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Murine Double Minute 2 characterization in Epstein-Barr Virus associated Gastric Cancers

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Murine Double Minute 2 characterization in Epstein-Barr Virus associated Gastric Cancers

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PREFACE

This study was performed at Molecular Oncology & Viral Pathology Group of the Portuguese Oncology Institute of Porto (IPO-Porto), under the supervision of Professor Hugo Sousa and Professor Rui Medeiros.

Part of this study was submitted for publication in an international peer-reviewed journal and the results were partially presented as poster at the ESCV 2019 International Conference (11-14$_{th}$ September 2019, Copenhagen, Denmark).

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RESUMO

Introdução: O cancro gástrico tem sido associado ao Vírus Epstein-Barr (EBVaGC) em cerca de 10% de todos os cancros gástricos e é caracterizado por um perfil molecular distinto. Recentemente, foi demonstrado que EBVaGC não contém mutações no *TP53*, apesar disso, pode ser observada uma acumulação de p53 nestes tumores. No presente estudo temos como objetivo analisar o potencial papel da proteína mdm2, o principal regulador negativo da p53, como explicação para a desregulação da p53 em EBVaGC. A regulação dos níveis de mdm2 tem sido associada a um comum polimorfismo de alteração de um único nucleótido (SNP), que aparenta estar correlacionado com um aumento de risco para o desenvolvimento de cancro gástrico.

Métodos: Fizemos uma revisão da literatura no que diz respeito ao impacto de SNPs em cancro gástrico, através de uma revisão sistemática, utilizando as normas PRISMA e um *software* de análise de risco específico. Além disso, foram utilizadas amostras de tumores coletadas de blocos de tecido fixado em formalina e embebidos em parafina (FFPE), de pacientes do IPO-Porto com cancro gástrico positivo (EBVaGC, n=12) e negativo (EBVnGC, n=27) para VEB. A expressão de *MDM2* foi obtida através de PCR em tempo real. O *status* da proteína no tecido foi analisado através de imunohistoquímica (IHC), utilizando um anticorpo monoclonal específico. O polimorfismo SNP309 do *MDM2* foi genótipado por PCR, seguido de análise do comprimento de fragmentos de restrição.

Resultados: A revisão sistemática revelou um total de 11 estudos, que incluem três polimorfismos: rs937283 (n=1), rs3730485 (n=1) e rs2279744 (n=9). A meta-análise só foi possível de ser realizada para os estudos de rs2279744, em que o alelo G é associado a um aumento de risco para o desenvolvimento de cancro gástrico (modelo dominante, OR=1,45, p<0,001; ou modelo recessivo, OR=1,73, p<0,001). Relativamente ao trabalho prático, IHC demonstraram que a mdm2 está presente em 5/12 dos EBVaGC e 10/27 dos EBVnGC (p=1,000), com 80% dos EBVaGC com uma expressão de mdm2 em mais de 50% das células, comparando com 20% nos EBVnGC (p=0,089). Foram encontradas diferenças estatisticamente significativas quando considerados os casos com expressão acima de 50%, comparado EBVaGC com EBVnGC do tipo difuso (p=0,048). Quando realizada a análise combinada da expressão de mdm2 com a de p53, observou-se que, nos casos com expressão de mdm2, é mais comum a alta expressão de p53. No que diz respeito aos níveis de mRNA de *MDM2*, foi visto um aumento significativo de

expressão nos EBVaGC, quando comparado com os EBVnGC do tipo intestinal (p=0,034) e uma tendência para sobrexpressão quando comparado com o tipo indeterminado (p=0,057). Quando comparada a percentagem de casos com expressão de mRNA detetada entre casos positivo e negativos para mdm2 por IHC, verificou-se que a expressão de mRNA parece não estar relacionada com a acumulação de proteína. A genótipagem do SNP309 do *MDM2* não revelou qualquer diferença significativa na frequência alélica quando comparado os casos positivos e negativos para VEB (p=0,577). Quando comparámos os níveis de mRNA de *MDM2* de acordo com o genótipo do SNP309, observámos que, em EBVaGC, o genótipo TT aparenta uma maior expressão do gene.

Conclusão: O nosso estudo demonstrou que a mdm2 pode ser um importante marcador para EBVaGC, o que, nestes casos, é refletido numa maior acumulação de proteína no tecido. Além disso, isto é provavelmente explicado por um aumento da transcrição, devido a alguma proteína viral ou miRNA que pode atuar em concordância com a presença de SNPs específicos. Ainda, o aumento de mdm2 não é concordante com a acumulação de p53, indicando desregulação no balanço entre mdm2 e p53.

PALAVRAS-CHAVE: Cancro gástrico, Vírus Epstein-Barr, cancro gástrico associado a VEB, carcinogénese, Murine Double Minute 2, polimorfismo, SNP309, p53.

ABSTRACT

Background: Gastric cancer has been associated with Epstein-Barr Virus (EBVaGC) in 10% of all gastric cancers and is characterized by a distinct molecular profile. Recently it has been shown that in EBVaGC there are no *TP53* mutations, nevertheless it may be observed accumulation of wildtype p53 in these tumors. In this study we aimed to analyze the potential role of mdm2 protein, the major p53 negative regulator, as the explanation for the p53 dysregulation in EBVaGC. The regulation of *MDM2* levels has been associated with a common single nucleotide polymorphism (SNP), which seems to be correlated with increased risk of cancers development.

Methods: We have reviewed the literature concerning the impact of SNPs in gastric cancer by performing a systematic review using PRISMA guidelines and specific software for the analysis of risk-association. Furthermore, we used tumor samples collected from formalin-fixed paraffin-embedded (FFPE) tissue blocks collected from IPO-Porto patients with EBV-positive (EBVaGC, n=12) and EBV-negative (EBVnGC, n=28) gastric cancers. *MDM2* expression levels was assessed by two-step real-time PCR. Protein status in the tissue was analyzed by imunohistochemistry (IHC), using a specific monoclonal antibody. *MDM2* SNP309 polymorphism was genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

Results: The systematic review revealed a total of 11 manuscripts which include three different polymorphisms: rs937283 (n=1), rs3730485 (n=1) and rs2279744 (n=9). A meta-analysis was only possible for rs2279744 studies, with the G allele associated with increased risk of gastric cancer development (dominant model, OR=1.45, p<0.001; or recessive model, OR=1.73, p<0.001).

