular characterization of “orphan” cancers, including some pediatric tumors where sufficient tissue is often lacking. To explore this hypothesis, OncoMap was used to query a series of pediatric low-grade gliomas (LGGs) whose mutation spectrum is incompletely defined. This analysis included genomic DNA from 127 pediatric and 28 adult gliomas (for comparison) spanning five histologic subtypes of pilocytic astrocytoma, ganglioglioma, and diffuse astrocytoma.

Candidate Prognostic or Therapeutic Targets in Pediatric Brain Tumors

Several potentially “actionable” cancer genes were found mutated in pediatric LGAs. Examples include *PDGFRA* (n = 1) and *PIK3CA* (n = 2), which may predict response to existing drugs (e.g., imatinib or nilotinib) or agents in development (e.g., PI3 kinase inhibitors). Two pediatric LGAs harbored mutations in *MYC*, a well established oncogene homologue of *NMYC*, which is amplified and indicative of poor prognosis in pediatric neuroblastoma [20,21]. Thus, tumor mutation profiling may refine patient stratification and/or disease prognosis in some pediatric brain cancers.

BRAF^{V600E} Mutations Are Common in Pediatric Gangliogliomas

Duplication of the *BRAF* locus has been reported as the most frequent aberration in pediatric LGAs (66% [18]), whereas activating point mutations in *BRAF* occur less commonly (4–6%) [22], [23]. We identified activating *BRAF*^{V600E} mutations in 11% (10/88) of pediatric LGAs—a higher percentage than previously reported [22,23]. Interestingly, the *BRAF*^{V600E} mutation was most prevalent within the ganglioglioma subtype of pediatric LGAs (classical and non-classical; 8/14 tumors, p = 0.00005), as shown in **Fig. 2**. *BRAF*^{V600E} mutations were not identified in any of the adult tumors examined, although *TP53* mutations commonly occurred in this setting (10/28 cases). Conversely, only 2 pediatric gliomas harbored a *TP53* mutation; in both cases, this was coincident with another mutation (*EGFR* and *FLNB*, respectively). These results suggest that OncoMap might facilitate classification of low-grade astrocytomas and other rare tumor types based on genetic criteria.

Discussion

Clinical oncology is in the midst of transition from a treatment paradigm dictated primarily by the anatomic site of tumor origin to one in which genetic and/or molecular characteristics play a decisive role in guiding choice of therapy. Moreover, the

Table 2. Druggable or actionable mutations identified in this study.

Tissue Type	Gene	Amino acid	N	%
Brain (astrocytoma, ganglioglioma, glioblastoma)	BRAF	V600E	22	14.2
	EGFR	T263P	1	0.6
	PDGFRA	D842V	1	0.6
	PIK3CA	R88Q	1	0.6
		H1047R	1	0.6
Breast (lobular and ductal carcinoma)	AKT1	E17K	1	1.9
	PIK3CA	R88Q	1	1.9
		N345K	1	1.9
		E542K	1	1.9
		E545K	2	3.8
		H1047R	9	17.0
Colon (adenocarcinoma)	AKT1	E17K	1	0.6
	BRAF	D594G	2	1.3
		L597Q	1	0.6
		V600E	13	8.2
	KRAS	A146T	1	0.6
		G12A/C/D/S/V	45	28.3
		G13C/D	13	8.2
		Q61H/L	2	1.3
	NRAS	Q61K	1	0.6
	PIK3CA	R88Q	1	0.6
		E542K	2	1.3
		E545G/K	13	8.2
		H1047R	2	1.3
	PTEN	R130Q/*	2	1.3
		R233*	2	1.3
		K267fs*9	2	1.3
Endometrium (adenocarcinoma)	AKT1	E17K	1	4.3
	BRAF	G466A	1	4.3
	FGFR2	S252W	1	4.3
		C382R	1	4.3
	KRAS	G12A/V	3	13.0
		G13D	1	4.3
	PIK3CA	R88Q	1	4.3
		C420R	1	4.3
		H701P	1	4.3
		H1047R	4	17.4
	PTEN	R130G/Q/*	6	26.1
		R173H	1	4.3
		N323fs*2	1	4.3
Esophagus (adenocarcinoma and squamous carcinoma)	AKT1	E17K	1	0.9
	PIK3CA	E542K	2	1.7
		E545K	1	0.9
Gastric (adenocarcinoma)		H1047R	1	0.9
	AKT1	E17K	1	0.5
	EGFR	L858M	1	0.5
	ERBB2	L755S	3	1.5

Table 2. Cont.

