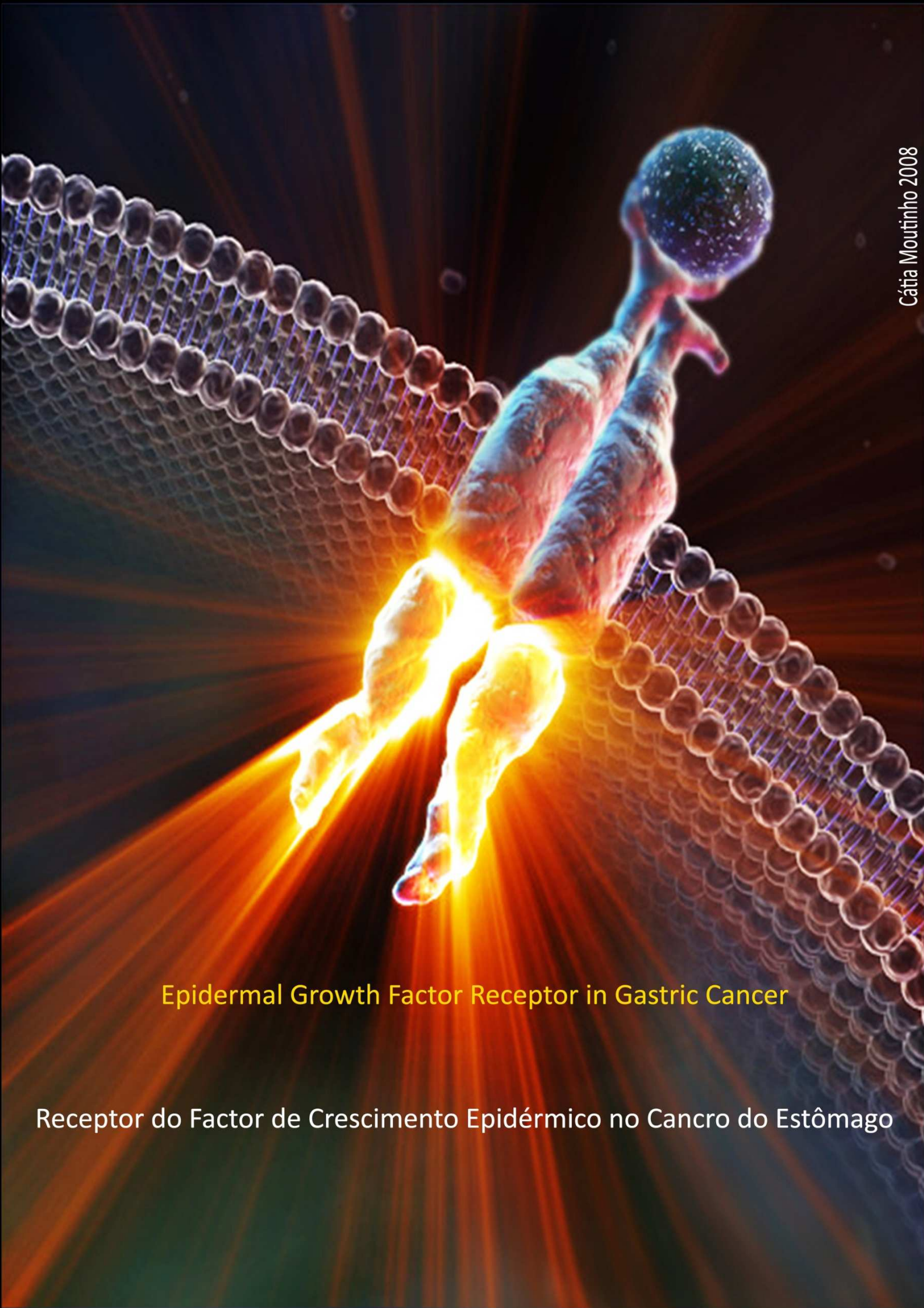

Epidermal Growth Factor Receptor in Gastric Cancer

Receptor do Factor de Crescimento Epidérmico no Cancro do Estômago

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CÁTIA ALEXANDRA MARTINS FREITAS MOUTINHO

Porto 2008

A ilustração da capa foi retirada e adaptada de *http://hybridmedicalanimation.com*

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- A- Adenine
- A- Alanine
- **A**- Aspargine
- ADAM - A disintegrin and metalloproteinase domain
- Ala- Alanine
- AR- Amphyregulin
- ATP- Adenosine triphosphate
- ATRS- AT-Rich consensus Sequence
- BCL2- B-cell CLL
- BTC- Betacellulin
- Ca- Calcium
- C-Cytosine
- CD44- CD44 molecule (Indian blood group)
- CDH1- Cadherin 1, type 1, E-cadherin
- CDK- Cyclin Dependent Kinases
- CDKN1B- Cyclin-Dependent Kinase inhibitor 1B
- CT- Carboxil Terminal
- D- Aspartic acid
- DAPI- 4', 6-diamidino-2-phenylindole
- DCC- Deleted in Colorectal Carcinoma
- DDX53 -DEAD (Asp-Glu-Ala-Asp) box polypeptide 53
- DNA- Desoxyrribonucleic Acid
- E- cadherin- Epitelial cadherin
- E2F-E2F transcription factor
- EGF- Epidermal Growth Factor
- EGFR, ERBB1, HER1- Epidermal Growth Factor Receptor
- EPG- Epigen
- EPR- Epiregulin
- EPS8- Epidermal growth factor receptor pathway substrate 8
- ERB4, HER4- Erythroblastic leukemia viral oncogene homolog 4
- ERBB- Avian Erytroblastosis Virus B
- ERBB2, HER2- Erythroblastic leukemia viral oncogene homolog 2

- ERBB3, HER3- Erythroblastic leukemia viral oncogene homolog 3
- F- Female
- FAP- Familial adenomatous polyposis
- FGFR2- Fibroblast growth factor receptor 2
- FHIT- Fragile Histidine Triad gene
- FISH- Fluorescence *in situ* Hybridization
- G- Guanine
- GAP- GTPase activating protein
- GC- Gastric cancer
- GDP- Guanosine-5'-diphosphate
- GH- Growth Hormones
- Gln- Glutamine
- GPCRs- G-Protein- Coupled Receptors
- GRB2- Growth factor receptor- bound protein 2
- GTP- Guanosine-5'-triphosphate
- H&E- Haematoxylin and Eosin
- H. Pylori- Helicobacter pylori
- HB-EGF-Heparin- Binding Epidermal Growth Factor
- HCl- Hydrocloric acid
- HDGC- Hereditary Diffuse Gastric Cancer
- HGF/SF -Hepatocyte Growth Factor/Scatter Factor
- His- Histidine
- HNPCC- Hereditary Nonpolyposis Colon Cancer
- IARC- International Agency for Research on Cancer
- JaK2-Janus tyrosine kinase 2
- KRAS- Kirsten rat sarcoma viral oncogene homolog
- LOH- Loss of Heterozygosity
- M- Male
- MAPK- Mitogen Activated Protein Kinase
- MET- Met proto-oncogene (Hepatocyte Growth Factor Receptor)
- miRNAs- MicroRNA
- MLH1- mutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)

- MMR- Miss Match Repair
- mRNA- Messenger RNA
- MSH2- mutS homolog 2, colon cancer, nonpolyposis type 1
- MSH6- mutS homolog 6 (E. coli)
- MSI- Microssatelite Instability
- MYC- Myelocytomatosis viral oncogene
- NaSCN- Sodium thiocyanate
- N-Asparagine
- NCK- Non-Catalytic region of tyrosine Kinase protein adaptor family
- NM23A -Non-metastatic cells 23A
- NP40- Nonidet P-40
- NSCLC- Non small cell lung cancer
- p- Long chromosome arm
- P85- Phosphatidylinositol 3-kinase, regulatory subunit
- PBS- Phosphate Buffered Saline
- PCR- Polymerase Chain Reaction
- pH- Potencial of hydrogen
- PI3K- Phosphatidylinositol 3-Kinase
- PKB, Akt- v-akt murine thymoma viral oncogene homolog 1
- PKC- Protein Kinase C
- PLC- Phospholipase C
- PMS1- Postmeiotic Segregation Increased 1
- PMS2- Postmeiotic Segregation Increased 2
- Prl- Prolactin
- PSMD9-Proteasome (prosome, macropain) 26S subunit, non-ATPase, 9
- PTB - Phosphotyrosine-Binding signalling molecules
- PTK- Protein Tyrosine Kinase
- q- Short chromosome arm
- RARβ- Retinoic Acid Receptor, beta
- RAS- Rat sarcoma viral oncogene homolog
- RNA- Ribonucleic Nucleic Acid
- RT- Room Temperature

ABREVIATIONS

- RTK- Receptor Tyrosine Kinase
- RUNX3- Runt-related Transcription Factor 3
- SH2 – src homology 2 motifs
- Shc- Srchomology and collagen
- Sos- Son of sevenless
- SPP1- Secreted phosphoprotein 1
- STAT1- Signal transducer and activator of transcription 1
- SYP-Synaptophysin
- TFF1- Trefoil factor 1
- TGFα- Transforming Growth Factor α
- TK- Tyrosine Kinase
- TP53 -Tumour protein 53
- TP73- Tumour protein p73
- T-Thymine
- Tyr- Tyrosine
- UV- Ultra Violet
- Val- Valine
- VEGF- Vascular Endothelial Growth Factor
- V-Valine

INTRODUCTION

1 - Cancer

Cancer is a disturbance of one or more cellular activities that are crucial for the development and the maintenance of multicellular organisms, namely, growth, differentiation, programmed cell death, and tissue integrity (Mareel and Leroy, 2003). In most cases it is a malignant disease because the cancer cells invade into neighbouring tissues and survive in this ectopic site. These cells invade beyond the constraints of the normal tissue from which they originate, permitting them to enter into the circulation from where they can reach distant organs and eventually form secondary tumours, called metastases (Trelstad and Revel, 1967; Edelman et al., 1983; Van Roy et al., 1986; Mareel et al., 1993; Mareel and Leroy, 2003; Thiery, 2003).

To perform a cancer diagnosis several parameters should be taken in consideration; the site of the tumour, the histological type of the cancer, its grade of differentiation and its extent of growth and invasion. Attention is also paid to the host cell reaction evidenced by the stroma, blood vessels and leukocytes. Because cancers are known to metastasize, it is mandatory to search for secondary tumours in the lymph nodes and in distant organs (Wittekind, 1997).

Qualitative and quantitative criteria are used to stage and grade cancers for therapeutic and prognostic purposes. Staging of tumours is done following the volume of the primary tumour and its depth of invasion (T stage), the number of lymph nodes with invasion (N stage) and the presence of distant metastases (M stage)- TNM system (Wittekind, 1997).

Individual cancers are currently portrayed by deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein analysis, covering as many characteristics of malignancy as possible (Kallioniemi et al., 2001).

The above-mentioned clinical and molecular observations indicate that cancer is a disease caused by the accumulation of modified cells, disturbing differentiation and in most cases, causing loss of structure and function, of the tissue and organ, leading to cancer cell invasion and cancer cell survival in an ectopic environment (Carter, 1984).

In order to get cell and tissue alterations a series of genetic changes occur in cancer cells (fig.1) (Fearon and Vogelstein, 1990; Bishop, 1991) These genomic

alterations occur in oncogenes and tumours suppressor genes. In oncogenes or tumour-promoter genes, one allele is activated leading to gain-of-function, and in tumour-suppressor genes or anti-oncogenes, both alleles need to be inactivated leading to loss-of-function events (Blume-Jensen and Hunter, 2001). Mechanisms of activation of oncogenes implicate mutation, gene amplification, and promoter activation. Mechanisms of tumour-suppressor genes inactivation are exemplified by Loss of Heterozygosity (LOH) plus silencing of the second allele genetically, through mutation; or epigenetically, through methylation (Chen et al., 2001; Lukas, 2001; Zajchowski et al., 2001).

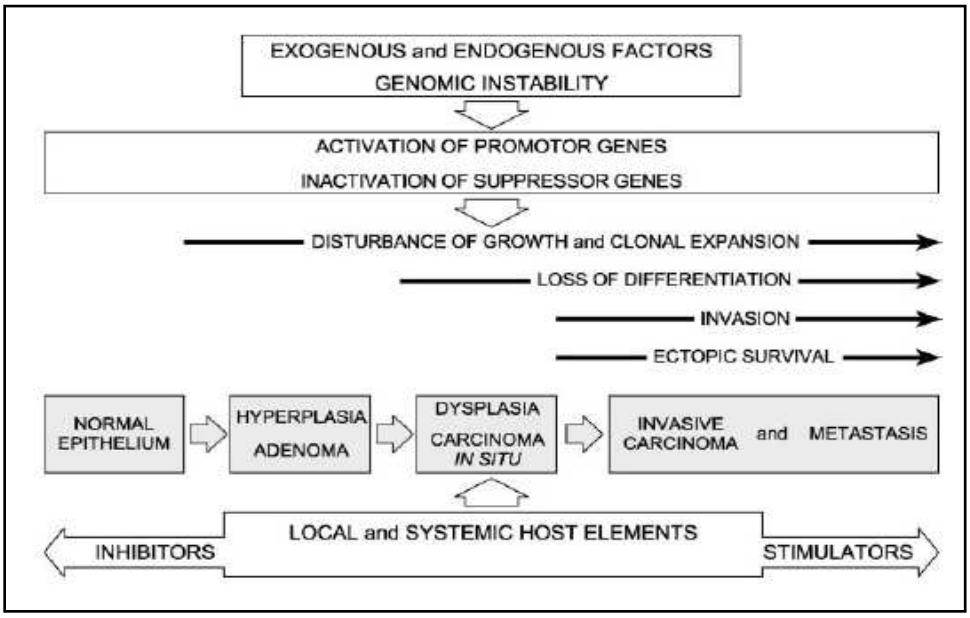

Figure 1: Schematic representation of genetic, epigenetic, and phenotypic aspects of cancer development. Adapted after Mareel (2002).

2 - The Stomach

The stomach is a single organ that can be divided in several different parts: the cardia, where the contents of the oesophagus empty into the stomach; the fundus, formed by the upper curvature of the organ; the body or corpus, the main central region and the antrum or pylorus (fig. 2), the lower section of the organ that facilitates emptying the contents into the small intestine (Owen, 1986).

What concerns to the stomach wall, as we can see in figure 1 it is constituted by four layers. From inside to outside, the first layer is the gastric mucosa, that is

composed by an epithelium with millions of microscopic glands, the lamina propria underneath, and a thin layer of smooth muscle called muscularis mucosae (Owen, 1997). The latter is formed of an inner circular and an outer longitudinal layer; it is continuous with thin fascicles of smooth muscle that go up inside the lamina propria to reach beneath the surface epithelium. The other layers of the stomach are the same as for the rest of the gastrointestinal tract (i.e. submucosa, muscularis externa [propria] and serosa). The submucosa consists of loose connective tissue with numerous elastic fibres; it contains plexuses of arteries, veins, lymph vessels, and the Meissner's nerve plexus. The muscularis externa is composed by three layers of smooth muscle: the inner oblique layer, the middle circular layer and the outer longituditinal layer. It is responsible for stomach contractions and emptying. Finally there is a thin outer layer covering all the stomach, known as the serosa (Owen, 1986).

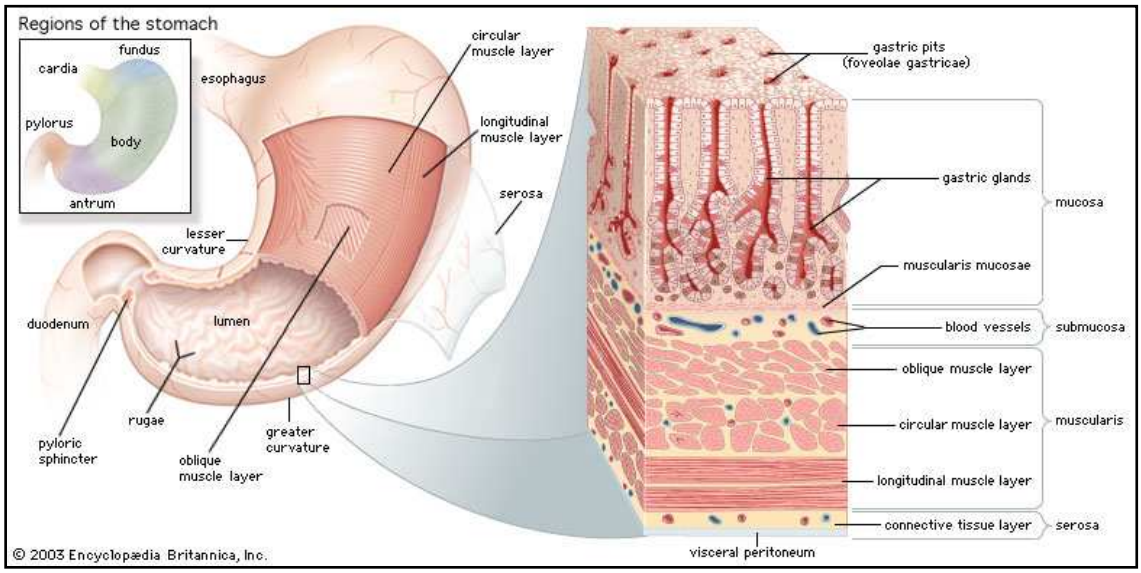

Figure 2: Histology of the stomach wall. Adapted from http://www.peptic-ulcers.co.uk

The three major types of gastric mucosa are: cardiac, fundic, and antral, which exhibits transitional areas in between. All of the gastric glands have two major components: foveolae (crypt, pit) and secretory portion (glandular). The foveolae represent the most important area for the genesis of gastric cancer (GC), in particular the layer of generative cells located at their base. The differences among the various types of gastric mucosa depend on the relative proportions between foveolae and secretory portions and the microscopic composition of the latter (Owen, 1986, 1997).

The gastric glands are composed by different cells types with distinct functions, found in different layers. In the isthmus of the glands we observe goblet cells responsible for the secretion of a mucous layer that protects the stomach epithelium from external aggressors (i.e. bacteria, acid juice, etc). In the neck of the glands there are parietal (oxyntic) cells that produce gastric acid and intrinsic factor (important in the absorption process of vitamin B12 by the small intestine). Generally in the base of the glands we have enteroendocrine cells, producers of hormones. Specifically in the base of the fundic glands we can also see chief (zymogenic) cells, producers of pepsinogen (this substance turns into pepsin under low potential of hydrogen [pH] conditions and is necessary in protein digestion) and rennin (Rosai, 2004).

2.1 - The Gastric Cancer

2.1.1 - Epidemiology

Until recently, GC was the second most common cancer worldwide, but now, with an estimated 934,000 of new cases per year in 2002 (8.6% of new cancer cases), it is in fourth place behind lung, breast and colorectal cancer. It is the second most common cause of death from cancer (700,000 deaths annually) (Parkin et al., 2005).