IHC demonstrated that mdm2 is present in 5/12 EBVaGC and 10/27 EBVnGC (p=1.000), with 80% of EBVaGC showing expression in the majority of cells, compared with 20% in EBVnGC (p=0.089). A significative difference was found when compared high mdm2 expression in EBVaGC and EBVnGC diffuse histological subtype (p=0.048). When we combined mdm2 and p53 expression, we observed that in cases with mdm2 expression, high p53 expression is more common. Regarding the *MDM2* mRNA levels, a significative increased expression was observed in EBVaGC, when compared with EBVnGC intestinal type (p=0.034) and a trend when compared to EBVnGC indeterminate type (p=0.057). The

comparison of percentage of cases expression *MDM2* mRNA among cases positive and negative for mdm2 IHC showed that the mRNA expression do not seem to be associated with the mdm2 accumulation. *MDM2* SNP309 genotyping revealed no significant differences in the allele frequency between EBVaGC and EBVnGC (p=0.577). When we compared the level of *MDM2* mRNA according to SNP309 genotype, we observed that in EBVaGC the TT genotype seems to lead to higher gene expression.

Conclusion: Our study shows that mdm2 may be an important marker for EBVaGC, which is reflected in the higher accumulation in tissue, in these cases. Furthermore, this is probably explained by the increased transcriptional activity, enhanced by some viral protein or miRNA that may act in accordance to the presence of specific SNPs. Also, mdm2 increase does not impair with p53 accumulation, indicating that there is some dysregulation in the p53-mdm2 balance.

KEY WORDS: Gastric cancer, Epstein-Barr virus, EBV associated gastric cancer, carcinogenesis, Murine Double Minute 2, polymorphism, SNP309, p53.

ABREVIATION LIST

A

Apaf -1 – Apoptotic protease activating factor 1

B

BART - BamHI-A Rightward Transcript

BER – Base Excision Repair

bp- Base pairs

β2M – Beta-2-Microglobulin

C

CA - Conventional-Type Adenocarcinoma

CD1 – Cyclin D1

CD2 – Cyclin D2

CD21- Cluster of Differentiation 21

CDK - Cyclin-Dependent Kinase

Cdki - Cyclin-Dependent Kinase Inhibitor

CDKN1A – Cyclin Dependent Kinase Inhibitor 1A

CDX2 - Homeobox protein CDX-2

CI – Confidence Interval

CIMP - Cpg Island Methylator Phenotype

CIN - Tumors with Chromosomal Instability

CLR - Crohn's disease-like Lymphoid Reaction

D

DBD – DNA Binding Domain

Dbd2 – Damage-specific DNA binding-protein 2

DNA – Deoxyribonucleic Acid

E

EBER – Epstein-Barr encoded small RNAs

EBER-ISH – In Situ Hybridization for detection of EBER

EBF3 - Early B-cell Factor-3

EBNA – Epstein-Barr Nuclear Antigen

EBV – Epstein-Barr Virus

EBV1 – Epstein-Barr Virus type 1
EBV2 – Epstein-Barr Virus type 2
EBVaGC – Epstein-Barr Virus associated Gastric Cancer
EBVnGC - Epstein-Barr Virus non-associated Gastric Cancer
EGFR - Epidermal Growth Factor Receptor

F
Fancc – Fanconi anemia, complementary group C
FAP - Familial Adenomatous Polyposis
FFPE – Formalin-Fixed Parafin-Embedded

G
GC – Gastric Cancer
GERD - Gastro Esophageal Reflux Disease
gp – Glicoprotein
GS - Genomically Stable Tumors

H
H. Pylori – Helicobacter Pylori
HDCA1 – Histone Deacetylase 1
HDGC - Hereditary Diffuse Gastric Cancer
HER2 - Human Epidermal growth factor Receptor 2
HR – Homologous Repair
HWE – Hardy-Weinberg Equilibrium

I
IARC – International Agency for Research on Cancer
IHC - Immunohistochemistry
IM – Infectious mononucleosis

L
LELC - Lymphoepithelioma-like carcinoma
LMP - Latent Membrane Protein
lncRNA – Long coding RNA

M
MDM2 - Murine Double Minute 2

MDM4 – Murine Double Minute 4
MDMX – Murine Double Minute X
MHC – Major Histocompatibility Complex
miRNA – Micro RNA
miR - Micro RNA
MMR – Mismatch Repair
mRNA - Messenger RNA
MSI - Microsatellite Unstable Tumors
MeSH – Medical Subject Headings

N
NES – Nuclear Export signal
NHEJ – Non-homologous End-joining
NLS – Nuclear Localization Signal
NoLs – Nucleolar Localization Signal
NPC – Nasopharegeal Carcinoma

O
OncomiR – Oncogenic micro RNA
OR – Odds Ratio
ORF – Open Reading Frame

P
PCHD10 - Protochaderin 10
PCR – Polymerase Chain Reaction
PCR-RFLP – PCR followed by Restriction Fragment Length Polymorphism
PD-L - Programmed Death Ligands
PI3K – Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIK3CA - Pphosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PJS - Peutz-Jeghers Syndrome
pRB - Retinoblastoma protein
PRISMA – Preferred Reporting Items for Systematic Reviews and Meta-Analyses
PTEN – Phosphatidylinositol 3,4,5-triphosphate 3
PML – Promyelocytic Leukemia protein
R
RNA – Ribonucleic Acid
ROS – Reactive Oxygen Species

RPL – Ribosomal Protein L

S
SNP – Single Nucleotide Polymorphism
Sp1 - Specificity protein 1

T
TAD – Transactivating Domain
TCGA - The Cancer Genome Atlas
TET – Tetramerization domain
TP53 – Tumor Protein 53

U
USP7 – Ubiquitin-specific-processing protease 7
UTR – Unstraslated Region
UV – Ultra Violet

W
WHO - World Health Organization
Wt - Wild-type

X
Xpc – Xerodema pigmentosum, complementation group C

Z
ZREs – Z-response elements

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I. INTRODUCTION

1. GASTRIC CANCER

1.1. Epidemiology and etiology

Gastric cancer (GC) is the 5th most common cancer, affecting more than 1,000,000 people per year and leads to approximately 783,000 deaths each year, corresponding to 5.7% of new cases and 8.2% of all cancer related deaths – Figure 1 and 2. It is twice more prevalent in men and has the highest incidence rates in Eastern Asia and Central and Eastern Europe and the lower in Africa and Northern Europe and America(1).