Tissue Type	Gene	Amino acid	N	%
		V777L	1	0.5
	KRAS	G12C/D	11	5.5
		G13C/D	5	2.5
	PIK3CA	R88Q	1	0.5
		C420R	2	1.0
		E542K	8	4.0
		E545K	8	4.0
		H1047R	10	5.0
	PTEN	K267fs*9	3	1.5
		N323fs*2	1	0.5
		N323fs*21	1	0.5
GI tract (GIST)	KIT	Y503_F504insAY	7	21.2
		W557G	2	6.1
		W557_K558del	2	6.1
		W557_V559>C/F	4	12.1
		V559D	1	3.0
		V560D	2	6.1
		V560del	2	6.1
		L576P	1	3.0
		K642E	2	6.1
		V654A	3	9.1
		D816H	1	3.0
		N822K	2	6.1
		Y823D	1	3.0
	PDGFRA	D842V	1	3.0
Kidney (renal cell carcinoma)	VHL	L89H	1	3.8
Lung (adenocarcinoma)	EGFR	L858R	3	11.5
	HRAS	Q61L	1	3.8
	KRAS	G12C/D	4	15.4
		Q61H	2	7.7
Ovary (adenocarcinoma)	BRAF	V600E	1	11.1
Prostate (adenocarcinoma)	AKT1	E17K	1	1.1
	FGFR3	F384L	1	1.1
	PIK3CA	H1047R	1	1.1
		G1049R	1	1.1
	PTEN	R173H	1	1.1
Thyroid (papillary carcinoma)	BRAF	V600E	4	57.1

doi:10.1371/journal.pone.0007887.t002

proliferation of targeted agents in development and clinical practice necessitates concomitant implementation of companion diagnostic approaches that enrich for subpopulations most likely to respond to a drug. New diagnostic approaches are therefore needed to profile any tumor for pivotal genetic mutations in multiple cancer genes simultaneously, in contrast to most existing tests that focus on single genes (or proteins).

In this study, we adapted genotyping-based mutation profiling for the characterization of both frozen and FFPE-derived tumor specimens spanning 12 cancer types. We robustly detected cancer gene mutations that direct clinical use and predict resistance to existing agents such as tyrosine kinase inhibitors (e.g., *EGFR* and *KRAS* mutations). We also identified multiple mutations that may

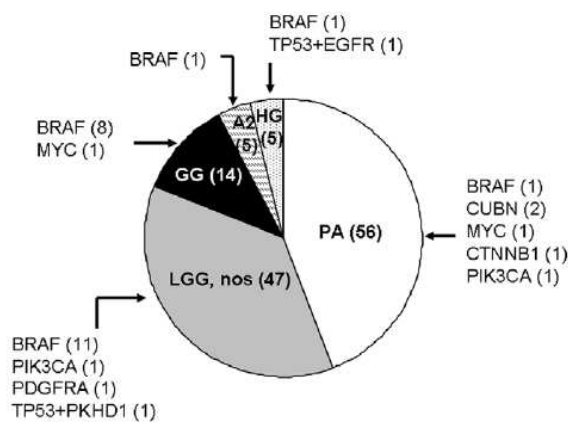


Figure 2. *BRAF*^{V600E} mutations detected in archival samples of pediatric gliomas. Abbreviations: PA - pilocytic astrocytoma, WHO grade I; LGG, nos - low-grade glioma, not otherwise specified, WHO grade I or II; GG - ganglioglioma; A2 - astrocytoma, WHO grade II; HG - high-grade glioma, WHO grade III or IV. Parentheses indicate total number of samples (in chart) or number of samples with indicated mutation (outside chart).

doi:10.1371/journal.pone.0007887.g002

guide the use of emerging agents. Finally, in a specific investigation of pediatric low-grade astrocytomas, we demonstrated the special value this platform may have for rare "orphan" cancers by identifying mutations that may inform molecular classification as well as new therapeutic avenues for children with these malignancies.