Almost two-thirds of the cases occur in developing countries and 42% in China alone. As we can see in figure 3, the geographical distribution of GC is characterized by wide geographic variations; high-risk areas include East Asia (China, Japan), Eastern Europe, and parts of Central and South America. Incidence rates are low in Southern Asia, North and East Africa, North America, Australia and New Zealand in men (Parkin et al., 2005).

Survival for GC is moderately good only in Japan (52%), where mass screening by photo fluoroscopy has been practiced since the 1960. Survival is also relatively high in North America, possibly due to early diagnosis following a greater number of endoscopic examinations performed for stomach disorders. In Western Europe is estimated a survival of 27%, while it is as low as 6% in sub-Saharan Africa (Parkin et al., 2005).

There is clearly a strong environmental component to the risk differences. Migrant populations from high risk parts of the world show a marked diminution in risk

when they move to a lower risk area, although this is quite gradual and seems to depend on the age at migration (McMichael et al., 1980; Kolonel and Hankin, 1980). The data fit with observations concerning the importance of childhood environment in determining risk (Coggon and Barker, 1990).

Figure 3: World-wide incidence of GC cancer. Adapted from Nature Rev. Cancer © 2004; Nature Publication Group. http://www.nature.com

There has been a steady decline in the risk of GC incidence and mortality over several decades in most countries (Muñoz, 1997). The worldwide estimates of age adjusted incidence are about 15% lower than the values estimated in 1985. This decline may be related to improvements in preservation and storage of food; it may also represent changes in the prevalence of *Helicobacter pylori* (*H. pylori*), perhaps as a result of reduced transmission in childhood, following a trend to improved hygiene and reduction of crowding (Banatvala et al., 1993; Roosendaal et al., 1997). If the observed secular decline continues, the expected number of new cases in 2010 will be around 1.1 million (19%), rather than the 21% additional cases due simply to a population growth and aging (Parkin et al., 2005).

Important also refer that between the two principal subtypes of GC, the intestinal type shows a greater frequency in the general population when compared to its diffuse counterpart, and it is more often related to environmental exposures (Sepulveda et al., 2000; El-Omar and Rabkin, 2001; Straus et al., 2002).

2.1.2 - Etiology of Gastric Cancer

The pathogenesis of GC represents a classic example of gene-environment interactions (Coussens, 2002; Peek and Martin, 2002; Malcolm et al., 2006).

The evidence linking *H. pylori* infection to CG was considered sufficient by International Agency for Research on Cancer (IARC) (IARC, 1994), to classify this bacterium as carcinogenic in humans. Its action is probably indirect by provoking gastritis, a precursor of stomach atrophy, metaplasia, and dysplasia. Infection is acquired in childhood, and prevalence within populations is certainly related to socioeconomic status (Sitas and Forman, 1992). The international variation in prevalence bears a certain similarity to that of GC; the overall estimate of *H. pylori* prevalence in adults is 76% in developing countries and 58% in developed countries. However, it is clear that with such high prevalence and relatively small international variation, there are other factors than *H. pylori* of major importance (Parkin et al., 2005).

Diet certainly plays an important role. Risk is increased by high intakes of some traditionally preserved salted food, especially meat and pickles, and with salt *per se*. Risk is decreased by high intakes of fruit and vegetables (WCRF, 1997; Palli, 2000) which may be in part related to their antioxidant content. Alcohol and tobacco smoking has also been clearly accepted as increasing the risk of GC (IARC, 2004).

Hypochlorhydria (85%-90%), pernicious anaemia and blood group A are also associated with a higher risk of gastric malignancy (Malcolm et al., 2006).

Hereditary factors clearly increase the risk of GC and this malignancy is part of a number of familial cancer syndromes (Woolf, 1961). Lending support to the genetic aetiological hypothesis is the recognition that patients with Hereditary Nonpolyposis Colon Cancer (HNPCC) and Familial adenomatous polyposis (FAP) are at an increased risk of developing malignancy in stomach (Malcolm et al., 2006).

Other factor thought to be in the pathogenesis of GC are gastric polyps, Menétrier's disease, gastric peptic ulcer, and gastric stump (Rosai, 2004).

2.1.2.1 - Pathology

The vast majority of gastric cancers are sporadic. However there is strong evidence that occasional cases have an inherited component (Carneiro, 2004).

2.1.2.1.1 - Classification

Stomach malignant tumours can be classified based on gross morphology and in histopathological features. They can be divided into tumour of the **proximal stomach** (cardia, fundus, and corpus), and of the **distal stomach** (antrum, anglus, pylorus) (Bottcher et al., 1993).

Macroscopically, advanced gastric cancers can be classified according to Borrmann (Borrman, 1926) in four types with distinct growth patterns, i.e., **type polypoid** (type I), well circumscribed polypoid tumours; **type fungating** (type II), polypoid tumours with marked central infiltration; **type ulcerated** (type III), ulcerated tumours with infiltrative and **type infiltrating** (type IV, linits plastic) (Borrman, 1926).

Microscopically, GC may assume different histological patterns. Several classifications have been proposed based on the morphologic features; however, the histological classification proposed by Laurèn is the most commonly used (Lauren, 1965).

In Lauren's classification gastric cancers are classified into two major types: **intestinal** or **diffuse**. This classification, based on tumour histology, characterizes two varieties of stomach adenocarcinomas, which have different pathology, epidemiology, etiologies and behaviour. The intestinal variant exhibits glandular, tubular and/or papillary structures. In contrast, in the diffuse variant, single cells or poorly cohesive clusters of cells infiltrate the gastric wall, often leading to widespread thickening and rigidity of the gastric wall, known as *linitis plastica* (Ming, 1977). About 16% of cases will be **unclassified** or of **mixed type** (in the same tumour there is intestinal and diffuse components) (Tahara, 2000).

Adenocarcinoma accounts for over 95% of all malignant stomach tumours, and generally the term GC refers to adenocarcinoma of the stomach. Other malignant tumours that can appear in the stomach are: lymphomas, sarcomas, squamous cell

carcinomas, adenosquamous, carcinoid tumours, and leiomyosarcomas, GISTs, etc (Ming S., 1977).

In contrast to the Laurèn´s classification that can be applied to small biopsies, Ming´s classification (Ming, 1977) requires histological examination of resection specimens. In the Ming scheme, the growth pattern is assessed at the invasion front of the tumour as being infiltrative or expansive. The expanding type is prognostically favorable, and the infiltrating type is of poor prognosis (Ming, 1977).

2.1.2.1.2 - Histogenesis

Gastric carcinomas do not raise *de novo* from normal epithelium, but they occur through successive changes. A hypothesis, about gastric carcinogenesis was proposed in 1975 by Correa *et al*. According to this hypothesis, gastric carcinogenesis is a multistage and a multifactorial process which involves irritant environmental and others factors, acid secretion, bacterial overgrowth, and bacterial production of nitrites or *N*-nitroso compounds from dietary nitrates. The result of a cascade of events is the progressive spectrum of histological states ranging from normal gastritis epithelium to stomach adenocarcinoma (GC) (Tahara, 2000; Malcolm et al. 2006).

As we can see in the figure 4, these different steps and successive changes from the normal gastric epithelium are well characterized for the intestinal GC human type, whereas, lesions predisposing to the development of the diffuse GC are not yet well understood (Malcolm et al. 2006).

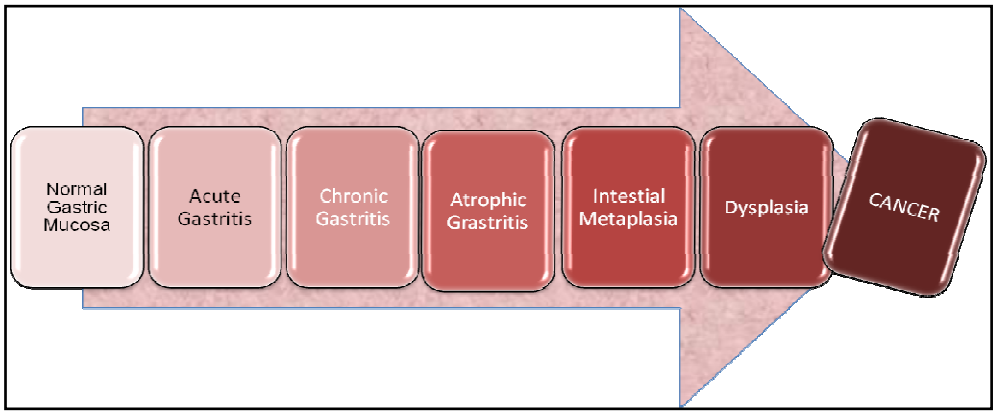

Figure 4: Different steps occurring in the transformation of the normal gastric mucosa to intestinal type gastric cancer. Adapted after Rogers and Fox (2004).

The development of the intestinal GC includes the transformation of the normal stomach mucosa into a mucosa that resembles intestinal epithelium (intestinal metaplasia). These steps begin with gastritis which progresses to mucosal atrophy (atrophic gastritis) followed by intestinal metaplasia, dysplasia and carcinoma with subsequent metastatic dissemination (Tahara, 2000).

No preceding steps have been identified in the pathogenesis of sporadic diffuse GC other than the obvious chronic gastritis that is the hallmark of *H. pylori* pathogenesis (Malcolm et al., 2006). Although the precursor lesion for sporadic diffuse GC is not known, histological analysis of early familial gastric cancers in the setting of Hereditary Diffuse Gastric Cancer (HDGC) has led to a development model for this disease (Carneiro, 2004).

In this model, showed in figure 5, loss of Epithelial-cadherin (E- cadherin) in cells within the lining of foveolae/ glands leads to the ‘‘Pagetoid’’ spread of signet ring cells through the glands and eventually to invasion of the lamina propria. The relevance of this model to sporadic diffuse gastric cancers has not been proven (Carneiro, 2004).

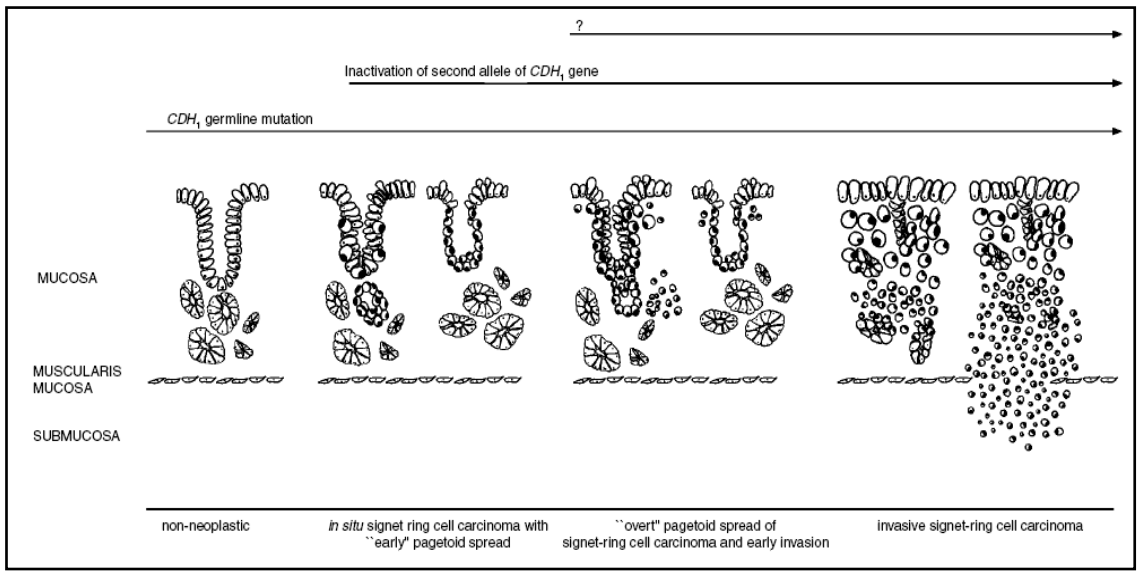

Figure 5: Model proposed for the early development of familiar diffuse GC in E-cadherin mutation carriers. Adapted after Carneiro (2004).

3 - Molecular Mechanisms of Gastric Carcinogenesis

The existing data concerning genotypic abnormalities in GC are confusing. No clear picture has emerged to indicate which abnormalities are pathognomonic for stomach carcinoma, at which stage of the process they appear, and what interpretation should be given to the rather disparate findings of the published series (Hirohashi and Sugimura, 1991; Tahara, 1993; Wright and Williams, 1993).

Both familial and sporadic gastric cancers are products of multiple genetic and epigenetic alterations that transform normal gastric epithelial cells into malignant neoplasms (Mario et al., 2004). These include abnormalities of oncogenes, tumour suppressor genes, cell adhesion molecules and cell cycle regulators. Additionally, genetic instability and alterations in growth factors and cytokines contribute to the complex pathways involved in gastric carcinogenesis. Differences exist in the pathways leading to diffuse and intestinal-type GC (Malcolm et al., 2006).

3.1 - Oncogenes

The Met proto-oncogene (MET), encoding a receptor for Hepatocyte Growth Factor/Scatter Factor (HGF/SF) is amplified in 19% of intestinal type and 39% of diffuse-type gastric cancers (Kuniyasu et al., 1992).

The fibroblast growth factor receptor 2 (FGFR2) oncogene is frequently activated in GC (Katoh et al., 1992), being preferentially amplified in 33% of advanced diffuse or scirrhous –type gastric cancers but not in intestinal-type cancers (Hattori et al., 1990). Overexpression of this gene in GC is associated with a poorer prognosis (Malcolm et al., 2006).

Another proto-oncogene, Erythroblastic leukemia viral oncogene homolog 2 **(**ERBB2), is preferentially amplified in 20% of intestinal-type GCs but this is not a feature of the diffuse type (Yokota et al., 1988). Overexpression of this gene is also correlated with poorer prognosis and liver metastases (Oda et al., 1990; Yonemura et al., 1991).

Mutations of the Kirsten rat sarcoma viral oncogene homolog (KRAS) are seen in intestinal type gastric adenocarcinomas (Sano et al., 1991; Lee et al., 1995; Isogaki et

al., 1999). The incidence of this mutation is low and it is not a feature of diffuse-type carcinomas (Malcolm et al., 2006).

3.2 - Tumour suppressor genes

The tumour suppressor gene Tumour protein 53 (TP53) is frequently inactivated in gastric carcinomas by LOH, missense mutations and frame shift mutations. This occurs in over 60% of gastric carcinomas, regardless of the histological subtype, and is frequently observed in precursor lesions such as intestinal metaplasia, dysplasia and adenomas (Sano et al., 1991; Yokozaki et al., 1992; Tohdo et al., 1993; Sakurai and Nakajima, 1995; Ochiai and Hirohashi, 1996).

LOH of Tumour protein 73 (TP73) is detected in 38% of gastric cancers, and alterations of this gene are predominant features of foveolar-type GCs with Trefoil factor 1 (TFF1) expression (Hiroshi, 1999).

TFF1 is normally expressed in gastric foveolar epithelial cells (Masiakowski et al., 1982; Lefebvre et al., 1996). Reduction or loss of the TFF1 gene by DNA methylation in the promoter region occurs in intestinal metaplasia and gastric adenomas, suggesting that this process may be important at an early stage in intestinal-type gastric carcinoma development (Tahara, 2004).

A further tumour suppressor gene is the nuclear Retinoic Acid Receptor beta (RARβ). Hypermethylation of this gene with reduced expression is observed in 64% of intestinal gastric cancers but this is not observed in the diffuse subtype (Hayashi et al., 2001).

Additional tumour suppressor gene alterations include those affecting distinct chromosomal loci. LOH at 1q and 7q are frequently associated with intestinal-type cancers while 1p is commonly affected in advanced diffuse cancers (Sano et al., 1991). LOH of the B-cell CLL (BCL2) gene is frequently observed in intestinal-type cancers (Ayhan et al., 1994).

Runt-related Transcription Factor 3 (RUNX3) is other tumour suppressor gene involved in gastric carcinogenesis, being necessary for the suppression of cell proliferation in the gastric epithelium. The loss of RUNX3 by hypermethylation is observed in several different cancers, including 64% of gastric carcinomas. RUNX3

methylation is also a feature of 8% of chronic gastritis, 28% of intestinal metaplasia and 27% of gastric adenomas (Li et al., 2002; Kim et al., 2004; Sakakura, 2005).

Other genes that appear to be affected in gastric carcinogenesis include the Fragile Histidine Triad gene (FHIT) gene and LOH at the Deleted in Colorectal Carcinoma (DCC) locus, which is a feature of intestinal-type cancers (Sano et al., 1991; Tamura, 1997)

Promoter hypomethylation of a novel cancer/testis antigen gene DEAD (Asp-Glu-Ala-Asp) box polypeptide 53 (DDX53, also known as CAGE) has recently been described in 35% of chronic gastritis and 78% of GC (Ono et al., 2002; Cho et al., 2003).

3.3 - Cell-adhesion molecules and metastasis-related genes

Mutations in Cadherin 1 (CDH1) occurs preferentially in 40–83% of diffuse type GC (Becker et al, 2006) and not in sporadic intestinal-type gastric carcinomas (Grady et al., 2000). In mixed gastric carcinomas, mutations and loss of expression of E-cadherin are seen only in the diffuse component of the tumour, suggesting that E-cadherin loss is the likely genetic basis for the divergence of a diffuse clone from an intestinal type GC (Machado et al., 1999). Since CDH1 is a tumour suppressor gene, it is the inactivation of its encoded protein that is believed to promote tumour formation in diffuse-type GC. Biallelic inactivation can occur through somatic mutations or LOH of the CDH1 promoter. This events were observed in both sporadic and inherited diffuse GC and appears to be present in approximately 50% of HDGC and 83% of sporadic diffuse GCs (Machado et al., 1999; Grady et al., 2000)

Mutations in β-*catenin* and γ-*catenin* together with E-cadherin mutations appear to be involved in the development and progression of diffuse and schirrhous type cancers (Kawanishi et al., 1995; Shibata et al., 1996; Caca et al., 1999).

Abnormal CD44 molecule (CD44) transcripts are frequently associated with GC and metastatic deposits, with the pattern of these abnormal transcripts varying between the intestinal and diffuse types (Yokozaki et al., 1994; Higashikawa et al., 1996).

Secreted phosphoprotein 1 (SPP1), a protein ligand of CD44, is overexpressed in 73% of gastric carcinomas and when co-expressed with CD44 it is correlated with lymphatic invasion and metastasis presence (Weber et al., 1996; Teruyoshi, 1998).

Reduced expression of the protein non-metastatic cells 23A (NM23A), involved in myelocytomatosis viral oncogene (MYC) transcriptional activation, and galectin-3, a galactoside-binding protein, are also implicated in metastatic gastric carcinoma (Nakayama et al., 1993; Lotan et al., 1994).

3.4 - Cell-cycle regulators

The cell-cycle regulator, cyclin E, is amplified in 15%-20% of gastric carcinomas that are associated with it's overexpression. Gene amplification or overexpression of cyclin E1 are associated with aggressiveness and lymph node metastasis (Akama et al., 1995).