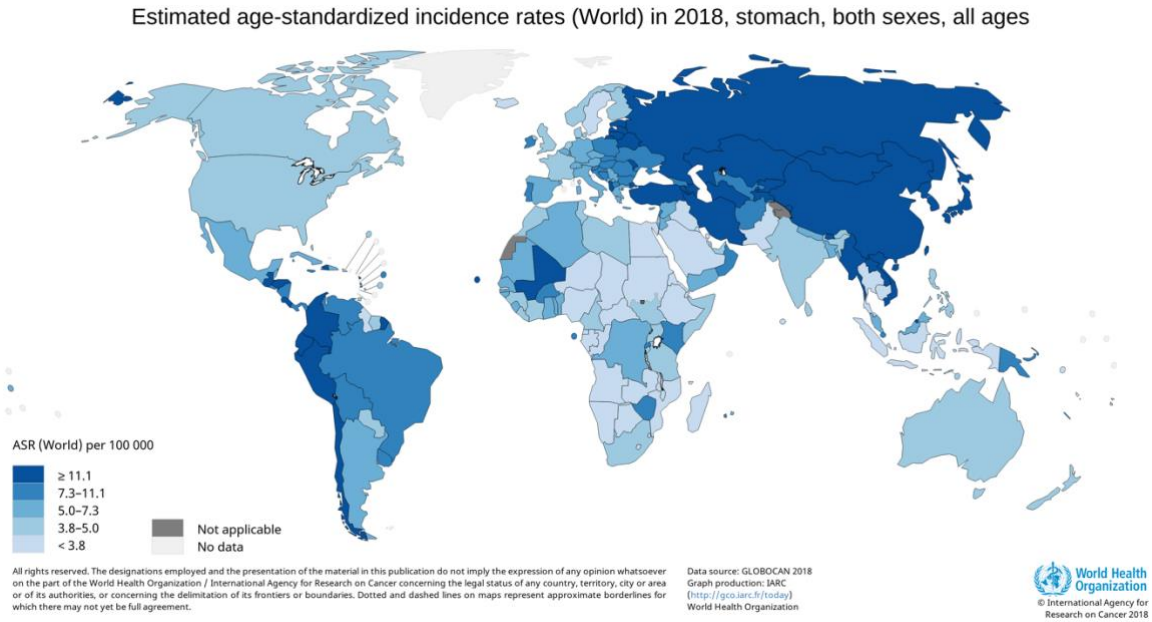


Figure 1 – Gastric cancer Age-Standardized Rate incidence worldwide, in both genders (GLOBOCAN 2018).

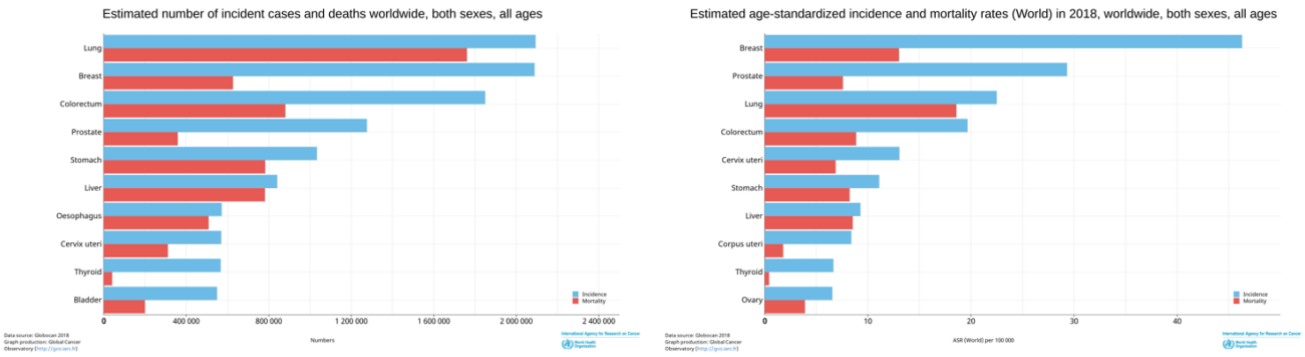
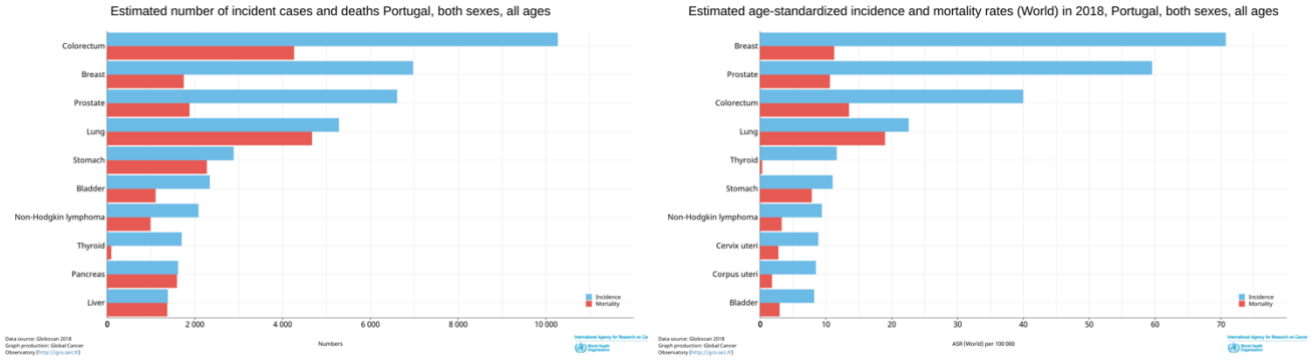


Figure 2 – Estimated number a) of incident cases and deaths of gastric cancer worldwide; and b)Age-standardized Rates (GLOBOCAN 2018).

In Portugal, we had about 2,885 new cases and 2,275 deaths per year due to gastric cancer, only in 2018 (1). GC was the fourth more common cancer in both genders, with 2885 new cases and a age-standardized mortality rate of 7.9 deaths per 100 000 cases - Figure 3. Both the incidence and the mortality are higher in males than females, with a distinct geographical distribution, being especially higher in two districts of the North of the country (2, 3).

Figure 3 – Estimated number a) of incident cases and deaths of gastric cancer in Portugal;



and b) Age-standardized Rates (GLOBOCAN 2018).

Worldwide, GC incidence has a distinct geographic distribution pattern as is the case of Portugal (2). In USA, ethnic/racial disparities have been consistently described, which can partially be explained by the immigration effect but it does not explain it entirely since high rates have been also observed in indigenous populations (4). It has been shown that when migrants from regions with *Helicobacter pylori* (*H. Pylori*) high incidence relocate to low incidence regions, maintain the risk as of their country of origin, while the descendance tend to have lower risk of GC development (5).