Diagnostic interventions that successfully introduce tumor mutation profiling to clinical practice must circumvent several technical and logistical hurdles. Chief among these is the attainment of robust performance in samples derived from FFPE and/or archival tumor material. We found that the OncoMap platform achieved nearly 100% specificity in both fresh/frozen and FFPE-derived tumor DNA, indicating that false positive mutation calls are likely to be relatively rare with this approach. It should be noted, however, that achieving this level of specificity required the implementation of an analytical sequence in which raw genotyping data is subjected to automated base calling followed by manual review of candidate mutations and validation of all candidates using alternative genotyping chemistries. Thus, clinical implementation of OncoMap must incorporate both genomic data generation and bioinformatic analytical expertise into a molecular pathology or clinical diagnostic setting.

The sensitivity of OncoMap is influenced by inherent technological parameters, individual mutation assay performance characteristics, and the quality and purity of tumor tissue. The 89–94% assay sensitivity observed in this study is sufficient for many translational and clinical applications; however, there are of course circumstances where even higher assay sensitivities will be desirable. Enrichment of tumor cells using core needle dissection or laser-capture microdissection prior to mutation profiling may offer one avenue to enhance sensitivity, particularly in tumors where the stromal or inflammatory content is high. At the same time, the sensitivity of OncoMap vastly exceeds that of Sanger sequencing, which remains the gold standard for many genetic diagnostic approaches. Furthermore, the breadth of cancer genes and specific mutations interrogated by OncoMap—supported by the aforementioned rigorous sensitivity and specificity determinations—substantially exceeds that of existing commercial mass spectrometric-based genomic profiling approaches[24].

Genomically guided therapies may play an especially prominent role in rare tumors where large randomized trials are often impractical. To test the ability of the OncoMap to identify uncommon and/or sample-limited tumors that may benefit from specific classes of therapeutic agents, we profiled a panel of pediatric low-grade gliomas for common cancer gene mutations. Knowledge of the genetic abnormalities present in pediatric LGAs is limited, though recent studies have identified *BRAF* translocations, chromosomal duplications, and occasional base mutations in low-grade astrocytomas[18,25], as well as diverse mechanisms for activating the ERK/MAPK pathway in pilocytic astrocytomas [23]. These findings suggest that the small molecule inhibitors of *BRAF* already in adult trials may also represent promising therapeutics for subsets of these tumors. Our results indicate that the frequency of *BRAF* point mutations in pediatric LGAs as a whole may be higher than previously reported, and specifically that gangliogliomas possess *BRAF*^{V600E} mutations at very high frequency. This observation may also aid in diagnostic identification of these tumors. Gangliogliomas, which tend to be indolent tumors, may therefore share some properties with cutaneous nevi, whose melanocyte precursors also derive from the nervous system (neural crest), exhibit indolent growth, and where the *BRAF*^{V600E} mutation is also highly prevalent. We also identified several mutations not previously reported in pediatric astrocytoma, some of which (*EGFR*, *PIK3CA*, *PDGFRA*) represent potentially actionable targets. In concordance with previous reports [22,26,27] we observe that mutations in genes frequently observed in adult anaplastic astrocytomas and/or glioblastomas, such as *TP53* or *PTEN*, are only rarely encountered in pediatric pilocytic and low-grade diffuse astrocytomas.

Although our findings support the clinical feasibility of high-throughput tumor mutation profiling, we recognize that the mass spectrometric genotyping approach has certain limitations that may preclude its implementation as a definitive cancer diagnostics platform. These include the finite number of specific point mutations that can be assayed (designated *a priori* within a subset of cancer genes), difficulties in designing genotyping assays that identify small insertions or deletions ("in-dels") larger than ~50bp in size, an inability to detect most TS gene mutations (which may occur anywhere within the gene, not just "hotspot" regions) or additional genomic alterations such as high-level gene amplifications or deletions that may also affect key cancer genes, and the somewhat labor-intensive nature of manual review and orthogonal assay validation. Over the long term, the adaptation of new genomics technologies such as second generation sequencing may offer a unifying approach to comprehensive tumor mutation profiling. However, the OncoMap platform may offer one immediate avenue by which systematic mutation profiling might be initiated to guide clinical trial design as well as use of existing targeted agents across many cancer types.