The expression of the Cyclin-Dependent Kinase inhibitor 1B (CDKN1B) that binds to a wide variety of cyclin/ Cyclin Dependent Kinases (CDK) complexes and inhibits kinase activity is frequently reduced in advanced GC (Yasui et al., 1997). Reduced Proteasome 26S subunit, non-ATPase, 9 (PSMD9) expression correlates with tumour invasion and nodal metastasis. This reduction in PSMD9 occurs at a post-translational level, and results not from genetic abnormalities but rather from ubiquitin-mediated proteosomal degradation (Yasui et al., 1998).

A family of E2F transcription factor (E2F) transcription factors is an important target of cyclin/CDKs at the G1/S transition (Yasui et al. 1999). Overexpression of E2F is observed in 40% of primary GC, and this tends to be co-expressed with cyclin E1 (Suzuki et al., 1999). Gene amplification and abnormal expression of the E2F gene may permit the development of GC (Malcolm et al., 2006).

3.5 - Microsatellite Instability

Microsatellite instability (MSI) is a hallmark of the DNA mismatch repair (MMR) deficiency that is one of the pathways of gastric carcinogenesis. Microsatellites are short DNA sequence repeats that are scattered throughout the human genome and

occur in nearly every case of GC associated with germline mutations of the MMR genes MutS homolog 2, colon cancer, nonpolyposis type 1 (MSH2), MutL homolog 1, colon cancer, nonpolyposis type 2 (MLH1), Postmeiotic Segregation Increased 1 (PMS1), Postmeiotic Segregation Increased 2 (PMS2), and MutS homolog 6 (MSH6) (Keller et al., 1996; Thibodeau et al., 1996). Errors that occur in DNA MMR mechanisms in tumour cells can cause expansion and contraction of these repeats (Malcolm et al., 2006).

MSI due to epigenetic inactivation of MLH1 is found in 15%-39% of sporadic intestinal type cancer, 70% of which are associated with loss of MLH1 by hypermethylation of the promoter (Leung et al., 1999; Fleisher et al., 1999). This tumours are associated with lymphocyte infiltration, multiple tumours and a potentially favourable prognosis (Malcolm et al., 2006).

Meanwhile MSI of the D1S191 locus is found in 26% of intestinal metaplasia and 46% of intestinal type GC. An identical pattern of this MSI of D1S191 is observed in adjacent intestinal metaplasia and intestinal type cancer that suggests the sequential development from the former to the latter (Hamamoto et al., 1997). Diffuse type cancers do not exhibit MSI phenotype (Santos et al., 1996).

4 - Receptors Tyrosine kinases

Receptor Tyrosine Kinase (RTK) is a subclass of cell-surface growth-factor receptors with an intrinsic, ligand-controlled tyrosine kinase (TK) activity. They regulate diverse functions in normal cells and have a crucial role in oncogenesis. Twenty years ago, the first primary structure of a RTK, the Epidermal Growth Factor Receptor (EGFR), was elucidated (Gschwind et al., 2004).

Moreover, substantial advances have been made in understanding the key roles of RTKs in the signalling pathways that govern fundamental cellular processes, such as proliferation, migration, metabolism, differentiation and survival, as well as those that regulate intercellular communication during development. RTK activity in resting, normal cells is tightly controlled (Gschwind et al., 2004). When they are mutated or structurally altered, RTKs become potent oncoproteins (Blume-Jensen and Hunter, 2001): abnormal activation of RTKs in transformed cells has been shown to be

causally involved in the development and progression of many human cancers (Gschwind et al., 2004).

The RTK class of cell-surface receptors now comprises 58 known members that are distributed among 20 subfamilies (fig.6) (Gschwind et al., 2004). More than half of these have been found to be overexpressed or mutated in human hyperproliferative or hypoproliferative diseases and are therefore considered being targets for cancer therapy (Zwick et al., 1999).

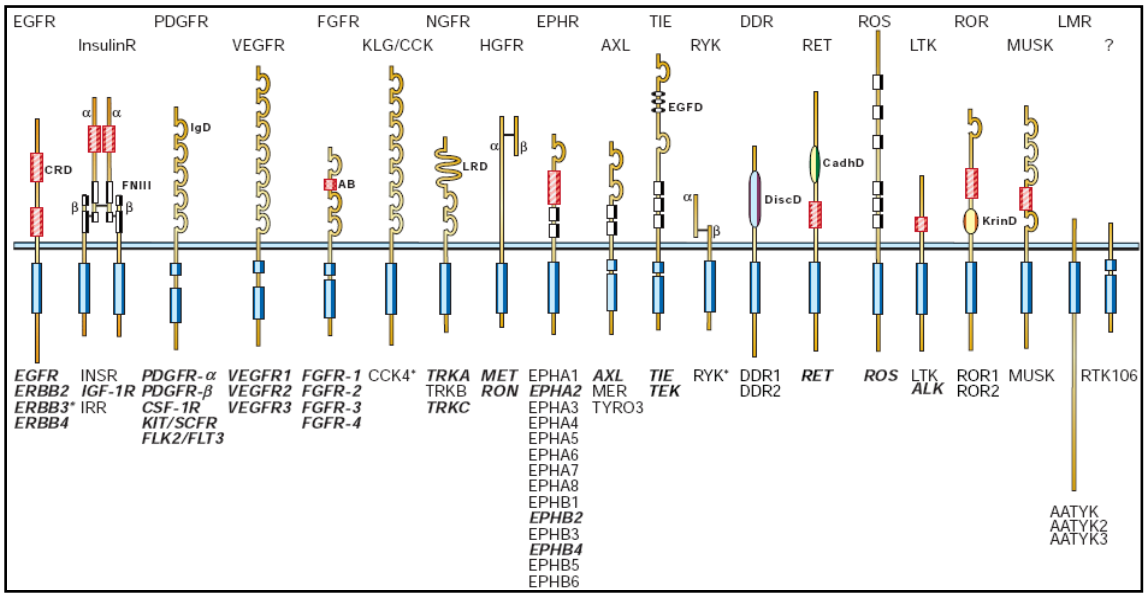

Figure 6: Human receptor RTK. The prototypic receptor for each family is indicated above the receptor, and the known members are listed below. The symbols α and β denote distinct RTK subunits. RTK members in **bold** and *italic* type are implicated in human malignancies. An asterisk indicates that the member is devoid of intrinsic kinase activity.

4.1 - The ERBB Receptor Family

Subclass I of the RTK superfamily consists of the Avian Erytroblastosis Virus B (ERBB) or Epidermal Growth factor (EGF) receptors and comprises four members: EGFR/ERBB1/HER1, Erythroblastic leukemia viral oncogene homolog 2 (ERBB2/HER2), Erythroblastic leukemia viral oncogene homolog 3 (ERBB3/HER3) and Erythroblastic leukemia viral oncogene homolog 4 (ERBB4/HER4). All members have an extracellular ligand-binding domain, a single membrane-spanning domain and a cytoplasmic domain containing a conserved protein tyrosine kinase (PTK) core (Yarden and Sliwkowski, 2001).

With few exceptions (i.e., haematopoietic cells), ERBB proteins are expressed in cells of mesodermal and ectodermal origins. They are expressed in various tissues of epithelial, mesenchymal and neuronal origin (Holbro and Hynes, 2004), where they play fundamental roles in development, proliferation and differentiation. Evidences show that the normal function of ERBB receptors and their ligands is to mediate cell–cell interactions in organogenesis and adulthood. So, this receptors' family is critical for the development of systems like the cardiovascular, nervous and gastrointestinal (Abud et al., 2005; Ciccolini et al., 2005; Mekada, 2006).

Under normal physiological conditions, activation of the ERBB receptors is controlled by spatial and temporal expression of their ligands (Harris et al., 2003), which are members of the EGF family of growth factors (Peles E, 1993). In the epithelium, the basolateral location of ERBB receptors enables them to mediate signals between the mesenchyme and the epithelium for cell growth. The mesenchyme serves as a storehouse for many ligands (Yarden and Sliwkowski, 2001).

Ligand binding to ERBB receptors induces the formation of receptor homo- and heterodimers and activation of the intrinsic kinase domain, resulting in phosphorylation on specific tyrosine residues within the cytoplasmatic tail (Holbro et al., 2003). ERBB3 is devoid of intrinsic kinase activity, due to substitutions in critical residues in its kinase domain (Holbro et al., 2003).

These phosphorylated residues serve as docking sites for a range of proteins, the recruitment of which leads to the activation of intracellular signalling pathways, like Rat sarcoma viral oncogene homolog- Mitogen Activated Protein Kinase (Ras-MAPK), Phosphatidylinositol 3-Kinase/ v-akt murine thymoma viral oncogene homolog 1 (PI3K-PKB/AKT) and Phospholipase C - Protein Kinase C (PLC-PKC) (Holbro et al., 2003).

The specificity and potency of the intracellular signals are determined by positive and negative effectors of ERBB proteins, as well as by the identity of the ligand, oligomeric composition and specific structural determinants of the receptors. The main determinant, however, is the vast array of phosphotyrosine- binding proteins that are associate with the tail of each ERBB molecule after the engagement into dimeric complexes (Yarden and Sliwkowski, 2001).

The potent cell proliferation signals generated by the ERBB network (fig. 7) are used by cancer cells to fix oncogenic mutations by clonal expansion. Hyperactivation of the ERBB network is implicated in multiple human pathologies. It is extremely useful to know whether a particular tumour has an overactive ERBB pathway because of mutation, overexpression or amplification of a component of the ERBB pathway, as it can tell us what the patient's chance of survival is and with which drug they should be treated (Yarden and Sliwkowski, 2001). Patients with cancer whose tumours have alterations in ERBB receptors tend to have a more aggressive disease, and one that is associated with factors that predict a poor clinical outcome. So, ERBB receptors have been intensely pursued as therapeutic targets (Holbro and Hynes, 2004).

There are two major classes of anti-ERBB therapeutics: ectodomain-binding antibodies and small-molecule TK inhibitors that compete with Adenosine Triphosphate (ATP) in the TK domain. Many of these therapies are either in clinical use or in advanced clinical development (Hynes and Lane, 2005).

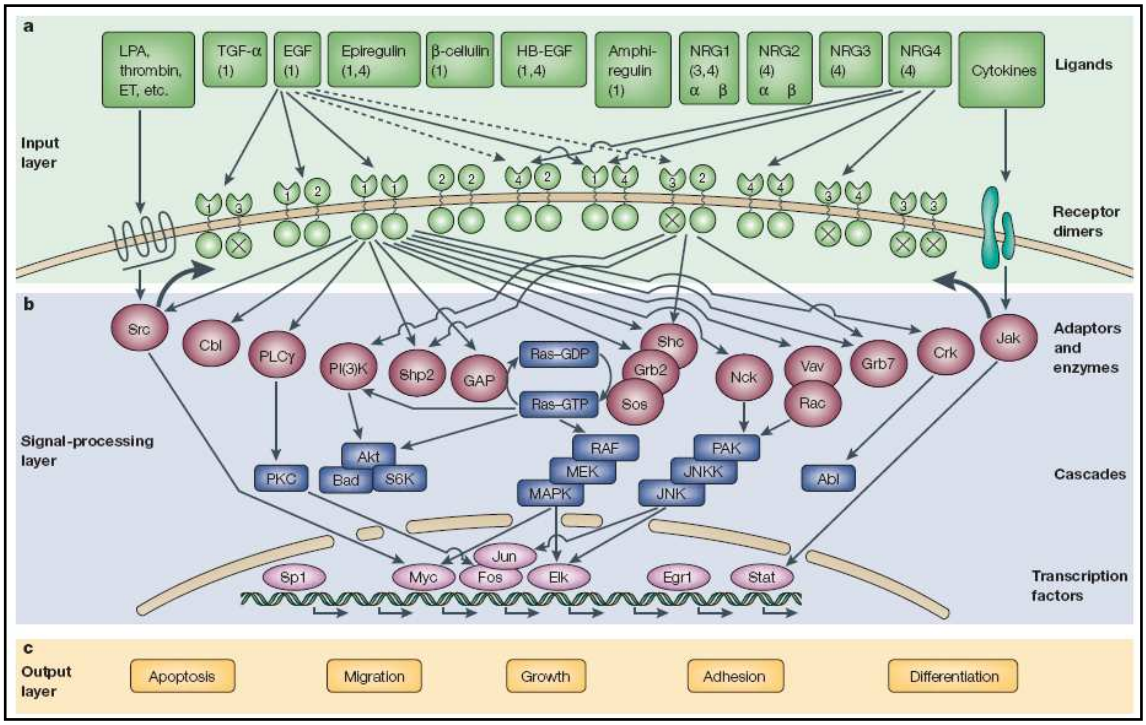

Figure 7: **The ERBB signaling network. a** Ligands and the ten dimeric receptor combinations comprise (input layer). **b** Signaling to the adaptor/enzyme layer is shown only for two receptor dimmers: the weakly mitogenic EGFR homodimer, and the relatively potent ErbB2–ErbB3 heterodimer. Only some of the pathways and transcription factors are represented in this layer. **c** How they are translated to specific types of output. Adapted after Yarden and Sliwkowski (2001).

4.1.1 - Epidermal Growth Factor Receptor

The EGFR is expressed in many normal tissues during the development and in adults, and its importance has been emphasised by the lethal phenotype of complete knock-out of the gene in mice (Miettiren et al., 1995; Gullick & Srinivasan, 1998). Inactivation of EGFR impairs epithelial development in many organs, including those involved in tooth growth and eye opening. Likewise, transgenic and *in vitro* studies implicate EGFR in promoting proliferation and differentiation of the epithelial component of skin, lung, pancreas and the gastrointestinal tract (Miettiren et al, 1995).

The EGFR pathway was first identified as a potential target for anticancer therapy in the early 1980s, and in recent years therapies directed at this target have been added to the arsenal available to the clinical oncologist (Normanno et al., 2006).

The gene for the EGFR maps to 7p11.2–p12 and comprises 28 exons. EGFR is synthesized from a 1210 residue polypeptide precursor after cleavage of the N-terminal sequence; an 1186 residue protein is inserted into the cell membrane. (Ullrich et al., 1984; Jorissen et al., 2003). Over 20% of the receptor's (170 kDa mass) is N-linked glycosylated and this is required for the translocation of EGFR to the cell surface and subsequent acquisition of function (Ullrich et al., 1984; Slieker et al., 1986). So, EGFR is a large glycoprotein expressed on the surface of the majority of normal cells.

This receptor consists of three regions (fig. 8): the extracellular ligand binding region (also called ectodomain), the transmembrane region either a single hydrophobic anchor sequence, by which the receptor traverses the cell membrane a single time and the intracellular or cytoplasmatic region with TK activity. The extracellular amino terminal end can be divided into four domains (I; II; III and IV). The EGF ligands bind between the I and III domains of the receptor (Garrett et al., 2002; Jorissen et al., 2003).

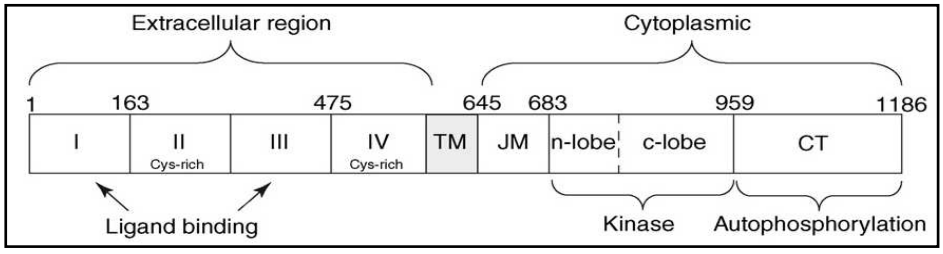

Figure 8: This diagram illustrates the structural features of the EGFR extracellular and intracellular region. Adapted after Linggi and Carpenter (2006).

The domains II and IV consist of a number of small molecules, each appearing to be held together by one or two disulphide bonds (Garrett et al., 2002; Ogiso et al., 2002). When the receptor dimerizes, loops from the domain II make contact with each other. The recent literature also suggests that receptor dimerization is a unique property of the receptors themselves, although the ligand may cause dimerization through some conformational changes in the receptor (Domagala et al., 2000 ; Burgess et al., 2003). The domain IV is involved in targeting EGFR to the caveolae/raft component prior to ligand-binding (Mineo et al., 1999).

Residues 626–647 of EGFR constitute the transmembrane domain and are α-helical. The juxtamembrane region appears to have a number of regulatory functions like downregulation and ligand-dependent internalization events (Song, 2000), basolateral sorting of the EGFR in polarized cells (Crepaldi et al., 1994; Hoschuetzky et al. 1994), and association with proteins such as epidermal growth factor receptor pathway substrate 8 (EPS 8) and calmodulin (which is competitive with PKC) (Li and Villalobo, 2002).

Just inside the cell membrane the justamembrane region is followed by PTK and autophosphorylation domains. The PTK activity plays a key role in the regulation of cell proliferation and differentiation (Yarden, and Sliwkowski 2001).

4.1.1.1 - EGFR Activation

EGFR exist in the cells both in a monomeric and dimeric state in the absence of ligand. Clearly, dimerization of the EGFR, while necessary, is not sufficient to activate the intracellular kinase (Jorissen et al., 2003).

As the concentration of ligand (EGF, Transforming Growth Factor α [TGF-α], Heparin- Binding Epidermal Growth Factor [HB–EGF], Amphyregulin [AR], Betacellulin [BTC], epigen [EPG] (Kochupurakkal, 2005), or Epiregulin [EPR] (Shelly et al., 1998) increases in the environment, an increasing proportion of receptors become occupied by ligand contingent on their extracellular domain. This provokes allosteric changes in the intracellular part of the receptor resulting in an increase of the proportion of dimerized receptor and in the activation of the intracellular TK enzymatic activity (Cohen et al., 1982; Yarden, 1987). This process is represented in figure 9.

The dimerization results in the autophosphorilation of five specific tyrosine (Tyr) residues (Tyr 1173, 1148, 1086, 1068 and 992) in the carboxyl-terminal (CT) end of the intracellular part of EGFR, with Tyr-1173 as the major autophosphorilation site (Downward and Waterfield, 1984; Hsuan, 1993).

Individual receptor pairings can consist of two molecules of the same type, called homodimers, or two molecules of different types, which has been termed as heterodimer. EGFR can interact with ERBB2, ERBB3 and ERBB4 in a ligand-dependent fashion to form heterodimers (Hynes, 2000; Yarden and Sliwkowski, 2001)

After the induction of tyrosine phosphorylation, some signalling pathways appear to start with the recognition of the CT phosphotyrosines by appropriate adaptor or signalling molecules (Jorissen et al., 2003).