The stomach is divided into different anatomic sites, such as cardia, fundus, body, antrum and the pylorus and it has been discussed the potential differential role of the anatomic sites in cancer development. The major distinction considers cancers originated in the cardia (cardia GC) and in other anatomical sites (non-cardia GC) and these two subgroups seem to have distinct epidemiological patterns and etiologies (6). Globally and in East/Central Asia and Eastern Europe, non-cardia GC is the predominant type, which is not seen in North America and Western Europe (4). The fact that GC incidence, and consequently the mortality, is declining in the majority of the world is mostly coincident with economic improvements, resulting in non-cardia gastric cancer decrease due to food storing improvements and *H. Pylori* decrease (1, 4, 5). Regarding cardia gastric cancer, the incidence has been stable or increasing, at least in Western countries (1, 7).

Regarding risk factors, non-cardia GC has been associated with *H. pylori* infection, low socioeconomic status and dietary habits, such as high salt intake and smoked foods consumption and low consumption of fruits and vegetables; while for cardia GC, the most frequent risk factors associated are obesity and gastro-esophageal reflux disease (GERD) (6). There are some common risk factors including aging, smoking, gender (male), familiar history, lack of physical activity and exposure to radiation (6). Inherited syndromes, such as hereditary diffuse gastric cancer (HDGC), familial adenomatous polyposis (FAP), and Peutz-Jeghers syndrome (PJS), may also contribute to GC development in 1-3% of the cases (6). Literature also suggests that host genetic polymorphisms may contribute to an increased risk pattern for GC development (8) – Figure 4.

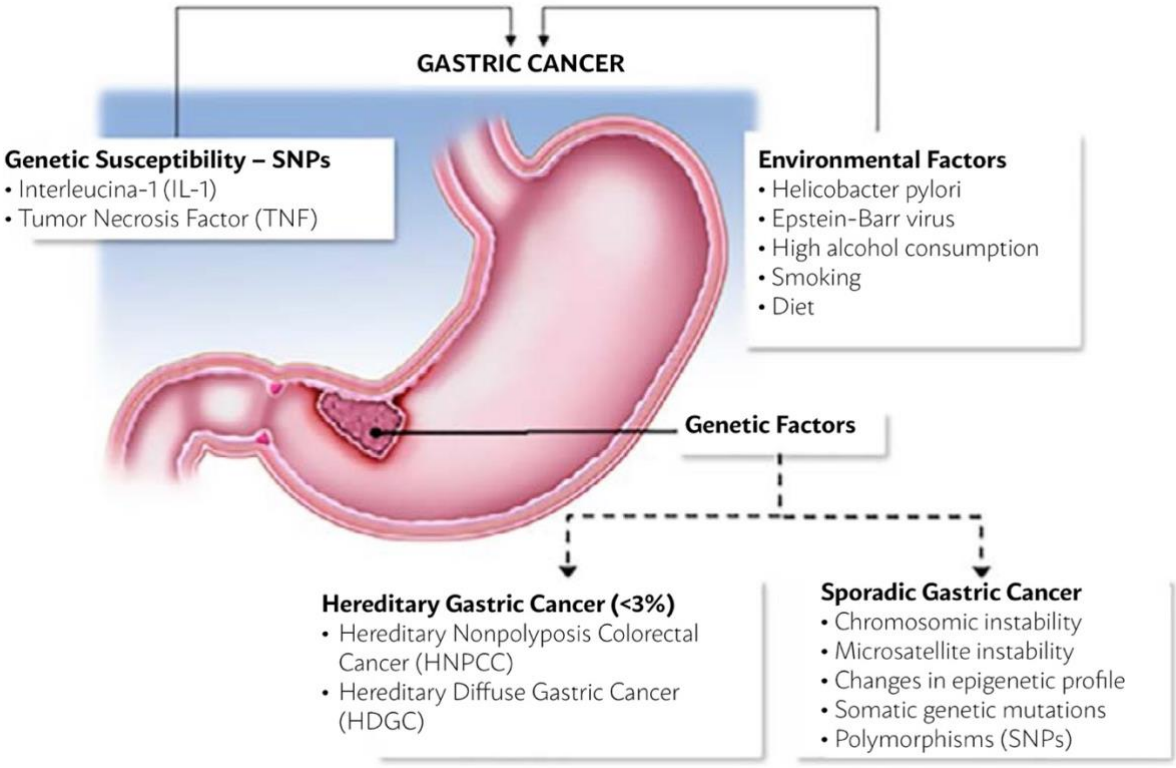


Figure 4 – Risk factors for gastric cancer development (From Ramos MFKP *et al*. 2018).

1.2. Classification

Gastric cancer has been classified according to its anatomical, histological or molecular features (9). The anatomical classification was for many years considered important since the two types (cardia and non-cardia GC) have distinct etiological and epidemiological characteristics (1, 4, 9). The two main histological classification systems used either for diagnosis and treatment decision, are the *World Health Organization* (WHO) and the *Lauren classification*, nevertheless the impact on the therapeutic and clinical outcome is very little (10, 11).

The Lauren classification was created in 1965 and divides gastric adenocarcinomas into diffuse, which includes the signet ring cell gastric adenocarcinoma variant, and intestinal type carcinomas, with distinct cell structure, secretion, growth and clinical features (12). Later, the classification included an intermediate type that combined cancers with uncommon histology(11). Among these histological subtypes some morphological characteristics can be highlighted. The specific characteristics of the intestinal type are: intracellular junctions, larger cells, morphologically more variable, and also, has well to moderate differentiation and forms glandular structures, usually associated with the loss of E-cadherin expression; while the diffuse type: does not have intracellular junctions, is poorly differentiated and the tumor cells are solitary and poorly cohesive, with no gland formation and, in the case of the signet ring cell gastric adenocarcinoma, contains abundant cytoplasmic mucin that displaces the nucleus towards the periphery which does not happen in the intestinal type (9, 12).

There are multiple evidences that indicate that the two principal subtypes may have distinct tumor development pathways (11, 13). The diffuse type is more aggressive and has worse prognosis, in part because it shows more characteristics of malignant tumor, such as cellular atypia and numerous mitotic figures, comparing with the intestinal type (12, 13); while the intestinal type is associated with *H. Pylori* infection, and a model of development characterized by chronic gastritis and gastric mucosa metaplasia (11). Moreover, intestinal type carcinoma has an abundant inflammatory cell infiltration that tends to ulcerate while the diffuse type tend to spread inside the mucosa (13).