In summary, this study represents the first large-scale application of the OncoMap platform for tumor mutation profiling in the clinical and archival setting. These results therefore enliven a framework wherein systematic tumor profiling might emerge as a widely feasible means to guide patient stratification for rational cancer therapeutics. Despite the inherent complexity of cancer, incorporating the growing knowledge of the molecular basis of cancer into both large-scale molecular epidemiologic studies and, ultimately, clinical decision making should ultimately speed the advent of more effective anticancer therapies.

Supporting Information

Methods S1 Profiling critical cancer gene mutations in clinical tumor samples.

Found at: doi:10.1371/journal.pone.0007887.s001 (0.07 MB DOC)

Table S1 OncoMap 3 core PCR primer and extension probe sequences.

Found at: doi:10.1371/journal.pone.0007887.s002 (0.13 MB XLS)

Table S2 *KRAS* G12 PCR primer sequences used to generate amplicons for Sanger and Illumina sequencing.

Found at: doi:10.1371/journal.pone.0007887.s003 (0.03 MB XLS)

Figure S1 Performance of OncoMap in fresh frozen and FFPE-derived DNA. Receiver operator characteristic curves (ROCs) are plotted for fresh frozen (left panel) and FFPE-derived (right panel) DNAs, against unidirectional OncoMap *KRAS* assays, using Illumina data as a truth-set (see Methods S1).

Found at: doi:10.1371/journal.pone.0007887.s004 (0.06 MB PPT)

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Acknowledgments

We thank Nicholas Wyhs, Benjamin Ebert and Sewit Tekki for technical expertise; Suelly Marie for providing carcinoma samples. Sanger sequencing was completed at Agencourt Bioscience Corporation, Illumina sequencing at Cofactor Genomics.

Author Contributions

Conceived and designed the experiments: LM CC AB CE AB RS WCH MLM LAG. Performed the experiments: CC SK MD KY MH CM LL MF JC SS. Analyzed the data: LM CC SK AB CH LN MD KY MH CM LL JP DG MAR KP JC YW JF GC FR RS MWK KLL CDS LAG. Contributed reagents/materials/analysis tools: AB CH LN MD KY MH CM LL CE AB JP DG MF MAR KP JC YW JF SS GC FR RS MWK KLL CDS MLM LAG. Wrote the paper: LM CC SK AB CH LN MD DG JF KLL CDS WCH MLM LAG.

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- GIOVANNI CORSO -

Programa Doutoral em Medicina e Oncologia Molecular

Nos termos do disposto do n.º 2 do art.º 17.º do Regulamento dos Terceiros Ciclos de Estudos da Universidade do Porto, a seguir se publicita o júri de doutoramento em Medicina e Oncologia Molecular, do licenciado Giovanni Corso, nomeado por despacho vice-reitoral de 20 de Setembro de 2011, com a tese "Searching for novel diagnostic-prognostic biomarkers in gastric cancer":

Presidente: Reitor da Universidade do Porto.

Vogais: Doutor Manuel Alberto Coimbra Sobrinho Simões, professor catedrático da Faculdade de Medicina da Universidade do Porto;

Doutora Maria de Fátima Machado Henriques Carneiro, professora catedrática da Faculdade de Medicina da Universidade do Porto;

Doutor António Amável Caldeira Fradique, professor associado convidado da Faculdade de Ciências Médicas da Universidade Nova de Lisboa;

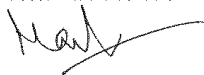
Doutor Lúcio José de Lara Santos, professor auxiliar convidado do Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto;

Doutor Franco Roviello, professor of Department of Human Pathology and Oncology, Unit of Surgical Oncology of University of Siena, Italy, co-orientador da tese;

Doutora Carla Isabel Gonçalves de Oliveira, investigadora principal do Instituto de Patologia e Imunologia Molecular da Universidade do Porto.

Divisão Académica, 31 de Outubro de 2011

O Director da Faculdade



(Doutor J. Agostinho Marques)