Another mechanism that induces EGFR phosphorylation and subsequent stimulation of intracellular signalling pathways is known as EGFR transactivation. For example, cytokines, such as growth hormones (GH) and prolactin (Prl), can indirectly activate EGFR through Janus tyrosine kinase 2 (Jak2) pathway, which phosphorylates specific tyrosine residues in the cytoplasmic domains of EGFR (Yamauchi et al., 1997; Yamauchi et al., 2000). The EGFR signal may also be released by non-physiological influences such as ultra violet (UV) and γ-radiation, osmotic shock, membrane depolarization, heavy metal ions and radical-

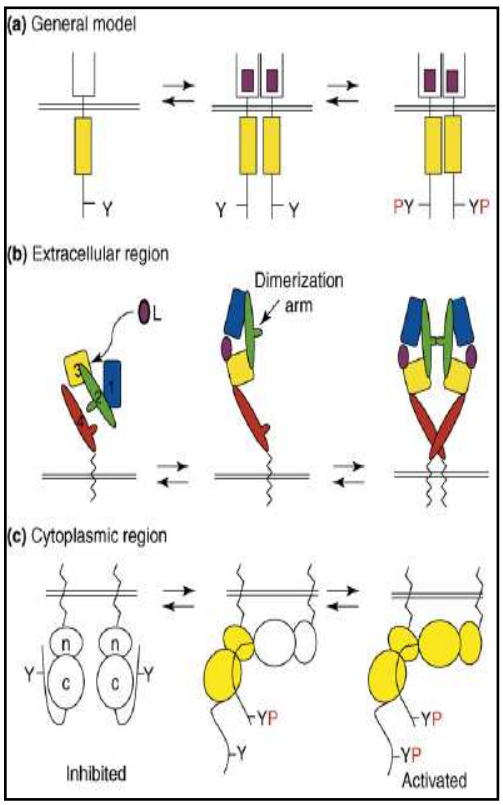

Figure 9: EGFR dimerization and activation. (a) A general scheme for ligand-dependent dimerization and activation of an EGFR. (b) The contribution of the dimerization arm to receptor association within the ectodomain. Before ligand binding, the arm is sequestered within a monomer by interactions with subdomain IV. Ligand binding alters this interaction such that the arm is now exposed to facilitate dimerization by intermonomer associations between dimerization arms. From the data available, the likely consequence of ectodomain dimerization is the asymmetric interaction of kinase domains such that activation occurs (c). P, phosphorylation; Y, tyrosine. Adapted after Linggi and Carpenter (2006).

generating agents such as hydrogenperoxide (Prenzel et al., 1999). This effect has been predominantly attributed to the inactivation of phosphatases that antagonize the intrinsic receptor kinase activity, thereby shifting the equilibrium of basal autophosphorylation and dephosphorylation towards the activated state (Fischer et al., 2003).

The strength and duration of intracellular signalling from the EGFR are also controlled by internalization and recycling of the receptor, which can be modulated by heterodimerization at the cell surface and by association with intracellular signalling molecules (Wiley, 2003).

4.1.1.2 - EGFR mediated signal transduction

EGFR–TK plays a key role in numerous processes that affect tumour growth and progression, including proliferation, dedifferentiation, inhibition of apoptosis, invasiveness and lack of adhesion dependence (Honegger et al., 1989).

Within the cell the phosphorylated receptors now can interact with proteins called second messengers. Again, a complex array of these proteins has been discovered, containing various structures which allow them to recognise receptors phosphorylated at specific sites. These fall into two main classes, those that possess structures called *src* homology 2 motifs (SH2) domains that directly or indirectly interacts with the autophosphorylated EGFR, including enzymes such as PLC, GTPase activating protein (GAP) and the synaptophysin (syp) phosphotyrosine phosphatise, as well as non-enzymatic adapter molecules such as the phosphatidylinositol 3-kinase, regulatory subunit(p85*)* of PI3K, the *Src* homology and collagen (Shc) protein, Growth factor receptor- bound protein (GRB2) and Non-Catalytic region of tyrosine Kinase (NCK) adaptor protein family; and a smaller group of proteins which interact with phosphorylated receptors via a structure called a phosphotyrosine-binding signalling molecules (PTB) domain (Gullick, 2001).

The stimulation of PLC-γ by EGFR mediated tyrosine phosphorylation causes the release of ion calcium (Ca2+) from intracellular compartments and the generation of diacylglycerol, the activator of PKC. PKC is a serine/ threonine kinase that possibly is responsible for the phosphorilation of serine/threonine residues (threonine 654 and

serine 1002, 1046 and 1047) involved in the desensitisation of EGFR (Voldborg et al., 1997).

When the Grb2/ Son of sevenless (Sos) complex that is recruited from the cytosol to the membrane, binds to tyrosine residues in EGFR normally occurs the activation of Ras, by catalyzing Guanosine-5'-diphosphate / Guanosine-5'-triphosphate (GDP/GTP) exchange. Activated Ras finally activates MAPKs cascade. Following activation MAPKs are imported to the nucleus where they activate transcription factors that regulate cell proliferation, apoptosis and differentiation (Chantry, 1995).

Recruitment of a second signaling pathway, the PI3K/AKT leads to inhibition of apoptosis mechanisms in tumour cells (Fernandes et al., 2001).

Other key downstream signalling molecules that are influenced by EGFR TK activity include Janus kinase signal transducer and activator of transcription pathway (Yamauchi et al., 2000; Jorissen et al., 2003).

Activity of EGFR-TK also influences tumour angiogenesis by up regulating (Baker et al., 2002) expression of Vascular Endothelial Growth Factor (VEGF) (Akagi et al. 2003) and interleukin 8 (Kitadai et al. 1998; Nair, 2005).

Recent reports also suggest that following EGF stimulation at the cell surface, the full-length EGFRs also migrates to the nucleus being a holoreceptor (Lin et al., 2001). The available reports suggest that these nuclear EGFR are not found in the nuclear envelope, but rather are present in the nucleoplasm in a non-membranous environment. The mechanism of traffic to the nucleus could involve endocytosis together with an unknown mechanism (Wells and Marti, 2002). In the nucleus EGFRs can bind an AT-Rich consensus Sequence (ATRS) via an undefined domain and enhance transcription via praline-rich region near their CT domain (Lin et al., 2001; Waugh and Hsuan, 2001 ; Carpenter, 2003). Since EGFR contain an enzymatic function, it is plausible that the modification of nuclear proteins is another consequence for nuclear-localized receptors or their cytoplasmic domain fragments. Finally, it is possible that EGFR carry other molecules into the nucleus and that these receptor-associated molecules are functional there. It has been proposed that EGFR may transport the signal transducer and activator of transcription 1 (STAT-1), a tyrosine-phosphorylated transcription factor, from the cytosol into the nucleus (Bild and Jove, 2002).

EGFR TK is also involved in the progression of cells through G1 phase into S phase. This progression is mediated by a family of protein kinases, the CDK and their corresponding activating partners, the cyclins (Hunter and Pines, 1994). Progression through G1 phase requires activation of the various cyclin-CDK kinase complexes (Voldborg et al., 1997). Some studies show an association between the EGFRs and the promoter region of cyclin D1, a protein that can play a key role in mitogenesis (Lin et al., 2001).

The identification of EGFR-TK substrates is far from complete (Voldborg et al., 1997).

4.1.1.3. Deregulation of EGFR in human cancer

EGFR overexpression and alterations are common in human malignancies. Increased signalling of EGFR may be caused by different mechanisms: high expression of the receptor; gene amplified copy number normally associated with structural rearrangements of the receptor; receptor mutations; heterodimerization with other members of this receptor family such as ERBB2 as well as with heterologus receptor systems (i.e. G-Protein- Coupled Receptors -GPCRs); increased expression of (autocrine/ paracrine) ligands, and alterations in molecules that control their function or regulation (Voldborg et al., 1997).

Overexpression of EGFR occurs in a variety of human tumours including non small cell lung carcinoma (NSCLC), breast, head and neck, brain, gastric, colorectal, esophageal, prostate, bladder, renal, pancreatic, and ovarian cancers (Arteaga, 2002). *In vitro* studies suggest that overexpression of the normal receptor leads to transformation only in the presence of a ligand. This receptor overexpression is associated with higher grade, higher proliferation, reduced survival and resistance to standard therapies (hormonal therapy, chemotherapy, and radiation) in various human malignancies (i.e. breast, head and neck, ovarian, bladder and esophageal cancers) (Gorgoulis et al., 1992 ; Voldborg et al., 1997; Baselga et al., 2000; Sirotnak et al., 2000; Ranson et al., 2002).

Gene amplification of EGFR has been demonstrated to occur in different tumour types and it is usually associated with EGFR protein overexpression, although

overexpression of EGFR in absence of gene amplification has been described (Salomon et al., 1995; Normanno et al., 2003). It is well established that EGFR gene amplification is associated with cancer aggressiveness, which ultimately leads to metastases (Yasmeen et al., 2006)

In addition to EGFR wild type, cancer cells have also been shown to express various mutated EGFR molecules. The most common mutant, named EGFRvIII, is one in which amino acids 6–273 (exons 2–7) of the extracellular domain are deleted. This in-frame deletion is common in glioblastomas and in several other types of cancer, including breast, ovarian, lung and medulloblastoma tumours (Weber and Spiess, 1984; Pedersen et al., 2001). EGFRvIII is constitutively active in a ligand-independent manner (Lorimer, 2002), and while its prognostic significance remains incompletely understood, it is important noting that the mutant confers radio-resistance, which exceeds the cytoprotective activity of wild type EGFR (Lammening et al., 2003). Other EGFR mutations were found in human cancers, like deletions and point mutations that are described that result in increased catalytic TK activity of the receptor (Humphrey, et al. 1990; Moscatello et al., 1995; Wikstrand et al., 1995; Voldborg et al., 1997). Mutations found especially in NSCLC appear to confer sensitivity to EGFR inhibition (Lynch et al., 2004; Pao et al., 2004).

The high expression of some EGFR ligands (such as EFR, AR and TGF- α) leads to the deregulated EGFR action and uncontrolled tumour growth (Gorgoulis et al., 1992; Cohen et al., 1994; Salomon et al., 1995).

The EGFR-homologus HER2 receptor, which is highly expressed in several human cancers, can potentiate EGFR function by increasing EGF binding affinity, stabilizing and recycling EGFR-HER2 heterodimers, and expanding the repertoire of receptor-associated substrates and signaling responses (Karunagaran et al., 1996; Worthylake et al., 1999). Cancers with high expression of EGFR or HER2 have a better prognosis than cancers that have high expression of both receptors (Tateishi et al., 1994).

As was already mentioned the excessive EGFR signaling can be activated by stimuli that do not directly interact with the structure or expression of the receptor.

EGFR signaling can be transactivated through GPCR in normal and cancer cells. In prostate cancer the deregulated activation of EGFR, GPCR agonists and their ligands

is correlated with tumour development (Prenzel et al., 1999; Gschwind et al., 2001). The A disintegrin and metalloproteinase domain (ADAM) molecules family and matrix metaloproteinases are involved in ectodomain shedding of EGFR (Izumi et al., 1998; Dong et al., 1999; Yan et al., 2002) . High activity levels of EGFR and ADAM17 are also found in primary breast tumours (Sternlicht et al., 2005). The ectodomain shedding causes the production of distinct soluble EGF-ligands that are associated with the activation of GPCRs (Hart et al., 2005).

Recent experimental results demonstrate an interaction between cadherins and EGFR, suggesting that changes in cadherin expression may not only modulate tumour cell adhesion, but also affect signal transduction and hence tumour malignancy (Christofori, 2003). The lack of cell-cell adhesion and increased migration are key characteristics of cancer cells. For this contribute the loss of expression of cell adhesion components (i.e. E-cadherin) and overexpression of components critical for cell migration, such as EGFR (Hodivala and Watt, 1994; Inada et al., 1999; Yasmeen et al., 2006).

RATIONALE

GC is one of the most frequent malignancies worldwide, associated with a variety of genetic alterations.

Much attention has been drawn to the oncogenic effect of EGFR and most of all to this success of EGFR target therapies in cancer, namely in NSCLC.

The EGFR system seems to be involved in regulation of gastric mucosa proliferation and progression of GC, however little is known about the molecular mechanisms underlying EGFR activation in this tumour type.

In GC, EGFR gene amplification is described as a rare event and activating mutations in the kinase domain were poorly investigated so far. Taking into account this lack of knowledge we considered that searching for EGFR alterations is an important subject to understand better the GC progression and to identify GC patients that can benefit from therapies targeting this molecule.

AIMS

General Aim

The present work aims to clarify the expression profile of EGFR in primary gastric carcinomas and to identify genetic alterations underlying aberrant expression profiles and activation in these tumours.

Specific Aims

1. Does EGFR overexpression occur in primary gastric carcinomas?

To address this question we evaluated the pattern of immunoexpression in a series of primary gastric carcinomas using antibodies against the extracellular and intracellular domain of the EGFR.

2. Is there any association between the overexpression of EGFR and the clinicopathologic features of the primary gastric carcinomas?

We analysed the relationship between the clinicopathologic features of the cases and the immuoexpression of EGFR.

3. Which are the genetic alterations underlying EGFR overexpression or activation in primary gastric primary carcinomas?

In attempt to answer this question we performed the following analysis:

3.1. Evaluation of the copy number of EGFR gene by flurescence *in situ* hybridization (FISH) analysis.

3.2. Screening of mutations in TK domain of EGFR by polymerase chain reaction (PCR) and direct sequencing of the exons, where alterations have been described in other neoplastic models.

4. Is there any association between structural alterations of EGFR and clinical features of the patients and histopathological features of primary gastric carcinomas?

To answer to this question we analysed the relationship between the clinicopathologic features of the cases and the EGFR alterations (gene amplification and mutations).

MATERIAL AND METHODS

1 - Case selection and histopathological classification of the tumours

Representative blocks of 77 formalin-fixed and paraffin embedded human gastric primary cancers were retrieved from the files of the Department of Pathology of the Hospital S. João. None of the patients included in the present series had a family history of GC. Haematoxylin and Eosin (H&E) stained sections were used to categorize tumours according the classifications of Lauren and Ming. Penetration of the gastric wall and the presence and localization of lymph node metastases were recorded for all patients using standard criteria for pathological staging. Orcein-stained sections were used for the detection of vascular invasion.

2 - EGFR immunohistochemistry

Immunohistochemistry was performed in 3 μm serial sections of the tumour samples using two different antibodies for EGFR. The antibodies used were the following: the EGFR clone 31G7 (Zymed Laboratories), recognising the extracellular domain, and the clone EGFR-384 (Novocastra Laboratories), recognising the intracellular domain.

For EGFR staining with the clone 31G7 antibody, sections were treated for antigen unmasking with Pepsine Digest All (Zymed Laboratories) 20 minutes at 37°C. The antibody was incubated for 1 hour (dilution 1:50) at Room Temperature (RT).

The EGFR antigen unmasking treatment for the antibody clone EGFR-384 staining was done by treating the sections in 0.01M citrate buffer, pH 6.0, during 20 minutes in a water bath at 98°C and cooled at RT for 20 minutes in the same solution. The incubation with the primary antibody was done for 1 hour at RT (dilution 1:50).

Immunohistochemistry was performed in every case using the Polimer Detection System Kit Picture™- *Plus* (Zymed ®).

A squamous cell breast carcinoma was used as positive immunostaining control for both anti-EGFR antibodies. As negative control we used the slides from the same tumour type, but instead of using the primary antibodies we used PBS (Phosphate BufferedSaline)

2.1 - Immunostaining Scoring

Three observers (Moutinho C., Carneiro F. and Weng) independently scored the immunostaining using a semi quantitative approach. The specimens immunoreactivity was classified as follow: a) according to the intensity of the staining, cases were classified as negative when there were no expression, (-); very weak staining, (+); medium staining intensity (+ +); strong staining, (+ + +); and b) according to the fraction of stained cells the cases were classified as: 0%, <25%, 25-50%, 50-75%, >75%. The immunostaining was also classified according to its sub cellular localization as membranous, cytoplasmatic, nuclear or absent.

3 - Fluorescence in Situ Hybridization for EGFR Gene

The paraffin blocks were sectioned at 5µm, and the sections were put in the drying oven at 60°C for 30 minutes. The slides were then deparaffinised with xylene (10 minutes), and washed 10 minutes with absolute ethanol.

A pre-treatment was performed with 2 X SSC solutions at room temperature for 3 minutes and 1M NaSCN solution at 80°C for 10 minutes, followed by washing in 2X SSC at RT for 4 minutes. A digestion step was performed using 4mg/ml of pepsin in a 0.2M HCL solution at 37°C for 22 minutes and then washed in a 2X SSC solution for 4 minutes. The slides were then dehydrated with increasing concentrations of ethanol (70%-96%-100%). After being completely dried, the slides were ready to receive the probe.

The LSI EGFR Dual Color Probe-Hyb Set (VYSIS®), optimized to detect the band region 7p12 in spectrum orange and the centromere of chromosome 7 (7p11.1-q11.1, D7Z1 locus) in spectrum green (fig. 10), both in interphase nuclei and on metaphase chromosomes, was used. Five µl of the probe were applied to each slide that were then covered with cover slips slides and sealed with rubber cement. The denaturation was performed in a Hybridiser at 80°C for 8 minutes followed by

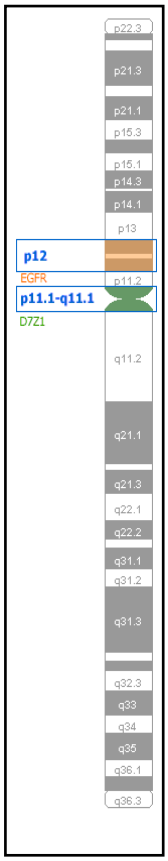

Figure 10: Chromosome 7. LSI EGFR Dual Color Probe-Hyb Set (VYSIS®) localisation. Adapted from http://international.abbottmolecular.com.

hybridization on a humid chamber at 37°C for 16 hours. The cover slips were removed and the slides were washed in a 2X SSC/ 0.1% NP40 solution at RT until the cover slip slides fall apart. Then the slides were washed in a 2X SSC/0.5% NP40 solution at 73°C for 5 minutes. Incubation with 4', 6-diamidino-2-phenylindole (DAPI) allowed nuclear staining. Afterwards, the slides were covered and kept in the dark at 4°C until fluorescence microscopy evaluation.

Sixty to one hundred intact interphase nuclei were analysed by two independent observers (Moutinho C and Milanezi F), in order to score the signals for the chromosome 7 centromere and the EGFR gene. Surrounding lymphocytes and normal mucosa were used as internal quality control for the assays. At least two or three representative areas of the neoplastic cells were selected, under a 100X/200X amplification field, to count the nuclei signals. After an overview under a 400X amplification, the signals were then counted using immersion oil (1000X).

4 - EGFR Mutation Screening

Genomic DNA was extracted from 10μm section after microdissection of the tumour areas with a sterile needle under a stereo microscope to ensure a purity of at least 70% of neoplastic cells. DNA extraction was performed using the Genomic DNA Purification Kit (Gentra System) according to the manufacturer's protocol. Exon-specific primers were designed and DNA was subjected to PCR amplification of exons 18, 19, 20 and 21. The four EGFR exons code for the TK domain of EGFR. Primers sequences are shown in Table I.