The WHO classification is based on the predominant histological pattern, dividing GC in multiple subtypes: papillary, tubular and mucinous adenocarcinomas, poorly cohesive, signet-ring cell and mixed carcinomas and some uncommon variants (14, 15). Epidemiologically, the tubular subtype is the most common,

followed by papillary and mucinous. Regarding prognosis, poorly differentiated and mucinous subtypes have worst prognosis and within the most common subtypes, the papillary has a poor prognosis and a higher tendency to metastatic disease (11). There is some correspondence between the Lauren´s and WHO classifications with the tubular and papillary subtypes of WHO classification to match the intestinal type; the mucinous, signet-ring cell and poorly cohesive carcinomas matching the diffuse type; and the mixed carcinomas to the indeterminate type – Table 1 (11, 15).

Table 1- Comparison of Lauren's and WHO classification systems

Lauren's Classification	**WHO classification**
Intestinal Type	Tubular adenocarcinoma
	Papillary adenocarcinoma
Diffuse Type	Mucinous adenocarcinoma
	Signet-ring cell carcinoma
	Poorly cohesive carcinoma
Indeterminate	Mixed Carcinoma
	Uncommon variants

More recently, in 2014, *The Cancer Genome Atlas* (TCGA) consortium group described a classification of gastric adenocarcinomas into 4 distinct subtypes: 1) microsatellite unstable tumors (MSI); 2) genomically stable tumors (GS); 3) tumors with chromosomal instability (CIN); and 4) tumors positive for Epstein-Barr Virus (EBVaGC) (10) – Figure 5. This classification is based on the hypothesis that molecular features may have a higher clinical impact, comparing with the classical classifications, in terms of treatment prediction and patient prognosis (15). The classification takes in account three parameters: 1) the high burden of EBV and extensive DNA promoter hypermethylation, distinguishing the EBVaGC; 2) the MSI subtype shows microsatellite instability and elevated mutation rates and hypermethylation, nevertheless the extreme CpG island methylator phenotype (CIMP) is distinct among these two subgroups; and 3) the presence or not of extensive somatic copy-number aberrations characterizes the CIN and GS subtypes, respectively (10). Globally, EBVaGC accounts for approximately 9% of the gastric adenocarcinomas, MSI for 22%, GS for 20% and CIN for 50% (9).

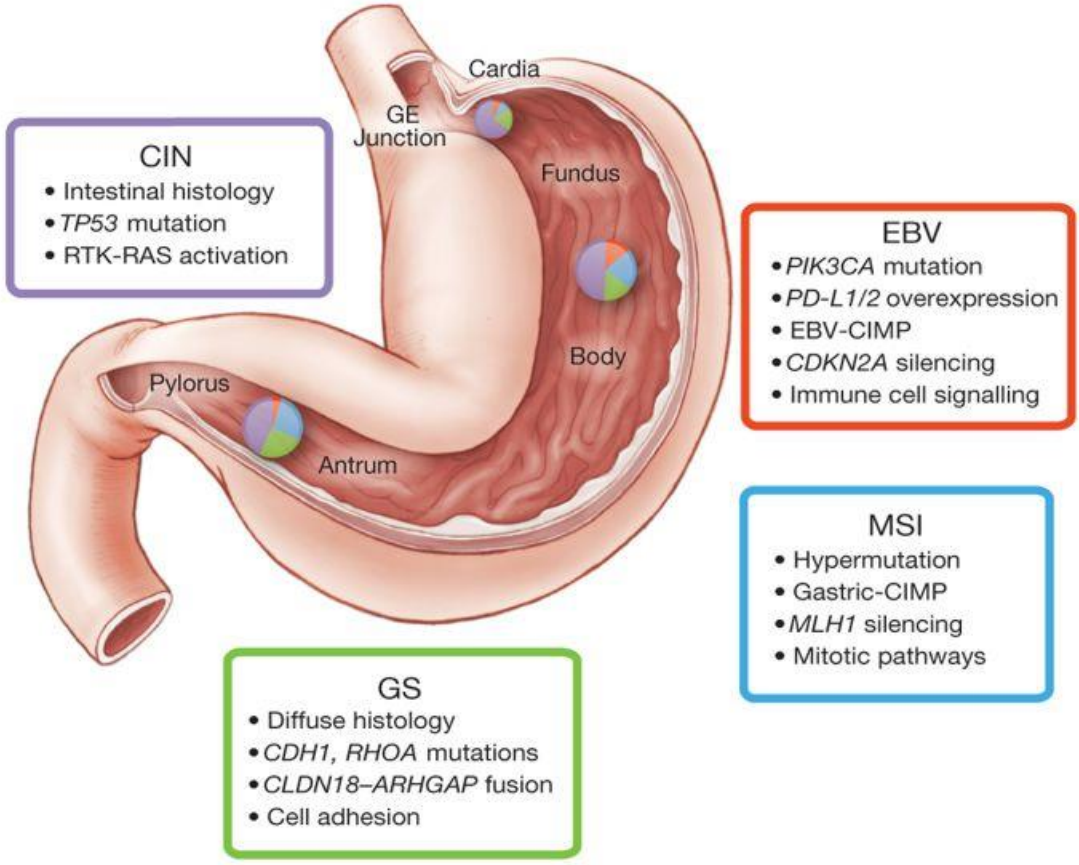


Figure 5 - Essential features of gastric cancer subtypes, according to the Cancer Genome Atlas Research Network, 2014.

1.3. Gastric carcinogenesis

Gastric cells malignant transformation is a multistep process in which genetic or epigenetic alterations can be observed, leading to the dysregulation of essential cell biological process, such as cell cycle regulation and apoptosis (16, 17). Both genetic mutations and/or epigenetic alterations lead to protein gain (oncogenes) or loss of their function (tumor suppressor). Epigenetic changes are mainly due to promoter hypermethylation and, in GC cases, can be increased due to *H. Pylori* infection or the infection-induced inflammation (16).