Tabela I: Primers used for PCR amplification of the EGFR TK

Exon		Primer Sequence	PCR product size (bp)
Exon 18	Forward	TGGGCCATGTCTGGCACTGC	283
	Reverse	ACAGCTTGCAAGGACTCTGG	
Exon 19	Forward	TCACTGGGCAGCATGTGGCA	241
	Reverse	CAGCTGCCAGACATGAGAAA	
Exon 20	Forward	CCTTCTGGCCACCATGCGAA	295
	Reverse	CGCATGTGAGGATCCTGGCT	
Exon 21	Forward	ATTCGGATGCAGAGCTTCTT	265
	Reverse	CCTGGTGTCAGGAAAATGCT	

PCR products were run on a 2% agarose gel and PCR amplified bands were extracted from the gel with the Gel Band Purification Kit (GE Healthcare). Samples were then purified and sequenced using the ABI Prism dGTP BigDye Terminator Ready Reaction Kit (Perkin Elmer, Foster City, CA) following manufacture's instruction and an ABI Prism 3100 Genetic Analyser (Perkin Elmer, Foster City, CA). The results were analysed using 3100 data collection software. Sequencing was performed in both strands. In cases with suspected mutations PCR amplification was repeated and the sample was re-sequenced to rule out PCR artefacts.

5 - Statistical Analysis

All statistical analyses were assessed by the x2 test or Student's *t* test. They were done to look for a possible correlation between EGFR overexpression and the clinicopathologic parameters of the patients and tumours; and between EGFR alterations and the clinicopathologic features. The association study for look for a correlation between the EGFR alterations and the clinicopathologic features of the cases was performed only in 30 cases (tumours analysed for EGFR mutations and EGFR copy number variation).

For both association studies a p value of <0.05 was considered statistically significant.

RESULTS

1 - EGFR immunoexpression

EGFR overexpression has been described in different types of tumors; however EGFR in primary GC has not been asseced in many series. In order to verify the frequency of EGFR expression in our series of primary gastric carcinomas we study the pattern of EGFR expression by immunohistochemistry using two different antibodies, one recognizing the extracellular domain (clone 31G7) and other recognizing the intracellular domain (clone EGFR-384) of EGFR protein. The results obtained are summarized in table II.

Table II: Results from EGFR immunohistochemistry. **M**: membrane; **C**: cytoplasm; **N**: nuclear; **A**: absent staining.

EGFR antibodies clones	Immunoreactivity					
	Positive (%)	Negative (%)	Localization (%)			
			M	C	N	A
Clone 31G7 (n=41)	61	39	46	5	10	39
Clone 384 (n= 38)	68	32	71	0	0	29

The percentage of positive cases is comparable using both antibodies, and the percentage of cases that show EGFR overexpression is ≈ 64.5%. What concerns to EGFR protein staining localization, it is predominantly present at the membrane (44%; 71%). Very few cases show EGFR nuclear staining (10%) or cytoplasmatic (5%). These cases were stained with the EGFR antibody clone 31G7.

In figure 11, we can see a picture set that represents different patterns of staining using the two antibodies. Pictures 11**A** and 11**B** show an EGFR positive intestinal and diffuse GC respectively, both with membrane immunoreactivity for the EGFR using the antibody that recognizes the extracellular domain (clone 31G7). The picture 11**C** and 11**D** are EGFR positive GC (intestinal and diffuse type, respectively) for the antibody recognizing the intracellular portion of EGFR (clone 384). The pictures 11**E** and 11**F** correspond to an EGFR positive atypical type GC using the clones 31G7 and 384, respectively.

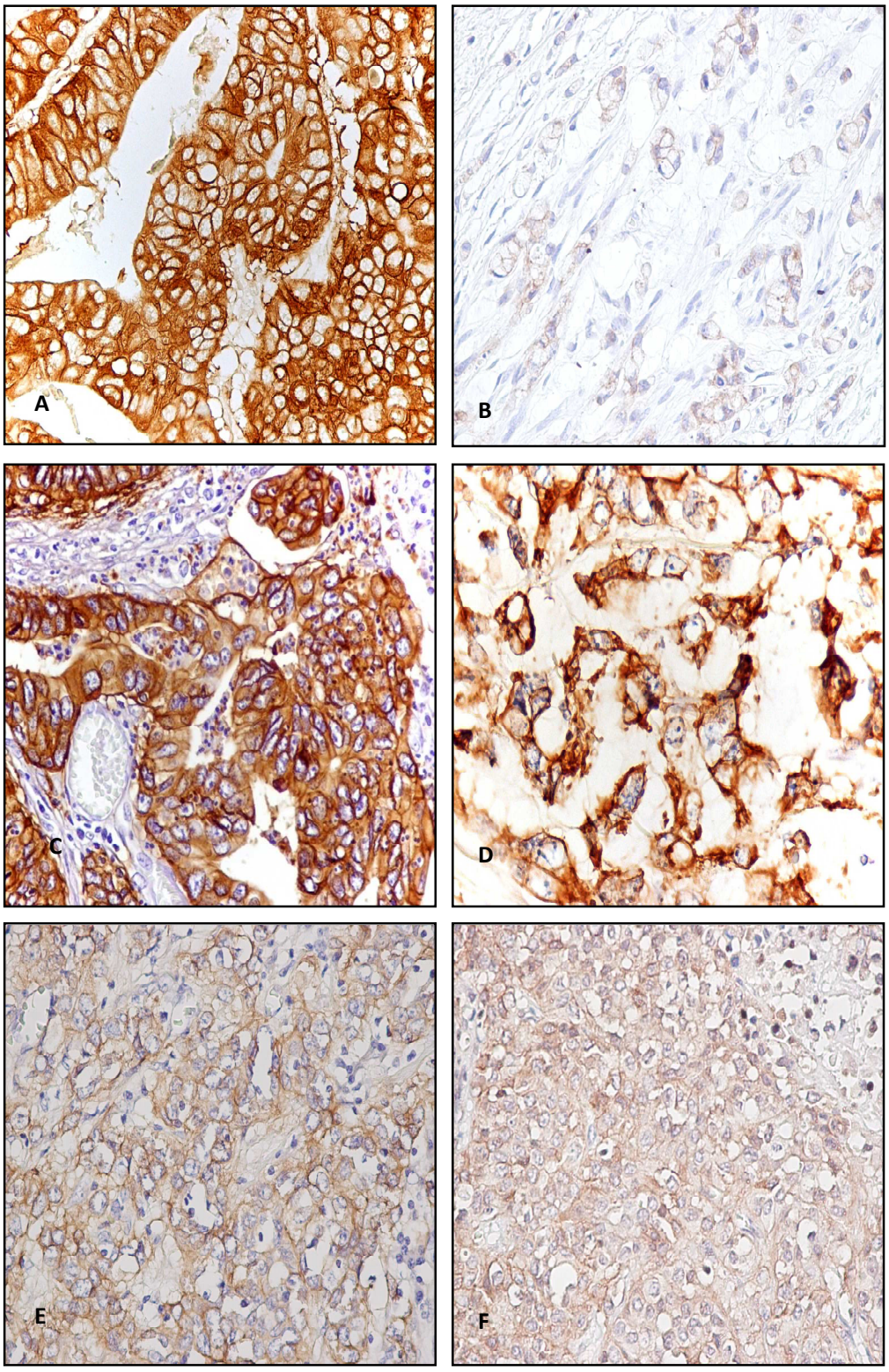

Figure12: Positive GC for EGFR immunostaining. **A**: Positive intestinal GC for EGFR (clone 31G7); **B**: Diffuse GC (clone 31G7); **C**: Positive intestinal GC for EGFR (clone EGFR-384); **D**: Diffuse GC (clone EGFR-384); **E** and **F**: Positive atypical GC for EGFR (clone 31G7 and clone EGFR-384). All pictures are at X400 magnification, except figure D that is X600.

In order to understand the clinical importance of EGFR overexpression in GC we performed association studies between the presence of EGFR expression (using the two antibodies recognizing both portions of EGFR) and the clinicopathologic features of the patients and tumours.

2 - EGFR overexpression and clinicopathologic parameters of the primary gastric carcinomas

Table III: Association studies results of EGFR imunoexpression with patients and tumours clinicopathologic parameters.

Clinicopathologic Parameters	**EGFR expression**					
	EGFR extracellular domain (n=41)			**EGFR cytoplasmatic domain (n=38)**		
	Positive	**Negative**	***P* value**	**Positive**	**Negative (**	***p* value**
Sex (F/M)	9/16	6/10	**0.92**	9/17	6/9	**0.72**
Age (SD)	61.4±11.4	50.8±16.2	**0.02**	50.0±15.1	58.5±12.7	**0.09**
Tumour localization			**0.84**			**0.10**
Proximal	13	7		14	8	
Distal	13	8		14	5	
Size (SD)	6.1±2.3	9.4±8.7	**0.09**	6.4±2.8	8.9±8.9	**0.20**
Lauren´s classification			**0.33**			**0.56**
Intestinal	13	7		12	8	
Diffuse	10	8		10	5	
Atypical	3	0		1	2	
Wall penetration			**0.74**			**0.94**
Early (T1)	1	1		1	1	
Advanced (T2-T4)	24	15		17	19	
Vascular Invasion			**0.51**			**0.89**
Absent (N0)	13	10		13	8	
Present (N≥1)	12	6		10	7	
Lymph node metastasis			**0.57**			**0.46**
Absent	10	5		4	5	
Present	15	11		17	12	
Staging			**0.51**			**0.80**
I	7	3		6	4	
II	4	2		2	3	
III	13	11		14	8	
IV	0	1		1	0	

The results of the association studies to see the relationship between EGFR overexpression and the clinical characteristics from patients and histological features from tumors are summarized in table III. We didn't found any significant statistical association between the presence of EGFR overexpression and the different parameters analysed.

3 - FISH for EGFR gene and for chromosome 7 centromere

In order to identify the molecular mechanisms underlying EGFR overexpression in this series of GC we determined the frequency of EGFR amplification using FISH analysis, since this phenomena has been described in other type of tumor models.

We performed FISH with an EGFR specific probe and with a centromeric probe for chromosome 7, in order to evaluate gene and respective chromosome copy number.

This analysis was only possible in 30 of the 41 cases analysed for EGFR pattern of expression. All EGFR signals were compared to signals for centromeric probes for chromosome 7. More than 2.0 EGFR copies per cell (balanced polysomy or gene amplification) were detected in 13.3% (4/30) of the cases. Of the four cases showing more than 2 copies of EGFR gene per chromosome 7, three had increased copy number due to polysomy (Fig. 12 **B**) and one had gene amplification, exhibiting the formation of clusters with numerous signals for EGFR (Fig.12 **C**).

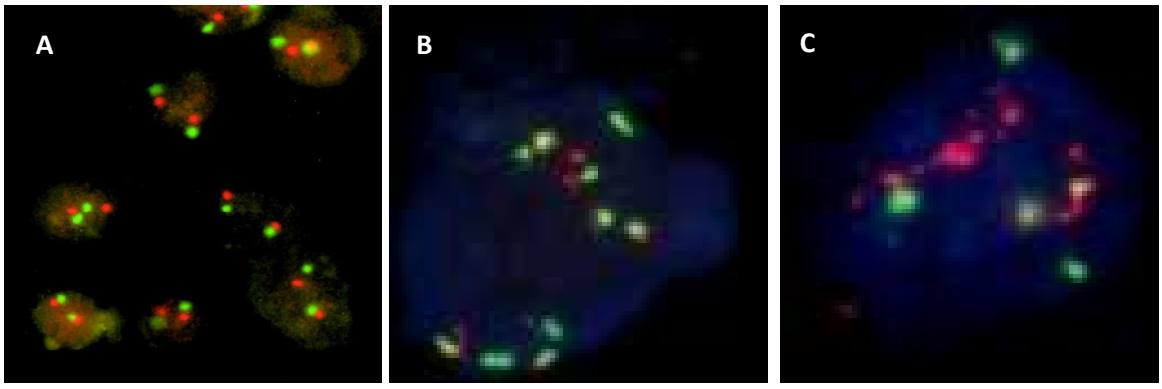

Figure 12: FISH for the centromere of chromosome 7 (green) and EGFR gene (red). **A**: Normal lymphocytes (2 copies #7, 2 copies of EGFR gene); **B**: Cancer cell with polyssomy (10 copies # 7, 7copies of EGFR gene; **C**: Cancer cell with EGFR gene. amplification (4 copies # 7; >10 copies of EGFR gene).

4 - Mutation screening by PCR and direct sequencing

Another mechanism of EGFR activation is the presence of EGFR mutations. This type of EGFR structural alteration can occur in the TK domain of this receptor, leading that to a modification of the receptor behaviour. We searched for EGFR mutations localized within the TK domain of the protein (exons 18, 19, 20 and 21), by direct sequencing.

From the 77 sporadic primary stomach carcinomas analysed, EGFR mutations in exons 18-21 were detected in 2.6% (2/77) of the cases. One mutation was found in exon 20 and the other was found in exon 21 (fig.13).

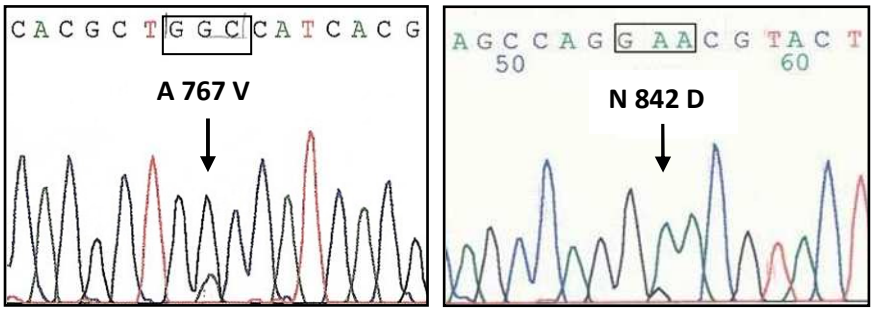

Figure 13: Missense mutations found in exon 20 (2300 C>T; A 767 V) and exon 21 (2524 A>G; N 842 D).

The mutation found in exon 20 of EGFR was a missense mutation (2300 C>T) leading to the substitution of the Alanine (A) 767 for a Valine (V). In exon 21 the single mutation found was also a missense mutation (2524 A>G) leading to the substitution of the Asparagine (N) 842 for an Aspartic acid (D). None of these mutations were previously described.

No sequence alterations were found in exons 18 and 19 from EGFR gene.

Several EGFR polymorphisms were found in exon 20 (table IV). The polymorphism 2361G>A, Glutamine (Gln) 787 Gln (fig. 14), previously described by Mu *et al.* (2005), was present in 43 of the 77 cases (55.8%) and in nine of the 43 cases in a homozygous state.

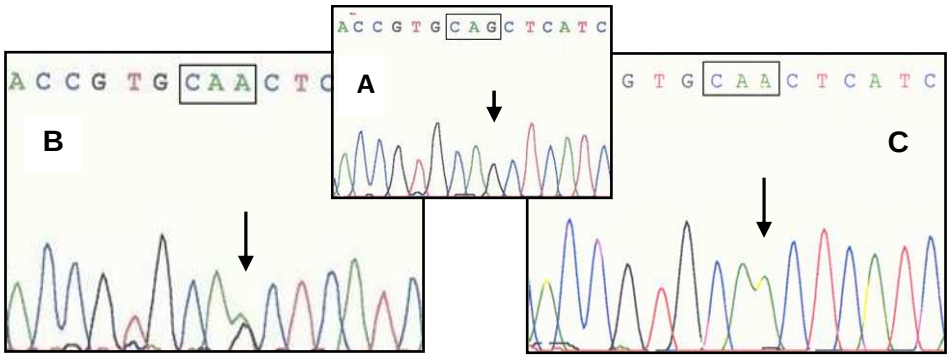

Figure 14: Silence alteration (2361G>A) found in exon 20. **A**: Normal sequence; **B**: Polymorphic sequence showing the heterozygous state; **C**: Polymorphic sequence showing the homozygous state.

Two other rare EGFR polymorphisms were found in exon 20; none of them was previously described. Both polymorphisms, the 2301 C> T, Alanine (Ala) 767 Ala, and the 2415 C>T, Histidine (His) 805His, were found in a single case (fig. 15).

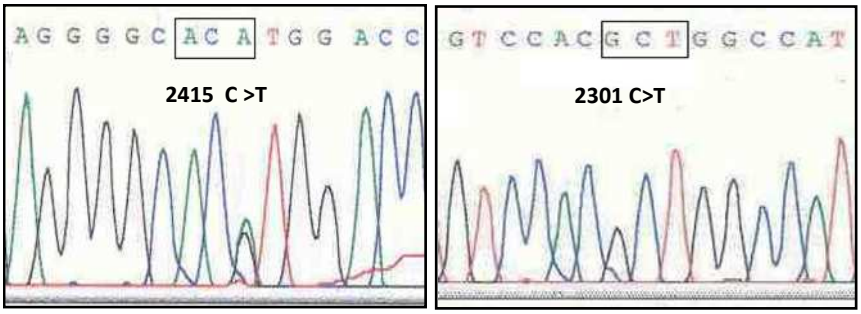

Figure 15: Silence alterations (2415 C> T; 2301 C> T) not described found in exon 20.

We screened 50 normal controls for exon 20 and we found the 2361G>A Gln787Gln silence alteration in 41 cases. The other two silent alterations (2301 C>T Ala767Ala and the 2415 C>T His805His) were absent in normal controls.

We also found six different sequence variants localized in intronic regions of EGFR, two of them described previously in Ensemble. They are represented by the figure 16.

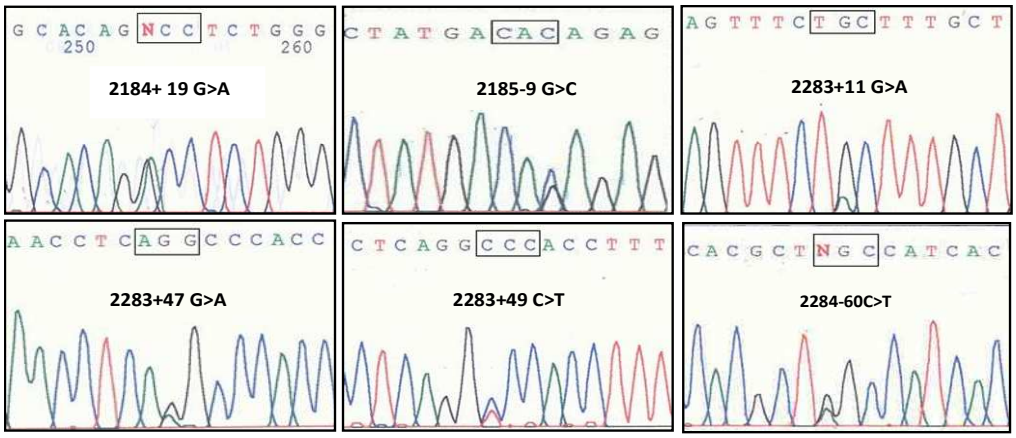

Figure 16: Intronic variants. **A**: exon 18; **B**, **C**, **D** and **E**: exon 20; **F**: exon 21.

Table IV summarizes all the sequences alterations found by direct sequencing of exons 18-20 of EGFR gene in the series analysed by us.

Table IV: Sequence alterations found by direct sequencing (exons 18-21 of EGFR gene).

	Alteration	Type	Frequency	References
Exon 18				
	2184+19 G>A	Intronic variant	2/77	Ensemble SNP rs17337107 (dbSNP126)
Exon 19				
	2185-9 C>G	Intronic variant	1/77	Not yet described
	2283+11 G>A	Intronic variant	1/77	Not yet described
	2283+47 G>A	Intronic variant	1/77	Not yet described
	2283+49 C>T	Intronic variant	1/77	Not yet described
Exon 20				
	2284-60 C>T	Intronic variant	2/77	Ensemble SNP rs10241451 (dbSNP126)
	2300 C>T	Missense Ala 767 Val	1/77	Not yet described
	2301 C>T	Silent Ala 767 Ala	1/77	Not yet described
	2361G>A	Silent Gln 787 Gln	43/77	Mu *et al* (2005)
	2415 G>T	Silent His 805 His	1/77	Not yet described
Exon 21				
	2524 A>G	Missense Asn 842 Asp	1/77	Not yet described

5 - EGFR structural alterations and clinicopathologic parameters of the patients and tumours

Table V: Association studies results of EGFR gene structural alterations with

Clinicopathologic parameters	EGFR status			
	Amplification/mutation (n=6)	Normal (n=24)	Total (n=30)	*p* value
Sex (F/M)	1/5	10/14	11/19	**0.2557**
Age (SD)	62.3 ± 14.1	56.3 ± 16.8	57.5 ± 16.2	**0.4271**
Tumour localization				**0.8548**
Proximal	3	11	14	
Distal	3	13	16	
Size (SD)	11,6 ± 9.8	5.8 ± 2.0	6.9±5.0	*** 0.0094**
Lauren´s classification				**0.1261**
Intestinal	1	12	13	
Diffuse	5	9	14	
Atypical	0	3	3	
Wall penetration				**0.4642**
Early (T1)	0	2	2	
Advanced (T2-T4)	6	22	28	
Vascular Invasion				**0.7125**
Absent (N0)	3	14	17	
Present (N=1)	3	10	13	
Lymph node metastasis				**0.1921**
Absent	1	11	12	
Present	5	13	18	
Staging				**0.3845**
I	1	7	8	
II	0	5	5	

Table V shows the associations between EGFR alterations and the clinicopathologic characteristics of the patients and tumours. When comparing gastric carcinoma harbouring EGFR alterations with carcinomas without EGFR mutation or increase copy number, we observed a significant association between EGFR structural alterations and increased tumour size (p=0.0094). No significant associations were found between EGFR alterations and other clinicopathologic parameters of the patients and tumours, namely gender and age of the patients, tumour localization, histological type, wall penetration, the presence of lymph node metastasis, vascular invasion and tumour staging of the tumour.

DISCUSSION

1 - Does EGFR overexpression occur in primary gastric carcinomas?

We accessed EGFR expression in a series of GC and found that about 65% of the cases show EGFR overexpression by immunohistochemical methods. This frequency obtained by us is within the range previously reported in GC that can vary from 19-83% of the cases (Yasui et al. 1988; Yonemura et al., 1992; Salomonet al., 1995).

In the majority of the cases, in our series, the EGFR antibody (clone 31 G7) for the extracellular domain exhibit fainter staining when compared with the EGFR antibody (clone EGFR-384) for the cytoplasmatic domain. This fact was clear when we compare the immunoreactivity between the two antibodies. We obtained 61% of positive gastric cancers for the EGFR extracellular domain staining, and 68% of positive gastric cancers for the EGFR antibody against the cytoplasmatic domain. One hypothesis to explain the different percentage between the two antibodies (7%) is the presence of EGFRvIII deletions in 4 cases. In the case of EGFRvIII deletions, EGFR misses the extracellular domain (exons 2-7) where the EGFR antibody recognizing the extracellular domain of the protein binds (Moscatello et al., 1995).

The EGFR staining for both antibodies is predominantly localised in the membrane, place where normally this receptor is located. However, we found some cases with cytoplasmic or nuclear staining for the antibody that recognizes the extracellular domain of EGFR (clone 31 G7). This localization can be due to the fact that after EGFR dependent ligand activation, this system (EGFR/ligand) is internalized to the cytoplasm and then degraded in the lysosomes (Lo et al., 2005). What concerns to the nuclear staining, it was recent related that activated EGFR could be imported to the nucleus where he can play different roles (i.e. holoreceptor, transcription factor or/and transporters of other molecules) (Lin et al., 2000; Bublil & Yarden, 2007).

2 - Is there any association between the overexpression of EGFR and the clinicopathologic features of the primary gastric carcinomas?

We did not found significant associations between the presence of EGFR overexpression and the clinicopathologic features of patients and tumours. In our series EGFR overexpression was more frequently observed in well- differentiated adenocarcinomas than in poorly differentiated carcinomas, being the intestinal type, the GC histotype with higher cases of EGFR overexpression for both antibodies (13/41 and 12/38), followed by the diffuse GC. This observation is in accordance with previous published results (Salomon et al., 1995). Although several immunohistochemical reports indicated that EGFR overexpression in GC is significantly correlated with tumour size (Salomon et al., 1995), our findings did not confirm this association.

Concerning other pathological parameters we observed that EGFR overexpression occurred in cases with a higher depth of wall invasion, with more lymph node metastasis and advanced stage of disease but without statistically significance. This is in agreement with the data published by Salomon et al. (1995).

3 - Which are the genetic alterations underlying EGFR overexpression or activation in primary gastric primary carcinomas?

Overexpression of EGFR protein has been associated with genetic abnormalities, namely amplification and rearrangements in other tumour types (Hynes and Lane, 2005). In order to verify if these alterations could explain part of the EGFR overexpression observed in our study, we performed FISH for EGFR gene.

3.1 - Evaluation of the copy number of EGFR gene by FISH analysis.

Here we report the presence of EGFR increased copy number in 13.3% of the 30 cases in which FISH analysis was possible. Of the four cases with increased copy number, just one was due to gene amplification, whereas the remaining 3 cases had EGFR increased copy number by polissomy of chromosome 7. It should be noted that it is unclear whether high polissomy indicates the activation of the EGFR gene, resulting in effects similar to those caused by gene amplification (Tsao et al., 2005). This low percentage of cases with increased EGFR copy number is in agreement with the previous reports [4.9% or 8.6%] (Tokunaga et al., 1995; Hirono et al., 1995; Tsugawa et al., 1998).

3.2 - Screening of mutations in TK domain of EGFR by PCR and direct sequencing of the exons where alterations have been described in other neoplastic models.

The mutational analysis of the TK domain of EGFR revealed the presence of mutations in 2.6% of 77 gastric carcinomas.

The identified mutations were of the missense type and were present in exons 20 and 21 of the EGFR gene. One of the missense alterations identified was a transition at codon 767 (2300 C>T) leading to an amino acid substitution (Ala to Val). The remaining mutation was a transition at codon 842 (2524 A to G) in exon 21. None of the identified mutations had been previously described and their functional significance is not yet assessed. However, due to their localization in the kinase domain of EGFR, it is tempting to speculate that they will affect the activity of the receptor and is likely that patients harbouring these types of EGFR mutations may benefit from the use TK inhibitors as therapeutic approach.

Besides the found of these mutations, other sequence alterations have been identified. In exon 20, namely, we have identified three silent substitutions.

The EGFR polymorphism previously described in Ensemble (2361G>A) occurs in high percentage of cases and in Portuguese normal controls. In contrast, the other two not yet described silent variants in exon 20 (2301 C>T, 2415 C>T) were absent in normal control demonstrating that these variants are rare EGFR polymorphisms.

In addition to these EGFR sequence variant in coding regions, we have also identified variations in the intronic sequence flanking exons 18, 19 and 20, but their

functional effect remains unclear. Maybe because some of them are situated in the first 10 bases after the end of the exon, they can interfere in the transcription process. Another possibility for the function relevance of these variations is the presence of mutations at regions that harbour MicroRNAs (miRNAs) localized to non coding RNA transcripts or within the introns of coding genes (Rodriguez et al., 2004). miRNAs are small, non coding, single stranded segments of RNA containing 19 to 24 nucleotides that have been associated with the regulation of messenger RNA (mRNA) expression (Lee et al., 1993). miRNAs reduce the transcription and translation of mRNA, thereby down-regulating gene expression (Lee et al., 1993). Transcriptional profiling using genomic microarrays and beads has enabled the discovery of numerous miRNAs that are differentially expressed in normal tissues *vs.* tumors and associated with cancer development, diagnosis, and prognosis (Calin & Croce, 2006). Today miRNA signatures can be used to detect and classify cancer and predict the severity of disease, with certain profiles of miRNA expression linked to aggressive cancers with advanced disease present at diagnosis (Jeffrey S. Ross, 2007).

4 - Is there any association between structural alterations of EGFR and clinical features of the patients and histopathological features of primary gastric carcinomas?

The correlation of the clinical parameters for the cases with complete information what concerns FISH analysis and mutation, showed that EGFR alteration occurs mainly in diffuse carcinomas of the stomach, although this association was not statically significant. The presence of EGFR alterations mainly in diffuse carcinomas is in contrast to what was found for the other member of the ERBB receptors in GC. In gastric carcinoma ERBB2 amplification was detected preferentially in intestinal carcinomas of the stomach (David et al, 1992). Similarly to what was found for ERBB2 alterations in GC, all cases with EGFR alterations are advanced carcinoma. However, we can't conclude that EGFR induces a higher capacity of tumour cells to invade the gastric wall since the number of early carcinomas analysed in our series are too low to allow definitive conclusions.

We found a significant association between EGFR alterations and tumour size. This result comes in agreement with the reports from Hirono et al., 1995 and Tsugawa et al., 1998, suggesting that EGFR is involved in tumour growth and alterations may occur in advanced stages during the progression, however needing to be further supported by other groups.

In addition, EGFR gene amplification is not a frequent event in gastric carcinomas, demonstrating that overexpression of this receptor is probably due to alterations in translation of this gene or due to alterations in protein partners (Salomon et al., 1995). For example it was recent described an interaction between E-cadherin and EGFR, suggesting that changes in E- cadherin expression (Christofori, 2003) and structure (Mateus et al., 2007) may not only modulate tumor cell adhesion, but also affect signal transduction and hence tumor malignancy (Christofori, 2003). The lack of cell-cell adhesion and increased migration are key characteristics of cancer cells. For this contribute the loss of expression of cell adhesion components (i.e. E-cadherin) and over -expression of components critical for cell migration, such as EGFR.

SUMMARY AND CONCLUSIONS

EGFR overexpression has been described in many human tumours including GC. In NSCLC patients somatic EGFR mutations, within the kinase domain of the protein, as well as gene amplification were associated with a good clinical response to EGFR inhibitors.

In gastric tumours data concerning structural alterations of EGFR remains controversial. Given its possible therapeutic relevance, we aimed to determine the possible overexpression, frequency and type of structural alterations of the EGFR gene in a series of primary gastric carcinomas.

Does EGFR overexpression occur in primary gastric carcinomas?

We evaluated 41 primary gastric carcinomas by EGFR immunohistochemistry. We found high EGFR expression for both used antibodies (clone 31 G7 and clone EGFR-384) in about 65% of the cases, when compared with normal gastric mucosa.

The EGFR protein expression was predominantly at the cellular membrane level, although there were some cases with a cytoplasmatic or nuclear immunoreactivity pattern.

So as answer to this specific aim we can say that in our GC cases we have EGFR overexpression in about 61% of the cases.

Is there any association between the overexpression of EGFR and the clinicopathologic features of the primary gastric carcinomas?

The statistical analysis between the EGFR protein expression results and the clinicopathologic features of the primary gastric carcinomas was negative. We didn't found any statistic correlation.

Which are the genetic alterations underlying EGFR overexpression or activation in primary gastric primary carcinomas?

As it was said, in some cancers the presence of molecular structural alterations is correlated with EGFR overexpression. It doesn't happen in our case.

- **Evaluation of the copy number of EGFR gene by FISH analysis.**

EGFR gene increase copy number was found in 13.3% of the 30 analysed cases. However just one was due to gene amplification. The other 3 cases had EGFR gene increased copy number due to polyssomy of chromosome 7.

- **Screening of mutations in TK domain of EGFR by PCR and direct sequencing of the exons where alterations have been described in other neoplastic models.**

EGFR screening was done in exons 18-21 that belong to the TK domain, in 77 GC cases. We identified mutations in 2.6% of gastric carcinomas.

We identified mutations of the missence type in exons 20 (2300 C›T) and 21 (2524 A›G) of the EGFR gene. None of them had been previously described, so their functional significance is not yet assessed.

Other sequences alterations have been found. In exon 20 we identified three silent substitutions (2361G›A; 2301 C›T; 2415 C›T) and flanking exons 18, 19 and 20 we found intronic variants.

Based on our results we can conclude that EGFR gene amplification and TK mutations are a rare event in our GC cases, don't being the major cause for EGFR protein overexpression.

Is there any association between structural alterations of EGFR and clinical features of the patients and histopathological features of primary gastric carcinomas?

We performed association studies, to look for a possible correlation between the structural EGFR molecule alterations and the clinicopathologic features, only in 30 cases.

The only significant correlation found was between EGFR alterations and tumour size ($p < 0.0094$).

So, we can conclude that EGFR structural alterations are rare in GC, but whenever present, it may lead to tumour growth.

Final we can conclude that structural EGFR gene alterations are not a frequent event in gastric carcinomas, demonstrating that overexpression of this receptor is probably due to alterations in translation of this gene or due to alterations in protein partners. However we consider that our results indicate that searching for EGFR structural alterations in gastric cancer is likely to be clinically important in order to identify patients that might benefit from non conventional therapies, susceptible to respond to TK inhibitors.

SUMÁRIO E CONCLUSÕES

A elevada expressão do EGFR tem sido descrita em muitos tumores humanos, incluindo os gástricos. A presença de mutações somáticas no seu domínio tirosina cinase, bem como a amplificação génica em pacientes com NSCLC são eventos associados a uma boa resposta clínica, quando sujeitos aos inibidores TK do EGFR. Nos tumores gástricos os dados referentes a alterações moleculares deste receptor permanecem controversos. Dada a sua possível relevância terapêutica, neste trabalho pretendeu-se determinar o grau de expressão do EGFR, bem como possíveis alterações estruturais, numa série de carcinomas gástricos primários.

Existe um aumento da expressão do EGFR nos carcinomas gástricos primários?

Foram analisados para o EGFR, 41 carcinomas gástricos, através de imunohistoquímica. Foi observado um aumento da expressão para ambos os anticorpos utilizados (clone 31 G7 e clone EGFR - 384) em cerca de 65% dos casos. O padrão de expressão do EGFR era predominantemente membranar, embora houvessem alguns casos com um padrão citoplasmático ou nuclear.

Estes resultados levam-nos a concluir que o EGFR está desregulado no cancro gástrico.

Existe alguma associação entre o aumento da expressão do EGFR e as características clinicopatológicas dos carcinomas gástricos primários?

Não foi observada qualquer associação entre a sobre-expressão do EGFR e as características clinicopatológicas dos carcinomas em estudo.

Quais são as alterações genéticas subjacentes ao aumento da expressão do EGFR ou à sua activação nos carcinomas gástricos primários?

Como foi dito, em alguns tipos de cancros a presença de alterações estruturais no EGFR está relacionada com o aumento da sua expressão. No entanto nesta série de casos, tal não acontece.

- **Avaliação do número de cópias do gene do EGFR através de FISH.**

Foi encontrado um aumento do número de cópias do gene do EGFR em 13,3% dos 30 casos analisados. No entanto apenas um foi devido a amplificação génica. Os outros 3 tinham um número aumentado de cópias do gene, devido à polissomia do cromossoma 7.

- **Pesquisa de mutações no domínio tirosina cinase do EGFR por PCR e sequenciação directa dos exões onde foram descritas alterações em outros modelos neoplásicos.**

A pesquisa de mutações foi feita nos exões 18-21, que pertencem ao domínio tirosina cinase, em 77 casos. Identificamos cerca de 2,6% de mutações. Nenhuma delas foi descrita anteriormente, de modo que a sua importância funcional ainda não foi avaliada.

Foram também encontradas outras alterações tais como mutações do tipo missence no exão 20 (2300 C> T) e 21 (2524 A> G); três substituições silenciosas (2361G> A; 2301 C> T; 2415 C> T (exão 20)) e variantes intrónicas nas zonas circundantes dos exões 18, 19 e 20.

Na nossa série de carcinomas gástricos, a amplificação do EGFR e as mutações no seu domínio catalítico são raras, não sendo os principais responsávei pelo aumento da sua expressão.

Existe alguma associação entre as alterações estruturais encontradas no EGFR e os parâmetros clinicopatológicos dos carcinomas gástricos primários?

Realizamos estudos de associação, de modo a pesquisar uma possível correlação entre as alterações estruturais da molécula do EGFR e as características clinocopatológicas, isto em apenas 30 casos.

A única associação ($p < 0,0094$) encontrada foi entre as alterações estruturais do EGFR e o tamanho do carcinomas.

Podemos então dizer que apesar das alterações estruturais do EGFR serem raras no cancro gástrico, quando presentes podem levar ao crescimento tumoral.

Como conclusão final, podemos dizer que a ocorrência de alterações estruturais no gene do EGFR é um evento raro, indicando que a sobre-expressão deste receptor pode dever-se provavelmente a alterações no processo de tradução ou alterações nas suas moleculas de interação. Apesar disso, consideramos que os nossos resultados indicam que a pesquisa de alterações do EGFR no cancro gástrico é importante ao nível clínico, de modo a identificar alguns doentes que poderão beneficiar de terapias não convencionais, podendo ser susceptíveis aos inibidores TK do EGFR.

FUTURE PERSPECTIVES

The following specific problems will be addressed:

- **Is the EGFR overexpressed in gastric carcinomas activated, this means, is this phosphorylated EGFR?**

EGFR overexpression was present in gastric carcinomas. However we don't know if these receptors are in their active state, triggering different cell behaviours.

To address the question we will perform an immunohistochemitry with an antibody recognising the phosphorylated EGFR protein. Then we will confirm the immunohistochemistry results, by measuring the *in vitro* kinase activity to see EGFR activity in gastric carcinomas and adjacent normal mucosa.

- **Are there EGFRvIII mutants present in gastric carcinomas?**

As it was already mentioned, EGFRvIII mutants misses the extracellular domain of EGFR (exons 2-7), leading this to a constitutive activation of the receptor.

The answer to this question will be achieved by the direct sequencing of EGFR exons 2-7.