There are several proliferation-associated proteins described as being altered in GC, such as Her2 receptor, CDX2, early B-cell factor-3 (EBF3) and Protochaderin 10 (PCHD10) (18-20). The Her2 receptor belongs to the Epidermal Growth Factor Receptor (EGFR) which activates cell proliferation through spontaneous homo or heterodimerization with other EGFR family receptors (21). This receptor is not only frequently found mutated in GC, as is amplified and highly expressed in 6 to 30%

of all cases, especially in the intestinal type (19). CDX2, an homeobox protein, another frequently mutated gene in GC, has been described as being able to increase proliferation and differentiation of the intestinal type cells of the gastric epithelium. It also inhibits growth by activating a cyclin-dependent kinase inhibitor, WAF1, possibly acting as a tumor suppressor (19). This hypothesis is supported by the fact that the expression level is decreased in early onset of gastric cancer and it is the lowest in advanced GC (19). PCHD10, a member of the protocadherin family, is found epigenetically downregulated or even silenced in over twice the gastric tumors, when compared with non-tumor tissues (18). This protein inhibits cell proliferation and induces cell apoptosis through different pro-apoptotic genes, such as Fas and caspase 8 (16). EBF3 is detected in about 40% of GC but not in normal mucosa (20). It leads to cell progression and apoptosis, by upregulation of p21 and p27, and repression on cyclin D1 (CD1), Cyclin D2 (CD2), Rb and CDK2, being involved in both apoptosis and cell cycle regulation (22).

Regarding direct regulators of the cell cycle, when altered they have a significant impact in the GC carcinogenesis. Among them we can highlight p53, CD1, CD2, the retinoblastoma protein (pRb), its main target, p16, C-MYC and some cyclin-dependent kinases inhibitors (CDKI), such as p27 and p21 (16, 23, 24). Tumor protein 53 (*TP53)*, is a tumor suppressor frequently altered in cancer and in about 25-40% of GC, by both genetic and epigenetic alterations (25, 26). p21 expression decreases along the GC carcinogenesis progression due to epigenetic changes, showing its role as a tumor suppressor. This CDKI has the capacity to inhibit all cyclin/cyclin-dependent kinases (CDK) complexes and is p53 main target (19). Reports show that CD1 and pRb are overexpressed in carcinomas, when compared with nonneoplastic mucosa, indicating that its expression may be detected in early stages of carcinogenesis (27). CD1 and CD2 are overexpressed in over 30% of GC (28). Another CDKI with an important role in cell cycle control, slowing it down or even stopping it, is the p27 protein, associated with advanced gastric adenocarcinoma (29). The C-MYC transcription factor is frequently upregulated or mutated in several cancer types. It regulates several genes related with proliferation, differentiation and apoptosis and is overexpressed in 40% of the GC cases (24).

Besides genetic and epigenetic changes, there is rising evidence that micro RNAs (miRNAs) are also responsible for gene downregulation or silencing leading to cancer development (30). miRNAs are 19-25 nucleotides long non-coding RNAs, expressed in a tissue-specific manner, that have the ability to biding to the 3'UTR

of specific mRiNAs and inhibit its translation, in response to intrinsic and extrinsic stress (30-32). In cancer, miRNAs can be divided into two major groups according to their function, oncomiRs and tumor suppressor miRs. OncomiRs target tumoral suppressor genes, acting as an oncogene, while tumor suppressor miRs have the opposite behavior, downregulating oncogenes (33). There is evidence that points to the dysregulation of several miRNAs in gastric cancer, such as the miR-106b-25 (31), miR-222-2221(31) and miR21(30, 32) oncomiRs and tumor suppressor miRs, such as miR-145, miR-133a, miR-133b, miR-93, miR-106b (30, 34).

2. EPSTEIN-BARR VIRUS AND GASTRIC CANCER

2.1. Historical background

Since the report in 1911, by Peyton Rous, that a "filterable agent" from extracts collected from chicken that had a tumor were able to transmit the tumor when injected in wealthy chickens, that is a known fact that viruses can be involved in cancer. Since then more viruses were associated with these diseases (35).

The Epstein-Barr Virus (36), was observed in cancer in 1964, when Denis Parsons Burkitt, the surgeon who first described Burkitt's lymphoma, had the chance to meet Anthony Epstein and they observed the unmistakable icosahedral structure of a previously undescribed herpesvirus by micrographs (37, 38). Later, EBV was considered the first virus to be directly associated with human cancers and since 1997, included by the *International Agency for Research on Cancer* (39) in the Group-I carcinogen risk factors (40, 41).

2.2. EBV characteristics

EBV is the most well-studied gamma herpesvirus and reports describe it as present in more than 90% of the world population due its easy oral transition through saliva (42, 43). The age of primary infection has geographical variances, mostly due to socioeconomic factors (39). It usually happens in the early childhood and is often asymptomatic (44). Despite the first infection occuring early in life it establishes a latent infection, mostly in B-lymphocytes (35, 44). The latent infection allows the virus to be maintained throughout the host life without causing any symptoms (39). When the infection happens later in life, as in the adolescence or early adulthood, it is responsible for the development of infectious mononucleosis (IM) (35, 39).

EBV, also known as herpesvirus 4, has a 168 to 184kbp double-stranded DNA that encodes over 85 genes wrapped in a toroid-shaped protein core (35). Also, its capsid is constituted by 162 capsomeres and has proteins spikes in its outer envelope (39).

EBV has two different types, EBV type-1 (EBV1) and EBV type-2 (EBV2), distinguished essentially by differences found in the coding sequence of some viral genes (EBNA2, 3A/3, 3B/4 and 3C/5) (35, 45). Besides that, EBV1 immortalizes B lymphocytes cell lines more efficiently and EBV2 leads to more viable EBV-infected lymphoblastoid cell lines (35, 46). This difference seems to be explained by the differences found in EBNA2 coding sequence (46). Furthermore,

both EBV types can be subdivided into different strains due to different polymorphisms that are found in different or even in certain geographical areas (35).

Despite these facts, there is still not enough evidence if there is any disease/population-specific type/strain (39). There are authors that affirm that the EBV type is associated with the geographical area and some say that the EBV type which someone is infected with leads to different malignancies (45). EBV2 is more common in Africa, New Guinea, Alaska Eskimos, homosexual men and immunosuppressed patients, while EBV1 is more common in Western Countries (46, 47).

2.3. EBV life cycle

EBV infects B lymphocytes by binding to the host cells through the lymphocytes CD21 and the viral envelope glycoprotein gp350/220, followed by endocytosis - Figure 6 (48). This process requires the involvement of three other viral proteins, gp25, gp45 and gp85. Gp42 also acts as a co-factor in virus to cell binding since it can also bind to the B lymphocytes major histocompatibility complex (MHC) class II (39). EBV also has tropism for epithelial cells but it shows a different behavior on them, entering into lytic replication (35). Not only the two cell types lead to a different behavior but also have different infection mechanisms. The mechanism used for epithelial cells infection is not well understood, since this cell type does not express CD21, though the infection of these cell types is essential for viral replication (39, 48).