- **Have the new EGFR point mutations any biologic role in gastric carcinogenesis?**

For see this, we will use recombinant plasmids encoding either wild- type EGFR or this new mutated forms, into a cell line not expressing this gene. Then we will perform functional assays with those cells.

- **Have the intronic sequence variants in the regulation of EGFR overexpression?**

To investigate this question we will search for a possible role of the intronic regions where the sequences variants were present, like an alteration in a stop region important in the transcription process or in regions that harbour miRNAs.

REFERENCES

Bibliographic Sources

Abud H., Heath N., Joan K. (2005). "Growth of intestinal epithelium in organ culture is dependent on EGF signalling." Experimental Cell Research **303**: 252-262.

Akagi M., Liu W., McCarty M., Takeda A., Fan F., Stoeltzing O., Parikh A., Jung Y., Bucana C., Mansfield P., Hicklin D., Ellis L. (2003). "Induction of neuropilin-1 and vascular endothelial growth factor by epidermal growth factor in human gastric cancer cells." Br J Cancer **88**: 796-802.

Akama Y., Yokozaki H., Kuniyasu H., Kitahara K., Ishikawa T., Tahara E. (1995). "Frequent amplification of the cyclin E gene in human gastric carcinomas" Jpn J Cancer Res **86**: 617-621.

Arteaga C. (2002). "Epidermal Growth factor Receptor dependence in Human tumours: more than just expression? " The oncologist **7**: 31-39.

Ayhan A., Yokozaki H., Seto M., Ueda R., Tahara E. (1994). "Loss of heterozygosity at the bcl-2 gene locus and expression of bcl-2 in human gastric and colorectal carcinomas." Jpn J Cancer Res **85**: 584-591.

Baker C., Kedar C., et al. (2002). "Blockade of Epidermal Growth Factor Receptor Signaling on Tumor Cells and Tumor-Associated Endothelial Cells for Therapy of Human Carcinomas. Am J Pathol **161**: 929-938.

Banatvala N., Megraud F., et al. (1993). "The cohort effect and Helicobacter pylori." J Infect Dis. **168**: 219 -221.

Baselga J., Pfidter D., Cooper M., Cohen R., Burtness B., Bos M., D'Andrea G., Seidman A., Norton L., Gunnett K., Flacey J., Anderson V., Mendelsohn J. (2000). "Phase I studies of anti-epidermal growth factor receptor chimeric antibody C225 alone and in combination with cisplatin." J Clin Oncol. **18**: 904-14.

Becker J., Serve H., Domschke W., Pohle T. (2006). "Role of receptor tyrosine kinases in gastric cancer: New targets for a selective therapy." World J Gastroenterol **12**: 3297-3305.

Bild A., Jove R. (2002). "Cytoplasmic transport of Stat3 by receptor-mediated endocytosis." EMBO J **21**: 3255-3263.

Bishop J. (1991). "Molecular themes in oncogenesis." Cell **64**: 235-248.

Blume-Jensen P. and T. Hunter (2001). "Oncogenic kinase signalling." Nature **411**: 355-365.

Borrman R. (1926). Geschwulste des Magens and Duodenums. Handbuch der speziellen patholischen anatomie und histologie. L. O. Henke F. Heidelberg, Springer**:** 865-879.

Bottcher K., Busch R., Fink U., Siwert J., Hermanek P., Meyer H. (1993). "Epidemiologie des Deutschen, Magen-Karzinom studie 1992." Deutshe Medzinische Wochenschrift **118**: 729-736.

Bublil E. and Yarden Y. (2007). "The EGF receptor family: spearheading a merger of signaling and therapeutics." Current Opinion in Cell Biology **19**: 124-134.

Burgess A., Cho A., et al. (2003). "An Open-and-Shut Case? Recent Insights into the Activation of EGF/ErbB Receptors." Molecular Cell **12**: 541-552.

Caca K., Kolligs F., et al. (1999). "α- and γ- Catenin Mutations, but not E-Cadherin Inactivation, Underlie T-Cell Factor/Lymphoid Enhancer Factor Transcriptional Deregulation in Gastric and Pancreatic Cancer." Cell Growth Differ **10**: 369-376.

Calin G., and Croce C. (2006). "MicroRNA-Cancer Connection: The Beginning of a New Tale." Cancer Res **66**: 7390-7394.

Carneiro F. (2004). "Model of the early development of diffuse gastric cancer in E-cadherin mutation carriers and its implications for patient screening." The Journal of Pathology **203**: 681-687.

Carpenter G. (2003). "Nuclear localization and possible functions of receptor tyrosine kinases." Current Opinion in Cell Biology **15**: 143-148.

Carter R. (1984). “Precancerous States”. New York, Oxford Univ. Press.

Chantry A. (1995). "The Kinase Domain and Membrane Localization Determine Intracellular Interactions between Epidermal Growth Factor Receptors." J. Biol. Chem. **270**: 3068-3073.

Chen J., Hong K, Yang T., Sher S., Shih Y., Wu J., Cheng R., Roffler J., et al. (2001). "Global Analysis of Gene Expression in Invasion by a Lung Cancer Model." Cancer Res **61**: 5223-5230.

Cho B., Lee H., et al. (2003). "Promoter hypomethylation of a novel cancer/testis antigen gene CAGE is correlated with its aberrant expression and is seen in premalignant stage of gastric carcinoma." Biochemical and Biophysical Research Communications **307**: 52-63.

Chouhei S. (2005). "Frequent downregulation of the runt domain transcription factors RUNX1, RUNX3 and their cofactor CBFB in gastric cancer." International Journal of Cancer **113**: 221-228.

Christofori G. (2003). "Changing neighbours, changing behaviour: cell adhesion molecule-mediated signalling during tumour progression." Embo J **22**: 18-23.

Ciccolini F., Mandl C., et al. (2005). "Prospective isolation of late development multipotent precursors whose migration is promoted by EGFR." Developmental Biology **284**: 112-125.

Coggon D., Barker D. (1990). "Stomach cancer and migration with England and Wales." Br J Cancer **61**: 573-574.

Cohen D., Simak R., Fair W., et al. (1994) “Expression of transforming growth factor- alpha and the epidermal growth factor receptor in human prostate tissues.” J Urol. **152**: 2120-2124.

Cohen S., Ushiro H., et al. (1982). "A native 170,000 epidermal growth factor receptor-kinase complex from shed plasma membrane vesicles." J. Biol. Chem. **257**: 1523-1531.

Coussens L. (2002). "Inflammation and cancer." Nature **420**: 860-867.

Crepaldi T., Pollack A., et al. (1994). "Targeting of the SF/HGF receptor to the basolateral domain of polarized epithelial cells." J. Cell Biol. **125**: 313-320.

David L., Seruca R., Nesland J., Soares P., Sansonetty F., Holm R., Borresen A., Sobrinho-Simoes M. (1992) “c-erbB-2 expression in primary gastric carcinomas and their metastases.” Mod Pathol. **5**:384-90.

Domagala T., Smyth F., Jorissen R., Fabri L., Geleick D., Lax I., Schlessinger J., Sawyer W., Howlett G., Burgess A., Nice E. (2000). "Stoichiometry, kinetic and binding analysis of the interaction between epidermal growth factor (EGF) and the extracellular domain of the EGF receptor." Growth Factors **18**: 11-29.

Dong J., Opresko L., Dempsey P., et al. (1999) Metalloprotease-mediated ligand release regulates autocrine signaling through the epidermal growth factor receptor. Proc Natl Acad Sci. USA. **96**, 6235-6240.

Downward J. and Waterfield M. (1984). "Autophosphorylation sites on the epidermal growth factor receptor." Nature **260**: 14538-46.

Edelman G., Gallin W., et al. (1983). "Early Epochal Maps of Two Different Cell Adhesion Molecules." PNAS **80**: 4384-4388.

El-Omar E. and Rabkin C. (2001). "Gastric cancer and H. pylori: Host genetics open the way." Gastroenterology **121**: 1002-1013.

Fearon E. and Vogelstein B. (1990). "A genetic model for colorectal tumorigenesis." Cell **61**: 759-767.

Fernandes H., Cohen S., et al. (2001). "Glycosylation-induced Conformational Modification Positively Regulates Receptor-Receptor Association. A STUDY WITH AN ABERRANT EPIDERMAL GROWTH FACTOR RECEPTOR (EGFRvIII/Delta EGFR) EXPRESSED IN CANCER CELLS." J. Biol. Chem. **276**: 5375-5383.

Fischer O., Gschwind A., Ullrich A. (2003). "EGFR signal transactivation in cancer cells." Biochem Soc Trans **31**: 1203-1208.

Fleisher A., Esteller M., et al. (1999). "Hypermethylation of the hMLH1 Gene Promoter in Human Gastric Cancers with Microsatellite Instability." Cancer Res **59**: 1090-1095.

Garrett T., McKern M., et al. (2002). "Crystal Structure of a Truncated Epidermal Growth Factor Receptor Extracellular Domain Bound to Transforming Growth Factor α." Cell **110**: 763-773.

Gorgoulis V., Aninos D., Milkou P., et al. (1992) “Expression of EGF, TGF-α and EGFR in squamous cell lung carcinomas.” Anticancer Res . **12**: 1183-1187.

Grady W. Willis J., et al. (2000). "Methylation of the CDH1 promoter as the second genetic hit in hereditary diffuse gastric cancer." Nat Genet **26**: 16-17.

Gschwind A., Fischer O., et al. (2004). "The discovery of receptor tyrosine kinases: targets for cancer therapy." Nat Rev Cancer **4**: 361-370.

Gschwind A., Zwick E., Prenzel N., Leserer M., Ulrich A.: (2001). "Cell communication networks: epidermal growth factor receptor as the paradigma for inter-receptor signal transmission." Oncogene **20;** 1594-1600.

Gullick W. (2001). "The Type 1 growth factor receptors and their ligands considered as a complex system." Endocr Relat Cancer **8**: 75-82.

Gullick W. and Srinivasan R. (1998). "The type 1 growth factor receptor family: new ligands and receptors and their role in breast cancer." Breast Cancer Research and Treatment **52**: 43-53.

Hamamoto T., Semba H., Yasui Y., Yunotani W., Miyazaki S., Tahara E. (1997). "Altered microsatellites in incomplete-type intestinal metaplasia adjacent to primary gastric cancers." J Clin Pathol **50**: 841-846.

Harris R., Chung E., et al. (2003). "EGF receptor ligands." Experimental Cell Research **284**: 2-13.

Hart S., Fisher O., Prenzel N., et al. (2005). “GPCR-induced migration of breast carcinoma cells depends on both EGFR signal transactivation and EGFR- independent pathways.” Biol. Chem. **386**, 845-855.

Hattori Y., Odagiri H., et al. (1990). "K-sam, an Amplified Gene in Stomach Cancer, is a Member of the Heparin- Binding Growth Factor Receptor Genes." PNAS **87**: 5983-5987.

Hayashi K., Goodison H., Oue S., N. Suzuki, Lotan T., Yasui R., Tahara E. (2001). "Inactivation of retinoic acid receptor β, by promoter CpG hypermethylation in gastric cancer." Differentiation **68**: 13-21.

Higashikawa K., Ue T., Taniyama K., Ishikawa T., Tarin D., Tahara E. (1996). "Evaluation of CD44 transcription variants in human digestive tract carcinomas and normal tissues." Int J Cancer **66**: 11-17.

Hirohashi S. and Sugimura T. (1991). "Genetic alterations in human gastric cancer.” Cancer Cells. **3**: 49-52.

Hirono Y., Tsugawa K., Fushida S., Ninomiya I., Yonemura Y., Miyazaki I., Endou Y., Tanaka M., Sasaki T. (1995) “Amplification of epidermal growth factor receptor gene and its relationship to survival in human gastric cancer.” Oncology. **52**:182-8.

Hiroshi Y. (1999). "Alterations of p73 preferentially occur in gastric adenocarcinomas with foveolar epithelial phenotype." International Journal of Cancer **83**: 192-196.

Hodivala K. and Watt F. (1994). “Evidence that cadherins play a role in the downregulation of integrin expression that occurs during keranicyte terminal differentiation.” J Cell Biol. **124**: 589-600.

Holbro T. and Hynes N. (2004). "ERBB RECEPTORS: Directing Key Signaling Networks Throughout Life." Annual Review of Pharmacology and Toxicology **44**: 195-217.

Humphrey P., et al. (1990). “Anti-synthetic peptide antibody reacting at the fusion juntion of deletion-mutant epidermal growth factor receptors in human glioblastomas.” Proc. Nati Acad. Sci. USA. **87**: 4207-4211.

Hunter T. and Pines J. (1994). "Cyclins and cancer II: Cyclin D and CDK inhibitors come of age." Cell **79**: 573-582.

Hynes M. (2000). "The ErbB signaling network: receptor heterodimerization in development and cancer." The EMBO Journal. **19**: 3159 - 3167.

Hynes N. and Lane H. (2005). "ERBB receptors and cancer: the complexity of targeted inhibitors." Nat Rev Cancer **5**: 341-354.

IARC, Ed. (1994). “IARC Monograph on the Evaluation of Carcinogenic Risks to Humans. Schistosomes, Liver Flukes and helicobacter pylori.” Lyon, International Agency for Research on Cancer.

IARC, Ed. (2004). “IARC Monographs on the Evaluation of Carcinogenic Risk to Humans. Tobacco Smoke and Involuntary Smoking.” Lyon. International Agency for Research on Cancer.

Inada S., Koto T., Futami K., et al. (1999). “Evaluation of malignancy and the prognosis of esophageal cancer based on an immunohistochemical study (p53, E-cadherin, epidermal growth factor receptor).” Surg Today. **29**; 493-503.

Isogaki J., Yin W., Arai T., Koda K., Kimura T., Kino I., Sugimura H. (1999). "Microsatellite instability and K-ras mutations in gastric adenomas, with reference to associated gastric cancers." Cancer Detect Prev **23**: 204-214.

Izumi Y., Hirata M., Hasuwa H., et al. (1998). “A metaloprotease-desintegrin, MDC9/meltrin-γ/ADAM9 and PKC8 are involved in TPA- induced ectodomain shedding of membrane-anchored heparin-binding EGF-like growth factor.” EMBO J. **17**: 7260-7272.

Jeffrey S., Ross J., Graham B. (2007). "miRNA: The New Gene Silencer." American Journal of Clinical Pathology **128**: 830 - 836.

Jorissen R., Walker F., et al. (2003). "Epidermal growth factor receptor: mechanisms of activation and signalling." Experimental Cell Research **284**: 31-53.

Kallioniemi, O., Kononen U., Sauter J., et al. (2001). "Tissue microarray technology for high-throughput molecular profiling of cancer." Hum. Mol. Genet. **10**: 657-662.

Karunagaran D., Tzahar E., Beerli R., et al. (1996) “ErbB-2 is a common auxiliary subunit of NDF and EGF receptors: implications for breast cancer.” EMBO J. **15**: 254-264.

Katoh M., Hattori Y., et al. (1992). "K-sam Gene Encodes Secreted as Well as Transmembrane Receptor Tyrosine Kinase." PNAS **89**: 2960-2964.

Kawanishi, J., Kato J., et al. (1995). "Loss of E-cadherin-dependent cell-cell adhesion due to mutation of the beta-catenin gene in a human cancer cell line, HSC-39." Mol. Cell. Biol. **15**: 1175-1181.

Keller G, Vogelsang H., Bischoff P., Mueller J., Siewert J., Hofler H. (1996). "Analysis for microsatellite instability and mutations of the DNA mismatch repair gene hMLH1 in familial gastric cancer." Int J Cancer **68**: 571-576.

Kim T., Lee H., et al. (2004). "Methylation of RUNX3 in various types of human cancers and premalignant stages of gastric carcinoma." Lab Invest. **84:** 479-484.

Kitadai Y., Sumii K., Yamamoto S., Ue T., Yokozaki H., Yasui W., Ohmoto Y., Kajiyama G., Fidler I., Tahara E. (1998). "Expression of interleukin-8 correlates with vascularity in human gastric carcinomas." Am J Pathol **152**: 93-100.

Kochupurakkal B., Harari D., et al. (2005). "Epigen, the Last Ligand of ErbB Receptors, Reveals Intricate Relationships between Affinity and Mitogenicity." J. Biol. Chem. **280**: 8503-8512.

Kolonel L. and Hankin J. (1980). “Cancer Patterns among Migrant and Native-born Japanese in Hawaii in Relation to Smoking, Drinking and Dietary Habits: Genetic and Environmental factors in Experimental and Human Cancer.” Tokyo, Japan Scientific Societies Press**:** 327-340.

Kuniyasu H., Kitadai Y., Yokozaki H., Ito H., Tahara E. (1992). "Frequent amplification of the c-met gene in scirrhous type stomach cancer." Biochem Biophys Res Commun **189**: 227-232.

Lammening G., Hewit T., Valerie K., Contessa J., Amorino G., Dent P., et al. (2003).“ EGFRvIII-mediated radioresistence throught a strong cytoprotective response.” Oncogene; **22**: 5545-53.

Lauren P. (1965). "The two histological main types of gastric carcinoma: Diffuse and so-called intestinal-type carcinoma. An attempt at a histo-clnical classification." Acta Pathol Microbiol Scand **64**: 31-49.

Lee K., Suh C., Kim S., Kim S., Lee J., Lee M., Park M., Sun H., Kim S. (1995). "Clinicopathologic significance of the K-ras gene codon 12 point mutation in stomach cancer. An analysis of 140 cases." Cancer Res **75**: 2794-2801.

Lee R., Feinbaum R., et al. (1993). "The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14." Cell **75**: 843-854.

Lefebvre O., Chenard M., et al. (1996). "Gastric Mucosa Abnormalities and Tumorigenesis in Mice Lacking the pS2 Trefoil Protein." Science **274:** 259-262.

Leung S., Yuen S., et al. (1999). "hMLH1 Promoter Methylation and Lack of hMLH1 Expression in Sporadic Gastric Carcinomas with High-Frequency Microsatellite Instability." Cancer Res **59**: 159-164.

Li H. and Villalobo A. (2002). "Evidence for the direct interaction between calmodulin and the human epidermal growth factor receptor." Biochem. J. **362**: 499-505.

Li Q., Ito K., et al. (2002). "Causal Relationship between the Loss of RUNX3 Expression and Gastric Cancer." Cell **109**: 113-124.

Lin S., Makino K., et al. (2001). "Nuclear localization of EGF receptor and its potential new role as a transcription factor." Nat Cell Biol **3**: 802-808.

Linggi B. and Carpenter G. (2006). "ErbB receptors: new insights on mechanisms and biology." Trends in Cell Biology **16**: 649-656.

Lorimer I. (2002). "Mutant Epidermal Growth Factor Receptors as Targets for Cancer Therapy." Current Cancer Drug Targets **2**: 91-102.

Lotan R., Yasui W., Yokozaki H., Lotan D., Tahara E. (1994). "Expression of a 31-kDa lactoside-binding lectin in normal human gastric mucosa and in primary and metastatic gastric carcinomas." Int J Cancer **56**: 474-480.

Lukas J. (2001). "Are all cancer genes equal?" Nature **411**: 1001-1002.

Lynch T., Bell D., Sordela R., et al. (2004) "Activating mutations in lung cancer to gefitinib." N Eng J Med, **350**: 2129-39.

Machado J., Carneiro F., et al. (1999). "E-cadherin gene mutations provide a genetic basis for the phenotype divergence of mixed gastric carcinomas." Lab Invest **79**: 459-465.

Malcolm G., Tahara E., Omar M. (2006). "Cellular and molecular aspects of gastric cancer." World J Gastroenterol **19**: 2979-2990.

Mareel B. (2002). Molecular mechanisms of cancer invasion. Encyclopedia of Cancer. B. JR. New York, New York: Academic. **3:** 221-233.

Mareel M. and Leroy A. (2003). "Clinical, Cellular, and Molecular Aspects of Cancer Invasion." Physiol. Rev. **83**: 337-376.

Mareel M., Van Roy F., Vakaet L. (1993). "Expression of E-cadherin in embryogenetic ingression and cancer invasion." Int J Dev Biol **37**: 227-235.

Mario S., Eva G., et al. (2004). "Molecular biology of sporadic gastric cancer: prognostic indicators and novel therapeutic approaches." Cancer treatment reviews **30**: 451-459.

Masiakowski P., Breathnach R., et al. (1982). "Cloning of cDNA sequences of hormone-regulated genes from the MCF-7 human breast cancer cell line." Nucl. Acids Res. **10**: 7895-7903.

Mateus A., Seruca R., et al. (2007). "EGFR regulates RhoA-GTP dependent cell motility in E-cadherin mutant cells." Hum. Mol. Genet. **16**: 1639-1647.

McMichael A., Hartchorne J., Woodings T. (1980). "Patterns of gastrointestinal cancer in European migrants to Australia: the role of dietary change." Int J Cancer **5**: 431- 437.

Mekada R. (2006). "ErbB and HB-EGF Signaling in Heart Development and Function." Cell Structure and Function **31**: 1-14.

Miettiren P., et al. (1995) “Epithelial immaturity and multiorgan failure in mice lacking EGFR”. Nature. **376**: 337-341.

Mineo C., Gill G., et al. (1999). "Regulated Migration of Epidermal Growth Factor Receptor from Caveolae." J. Biol. Chem. **274**: 30636-30643.

Ming S. (1977). "Gastric Carcinoma: a pathobiological classification." Cancer **39**: 2475-2485.

Moscatello K., Holgado M., et al. (1995). "Frequent Expression of a Mutant Epidermal Growth Factor Receptor in Multiple Human Tumors." Cancer Res **55**: 5536-5539.

Mu X., LY L., Zhang X., Wang M., Feng R., Cui Q., Zhou H., Guo B. (2005). “Gefitinib-sensitive mutations of the epidermal growth factor receptor tyrosine kinase domain in chinese patients with non-small cell lung cancer.” Clin Cancer Res. **11**: 4289-94.

Muñoz N. (1997). "Epidemiology of gastric cancer and perspectives for prevention." Salud Publica Mex **39**: 318 -330.

Nair P. (2005). "Epidermal growth factor receptor family and its role in cancer progression." Current Science **88**: 890-98.

Nakayama H., Yokozaki H., Tahara E. (1993). "Reduced expression of nm23 is associated with metastasis of human gastric carcinomas." Jpn J Cancer Res **84**: 184-190.

Ni C., Golde T., Carpenter G. (2001). " c-Secretase cleavage and nuclear localization of ErbB-4 receptor tyrosine kinase." Science **294**: 2179-2181.

Normanno N., Bianco C., et al. (2003). "Target-based agents against ErbB receptors and their ligands: a novel approach to cancer treatment." Endocr Relat Cancer **10**: 1-21.

Normanno N., Luca A., et al. (2006). "Epidermal growth factor receptor (EGFR) signaling in cancer." Gene **366**: 2-16.

Ochiai A., Hirohashi S. (1996). "p53 mutations in the nonneoplastic mucosa of the human stomach showing intestinal metaplasia." Int J Cancer **69**: 28-33.

Oda N., Tsuda T., Yoshida K., Nakayama H., Yasui W., Tahara E. (1990). "DNA ploidy pattern and amplification of ERBB and ERBB2 genes in human gastric carcinomas." Virchows Arch B Cell Pathol Incl Mol Pathol **58**: 273-277.

Ogiso H., Ishitani R., et al. (2002). "Crystal Structure of the Complex of Human Epidermal Growth Factor and Receptor Extracellular Domains." Cell **110**: 775-787.

Ono S., Kuniyasu H., Suzuki T., Ito R., Matsusaki K., Ishikawa T., Tahara E., Yasui W. (2002). "Acetylated histone H4 is reduced in human gastric adenomas and carcinomas. ." J Exp Clin Cancer Res **21**: 377-382.

Owen D. (1986). "Normal histology of the stomach." Am J Surg Pathol **10**: 48-61.

Owen D. (1997). “Stomach. Histhology for the pathologists.” Philadelphia, Lippincott-Raven. p.p 481-494.

Palli D. (2000). " Epidemiology of gastric cancer: an evaluation of available evidence." J Gastroenterol **35**: S84 -S89.

Parkin D., Bray F., et al. (2005). "Global Cancer Statistics, 2002." CA Cancer J Clin **55**: 74-108.

Pedersen M., Meltorn M., et al. (2001). "The type III epidermal growth factor receptor mutation: Biological significance and potential target for anti-cancer therapy." Ann Oncol **12**: 745-760.

Peek R. and Martin J. (2002). "Helicobacter pylori and gastrointestinal tract adenocarcinomas." Nat Rev Cancer **2**: 28-37.

Peles E. (1993). "Neu and its ligands: from an oncogene to neural factors." BioEssays **15**: 815-824.

Prenzel N., Zwick E., Daub H., et al. (1999). “EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of pro-HB-EGF.” Nature. **402**: 884-888.

Ranson M., Hammond L., Ferry D., Kris M., Tullo S., Murray P., Miller V., Averbuch S., Ochs J., Morris C., Feyereislova A., Swaisland H., Rowinsky E. (2002). “ZD1839, a selective oral epidermal growth factor receptor-tyrosine kinase inhibitor, well tolerated and active in patients with solid, malignant tumours: results of a phase I trial.” J Clin Oncol. **20**: 2240-50.

Rodriguez A., Griffiths S., et al. (2004). "Identification of Mammalian microRNA Host Genes and Transcription Units." Genome Res. **14**: 1902-1910.

Rogers A. and Fox J. (2004) “Inflamation and cancer. Rodent mice of infectious gastrointestinal and liver cancer.” Am J Physiol. Gastrointestinal liver Physiol. **286**: 6361-6366.

Roosendaal R., Buiterwerf J., et al. (1997). "Helicobacter pylori and the birth cohort effect: evidence of a continuous decrease of infection rates in childhood." Am J Gastroenterol **92**: 1480 -1482.

Rosai, (Ed. 2004). Surgical Pathology. Edinburgh, Mosby.

Sakurai S. and Nakajima T. (1995). "Clinicopathological and molecular biological studies of gastric adenomas with special reference to p53 abnormality." Pathol Int **45**: 51-57.

Salomon D., Brandt R, et al. (1995). "Epidermal growth factor-related peptides and their receptors in human malignancies." Critical Reviews in Oncology/Hematology **19**: 183-232.

Sano T., Tsujino T., et al. (1991). "Frequent Loss of Heterozygosity on Chromosomes 1q, 5q, and 17p in Human Gastric Carcinomas." Cancer Res **51**: 2926-2931.

Santos N., Seruca R., et al. (1996). "Microsatellite instability at multiple loci in gastric carcinoma: Clinicopathologic implications and prognosis." Gastroenterology **110**: 38-44.

Sepulveda A., Shelton J., et al. (2000). "Predicting the risk of gastric cancer using H. pylori gastritis patterns associated with family history of gastric cancer." Am J Hum Genet **67**.

Shelly M., Pinkas-Kramarski R., et al. (1998). "Epiregulin Is a Potent Pan-ErbB Ligand That Preferentially Activates Heterodimeric Receptor Complexes." J. Biol. Chem. **273**: 10496-10505.

Shibata T., Kanai Y., Akimoto S,, Gotoh M,, Yasui N., Machinami R., Hirohashi S. (1996). "Dominant negative inhibition of the association between beta-catenin and c-erbB-2 by N-terminally deleted beta-catenin suppresses the invasion and metastasis of cancer cells." Oncogene **13**: 883-889.

Sirotnak F., Zakowski M., Miller V., Scher H., Kris M. (2000) Efficacy of cytotoxic agents against human tumour xenografts is markedly enhanced by coadministration of ZD1839 (Iressa), an inhibitor of EGFR tyrosine kinase. Clin Cancer Res. 12. 4885-92

Sitas F.and Forman D. (1992). "Helicobacter pylori infection rates in relation to age and social class in a population of Welsh men." Gut **33**: 1582.

Slieker L., Martensen T., et al. (1986). "Synthesis of epidermal growth factor receptor in human A431 cells. Glycosylation-dependent acquisition of ligand binding activity occurs post-translationally in the endoplasmic reticulum." J. Biol. Chem. **261**(32): 15233-15241.

Song Jae Kil C. (2000). "EGF receptor residues Leu (679) and Leu (680) mediate selective sorting of ligand-receptor complexes in early endosomal compartments." Journal of Cellular Physiology **185**: 47-60.

Sternlicht M., Sunnarborg S., Kouros-Mehr H., et al. (2005) “Mammary ductal morphogenesis requires paracrine activation of stromal EGFR via ADAM 17-dependent shedding of epithelial amphiregulin.” Development. **132**, 3923-3933.

Straus E., H. Patel, et al. (2002). "H. pylori Infection and Genotyping in Patients Undergoing Upper Endoscopy at Inner City Hospitals." Digestive Diseases and Sciences **47**: 1575-1581.

Suzuki T., Yokozaki H., Naka K., Ishikawa T., Tahara E. (1999). "Expression of the E2F family in human gastrointestinal carcinomas." Int J Cancer. **81**: 535-538.

Tahara E. (1993). "Molecular mechanism of stomach carcinogenesis. "J Cancer Res. Clin. Oncol. **119**: 265-272.

Tahara E. (2000). "Molecular aspects of invasion and metastasis of stomach cancer." Verh Dtsch Ges Pathol **84** (43-9).

Tahara E. (2004). “Genetic pathways of two types of gastric cancer.” IARC Sci Publ. pp. 327-349.

Tamura G. (1997). "Analysis of the fragile histidine triad gene in primary gastric carcinomas and gastric carcinoma cell lines." Genes, Chromosomes and Cancer **20**: 98-102.

Tamura G., Kihana T., et al. (1991). "Detection of Frequent p53 Gene Mutations in Primary Gastric Cancer by Cell Sorting and Polymerase Chain Reaction Single-Strand Conformation Polymorphism Analysis." Cancer Res **51**: 3056-3058.

Tateishi M., Ishida T., Kohdono S., et al. (1994) “Prognostic influence of the co-expression of epidermal growth factor receptor and c-erbB-2 protein in human lung adenocarcinoma.” Surg Oncol. **3**: 109-113.

Teruyoshi Ue H. (1998). "Co-expression of osteopontin and CD44v9 in gastric cancer." International Journal of Cancer **79**: 127-132.

Thibodeau S., French A., et al. (1996). "Altered Expression of hMSH2 and hMLH1 in Tumors with Microsatellite Instability and Genetic Alterations in Mismatch Repair Genes." Cancer Res **56**: 4836-4840.

Thiery J. (2003). "Epithelial-mesenchymal transitions in development and pathologies." Current Opinion in Cell Biology **15**: 740-746.

Tohdo H., Haruma K., Kajiyama G., Tahara E. (1993). "p53 gene mutations in gastric adenomas. ." Virchows Arch B Cell Pathol Incl Mol Pathol **63**: 191-195.

Tokunaga A., Onda M., Okuda T., Teramoto T., Fujita I., Mizutani T., Kiyama T., Yoshiyuki T., Nishi K., Matsukura N. (1995) “Clinical significance of epidermal growth factor (EGF), EGF receptor, and c-erbB-2 in human gastric cancer.” Cancer. **75**: 1418-1425.

Trelstad R. and Revel J. (1967). "Cell contact during early morphogenesis in the chick embryo." Dev Biol **16**: 78-106.

Tsao M., Sakurada A., Cutz J., Zhu C., et al. (2005). “Erlotinib in lung cancer-molecular and clinical predictors of outcome.” New Eng J Med. **353**: 133-144

Tsugawa K., Yonemura Y., Hirono Y., Fushida S., Kaji M., Miwa K., Miyazaki I., Yamamoto H. (1998). “Amplification of the c-met, c-erbB-2 and epidermal growth

factor receptor gene in human gastric cancers: correlation to clinical features." Oncology. **55**:475-81.

Ullrich A., Hayflick J., Dull T., Gray A., Tam A., Lee J., Yarden Y., Libermann T., Schlessinger J., et al. (1984). "Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells." Nature **309**: 418-425.

Van Roy F., Messiaen L., et al. (1986). "Invasiveness and Metastatic Capability of Rat Fibroblast-like Cells before and after Transfection with Immortalizing and Transforming Genes." Cancer Res **46**: 4787-4795.

Voldborg B., Spang-Thomsen L., Poulsen M., Skovgaard H. (1997). "Epidermal growth factor receptor (EGFR) and EGFR mutations, function and possible role in clinical trials." Ann Oncol **8**: 1197-1206.

Waugh M. and Hsuan J. (2001). "EGF receptors as transcription factors: ridiculous or sublime?" Nat Cell Biol **3**: E209-E211.

WCRF (1997). Diet, nutrition and the prevention of cancer: a global perspective. World Cancer Research Fund Panel. Washington, DC, World Cancer Research Fund.

Weber G., Glimcher M., Cantor H. (1996). " Receptor-ligand interaction between CD44 and osteopontin (Eta-1)." Science **271**: 509-512.

Weber W., Spiess J. (1984). "Production of an epidermal growth factor receptor-related protein." Science **224**: 294-7.

Wells A. and Marti U. (2002). "Signalling shortcuts: cell-surface receptors in the nucleus?" Nat Rev Mol Cell Biol **3**: 697-702.

Wikstrand C, Hale L., Batra S., Hill M., Humphrey P., Kurpad S., McLendon R., Moscatello D., Pegram C. (1995) "Monoclonal antibodies against EGFRvIII are tumour specific and react with breast and lung carcinomas and malignant gliomas." Cancer Res. **14:** 3140-8.

Wiley H. (2003). "Trafficking of the ErbB receptors and its influence on signaling." Experimental Cell Research **284**: 78-88.

Wittekind L., Ed. (1997). TNM. Classification of Malignant Tumours New York, Wiley.

Woolf C. (1961). "An analysis of 5 "stomach cancer families" in the state of Utah." Cancer Res **14**: 1005-1016.

Worthylake R., Opresko L., Wiley H. (1999). “ErbB-2 amplification inhibits down regulation and induces constitutive activation of both ErbB-2 and epidermal growth factor receptors.” J Biol Chem. **274**: 8865-8874.

Wright P. and Williams. G. (1993). "Molecular biology and gastric carcinoma." Gut **34**: 145-147.

Yamauchi T., Ueki K., et al. (1997). "Tyrosine phosphorylation of the EGF receptor by the kinase Jak2 is induced by growth hormone." Nature **390**: 91-96.

Yamauchi T., Yamauchi N., et al. (2000). "Constitutive Tyrosine Phosphorylation of ErbB-2 via Jak2 by Autocrine Secretion of Prolactin in Human Breast Cancer." J. Biol. Chem. **275**: 33937-33944.

Yan Y., Shirakabe K., Werb Z. (2002). “The metalloprotease Kuzbanian (ADAM 10) mediates the transactivation of EGF receptor by G-protein-coupled receptors.” J. Cell Biol. **158**, 221-226.

Yarden Y. (1987). "Self-phosphorylation of epidermal growth factor receptor: evidence for a model of intermolecular allosteric activation." Biochemistry **10**: 1434-42.

Yarden Y. and Sliwkowski M. (2001). "Untangling the ErbB signalling network." Nat Rev Mol Cell Biol **2**: 127-137.

Yasmeen A., Bismar T., et al. (2006). "ErbB receptors and epithelial-cadherin -β catenin complex in human carcinomas." Future Oncology **2**: 765-781.

Yasui W., Semba S., Yokozaki H., Tahara E. (1997). "Reduced expression of cyclin-dependent kinase inhibitor p27Kip1 is associated with advanced stage and invasiveness of gastric carcinomas." Jpn J Cancer Res **88**: 625-629.

Yasui W., Sumiyoshi H., Hata J., et al. (1988). “Expression of epidermal growth factor receptor in human gastric and colonic carcinoma.” Can Res. **48**: 137-141.

Yasui W., Tahara E., Fujimoto J., Nakayama J., Ishikawa F., Ide T., Tahara E. (1998). "Expression of telomerase catalytic component, telomerase reverse transcriptase, in human gastric carcinomas." Jpn J Cancer Res **89**: 1099-1103.

Yasui W., Yokozaki H., Nakatani H., Ochiai A., Ito H., Tahara E. (1988). "Interaction between epidermal growth factor and its receptor in progression of human gastric carcinoma." Int J Cancer **41**: 211-217.

Yokota J., Miyajima N., Toyoshima K., Nomura N., Sakamoto H., Yoshida T., Terada M., Sugimura T. (1988). "Genetic alterations of the c-erbB-2 oncogene occur frequently in tubular adenocarcinoma of the stomach and are often accompanied by amplifi cation of the v-erbA homologue." Oncogene **2**: 283-287.

Yokozaki H., Kitadai Y., Nishimura K., Todo H., Ayhan A., Yasui W., Ito H., Tahara E. (1992). "p53 point mutations in primary human gastric carcinomas. ." J Cancer Res Clin Oncol **119**: 67-70.

Yokozaki H., Nakayama H., Kuniyasu H., Taniyama K., Tahara E. (1994). "Expression of CD44 abnormal transcripts in human gastric carcinomas." Cancer Lett **83**: 229-234.

Yonemura Y., Sugiyama K., Nimomiya I., et al. (1993) “Evidence of autocrine mechanism in poorlu differentiated adenocarcinoma of the stomach.”Int J Oncol. 2_ 643-648

Zajchowski D., Bartholdi M., et al. (2001). "Identification of Gene Expression Profiles That Predict the Aggressive Behavior of Breast Cancer Cells." Cancer Res **61**: 5168-5178.

Zwick E., Hackel P., et al. (1999). "The EGF receptor as central transducer of heterologous signalling systems." Trends in Pharmacological Sciences **20**: 408-412.

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http://hybridmedicalanimation.com

http://international.abbotmolecular.com

http://www.ensemble.org

http://www.nature.cim

http://www.peptic-ulcers.co.uk