Upon EBV infection via saliva, the virus enters the epithelium in the oropharynx where the viral load is increased by lytic replication. Then the B naïve cells infection occurs in the lymphoid tissues underlying the oropharynx and become activated lymphoblasts. This latency allows cells to migrate to the follicle and a germinal center of reaction and a latency change takes place (III to II). Next, the B cells exit the germinal center as memory B cells and a latency 0 is stablished as the B cells become resting. The normal cell homeostasis maintains them in this state until new reactivation, when they undergo plasma-cell differentiation and the viral replication is triggered (39).

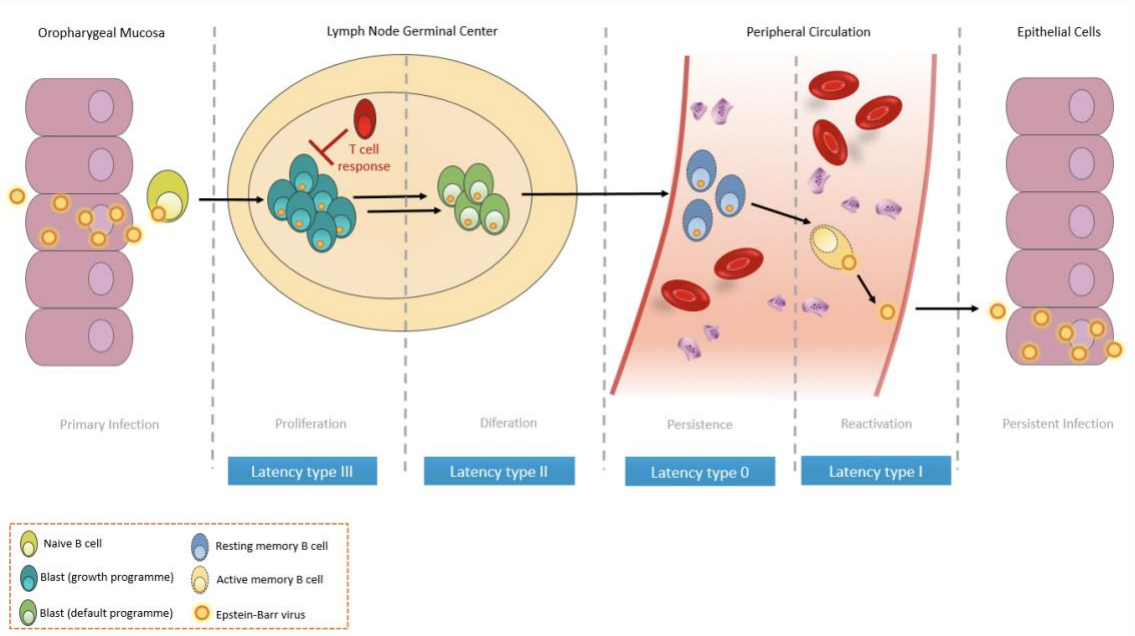


Figure 6 – EBV infection mechanism (From Malta M., 2016).

The lytic cycle occurs periodically to ensure the existence of new viral particles to maintain the pool of infected cells and allow the dissemination into new hosts. This process is observed in terminally differentiated and epithelial cells (49). To initiate the expression of the immediate early genes, BZLF1 and BRLF1 are necessary. They bind and activate promoters containing Z-response elements (ZREs) and enhance the transcription by binding to DNA GC-rich regions, respectively. This leads to a second phase of gene expression, some lytic-associated viral genes - the early lytic genes. Among them there are the ones necessary for viral DNA synthesis, immune invasion and apoptosis inhibition. After viral DNA replication, the late lytic genes are expressed, leading to the production of structural and packing viral elements (37).

At the same time, it is necessary that the virus expresses proteins associated to cell death, allowing the cell to burst and release the new viral particles. This has to happen at the right time, if it happens too soon, the viral progeny will not be properly synthetized and packed. This is possible due to the fact that the viral lytic genes have both pro- and anti-apoptotic features. For example, BZLF1 indirectly induces cell death and BHRF1, the viral Bcl-2 protein, indirectly blocks the previous toxicity (37).

2.4. EBV Latency Profiles

During latent infection, the virus' genome turns into an episome, allowing it to be maintained in a constant number in cells and to use the host machinery to express their own genes (35, 47). In this phase of the latent cycle, some latent genes are expressed such as EBV nuclear antigens (EBNAs 1, 2, 3A, 3B, 3C and EBNA-LP), EBV-encoded small RNAs (EBERs) 1 and 2, latent membrane protein (LMP 1, 2A and 2B) and 40 microRNAs from BamHI-A rightward transcripts, known as BARTs (41). Different protein combinations lead to different latencies and associated malignancies, since these proteins can influence cellular transformation and are essential for cancer initiation and progression (35, 47) - Table 2.

Depending on the infected cell type, differentiation and activation status, different EBV-encoded proteins are expressed, originating different latency profiles. Some of these proteins, such as EBNA1, EBNA 2, LMP1, LMP2, EBERs and BART are oncogenic, being able to lead to different malignancies, depending on the latency type (35, 39, 41, 50, 51) – Table 2. In particular, EBVaGC has been described as having a specific viral latency profile (Latency I), which corresponds to the expression of EBERs, BARTs and EBNA1, nevertheless some authors described that some cases present a latency II-like in EBVaGC, which does not express LMP1, comparing with latency II (52).

Table 2 – EBV associated diseases latency profiles

	EBNA1	EBNA2	EBNA3	LMP1	LMP2	EBERs	Malignancies
Latency 0							
Latency I	x					x	Burkitt's Lymphoma
Latency II	x			x	x	x	Nasopharyngeal Carcinoma, Hodgkin Lymphoma, T-cell non-Hodgkin Lymphoma
Latency II-like	x				x	x	Gastric Cancer
Latency III	x	x	x	x	x	x	Post-transplant lymphoma, AIDS-associated lymphoma

2.5. EBV Proteins

2.5.1. EBV encoded nuclear antigens

EBNA1 is one of the most important EBV proteins and is expressed in every EBV-infected cells, independently of cell type, status of activation and latency profile (39). It is a phosphoprotein that binds to specific DNA sequence and acts as a transcription factor being responsible for episomal maintenance, DNA replication and indirectly associated with cell transformation (35, 44, 53). Besides that, it also contributes, by a couple of mechanisms, to genomic instability and translocations, including increased levels of reactive oxygen species (ROS) (37, 39). Some authors defend that EBNA1 has the ability to stabilize p53 by binding to its regulator USP7, upregulating survivin and blocking caspase activation (37).

EBNA2, a nuclear phosphoprotein, is one of the earliest expressed genes in recently infected B cells and has a critical role in lymphocytes immortalization (37, 39, 47). It induces host genes expression by co-activating the transcription of several viral and host genes, without binding to DNA and it influences the promoter response by, with other cellular proteins, forming a complex that remodels the chromatin (39). It also regulates the expression of some latent membrane viral proteins (35, 39).

EBNA3 family is composed by 3 nuclear phosphoproteins (EBNA3A, 3B and 3C) that act as EBNA2-mediated transactivation activity inhibitors (37, 39). EBNA3C also associates physically with histone deacetylase HDCA1, activates the transcription of cellular and viral genes, such as CD21 and LMP1, and disrupts cell-cycle checkpoints on different levels (39). To activate the transcription, Sp1 transcription factor intact binding site and a totally functional EBNA2 protein are needed (37).

EBNA-LP, or EBNA5, is one of the earliest genes to be expressed in recently infected B cells and seems essential for cell immortalization (37, 39). When co-expressed with EBNA2, it enhances EBNA2 transcriptional activity allowing resting B cells to enter G1-phase (39). It has an effect of p53-Rb axis by targeting a p53 regulator, p14 ARF which is able to bind to Murine Double Minute 2 (MDM2) and suppress its activity of p53 degradation, thus EBNA5 leads to p53 degradation (39).

2.5.2. Latent Membrane Proteins

Three LMPs are expressed in both latency II and III: LMP1, LMP2 and LMP3. They are expressed at the cellular membrane surface and the intracellular membrane of the endoplasmic reticulum and Golgi complex. They are able to interact with multiple cellular signaling pathways and, in the case of lymphomas associated with EBV, LMP1 and 2 are essential for cell survival (39, 53).

LMP1 is the major EBV oncogene and has showed to be essential for B lymphocytes transformation. It also induces apoptotic genes, epithelial cells transformation and invasiveness and metastasis factors, leading to disruption of the basal membrane (53, 54). It also induces telomerase activity and avoids cells apoptosis by different pathways, including upregulating anti-apoptotic genes and block p53-mediated apoptosis by BCL-2 upregulation (39, 47). It has been described as being able to mediate the regulation of cellular proteins, like induction of adhesion molecules expression, and to activate EGFR in epithelial cells (39, 47).

LMP2A main role is to avoid the activation of the virus lytic cycle by maintaining latency in infected B lymphocytes and modulating epithelial cell growth (39, 47, 53). This protein also has some tissue-specific roles such as mimicking receptors signaling in B lymphocytes, contributing to its survival and has been shown to block cell differentiation by activation of the PI3K/AKT and β-catenin pathway in epithelial cells (53). EBV mutants for LMP2A lead to B cells death in the germinal center, showing the importance of this protein for these cells growth and transformation. It modulates cell growth and apoptosis, since it has been shown to activate PI3K pathway in B cells and epithelial cells, where can also induce cell mobility and invasion (39).

2.5.3. EBV non-coded RNAs

EBV was the first virus known to encode miRNAs and, during transformation, a number of viral non-coding ones are expressed, with some of them regulating cell death during both transformation and latency (46). They can be divided into two groups, EBV encoded RNAs and EBV miRs, which can be subdivided into two families, according to its location in the viral genome: BHRF1 and BARTs (37).

EBERs are noncoding RNAs found at high copy levels per cell, making them the target for EBV detection (47, 53). EBER1 and EBER2 are expressed in all latency profiles and may contribute to B cell transformation, even though there is no evidence that they are essential for transformation (37, 39). They seem to act as

signaling and transcription factor regulators by the production of cytokines and interferons (39).

EBV also encodes around 40 miRNAs, expressed in various levels in latency I, II and III (37, 39). It is also known that they bind, and might partially regulate, hundreds of cellular and viral transcripts, some of them involved in cell survival (37). BHRF1 role is not very understood yet but, according with some results, BHRF1 miRNAs and proteins cooperate to control cell cycle initiation and apoptosis during primary infection (37). Most of BARTs are described as downregulating host genes, such as tumor suppressors and pro-apoptotic genes, contributing to EBV-induced carcinogenesis, including several cell growth and cell cycle related (37, 53). Multiple evidence also suggests that, in the lack of viral transforming proteins, BART miRNAs are the major cause of expression pattern and cell growth changes (53).

2.6. EBV-associated Gastric Cancer

EBVaGC is defined by monoclonal proliferation of carcinoma cells with latent EBV infection, and studies have confirmed that every cell from the cancer clone carries the clonal virus genome, suggesting that the virus was acquired before the transformation, even though it seems that it is not detected in precursor lesions (55-57).

Epidemiological studies, including one from our group, suggest that EBVaGC corresponds to approximately 10% of all GC (57-59). Recently it has been described that there are three histological subtypes of EBVaGC based on the host cellular immune response status: the lymphoepithelioma-like carcinoma (LELC), the carcinoma with Crohn's disease-like lymphoid reaction (CLR) and the conventional-type adenocarcinoma (CA) (60).

The prevalence of EBVaGC subtype seems to be equally distributed worldwide, even considering regions with different GC prevalence, males are more affected than females, EBVaGC is more prevalent in younger patients and in Caucasian and Hispanic populations (61, 62). In addition, EBVaGC seems to be more frequently found in the proximal stomach and has a moderate to poor degree of differentiation, nevertheless it might not have impact on the overall survival and recurrence, even though there is a lower rate of lymph nodes metastasis, but this is still a controversial topic. EBV positivity is equally found in intestinal and diffuse

types of GC and therefore, the characteristics of these tumors are being discussed (10, 41, 56, 61, 63, 64).

EBVaGC has some distinctive features in terms of genome alterations, such as DNA hypermethylation, higher levels of programmed death ligands 1 and 2 (PD-L1/2) (10), different *PIK3CA* mutation patterns, extensive CpG island methylation and driver mutations (52, 65). *TP53* mutations are rare and in a recent study from our group, we have shown that EBVaGC presents a lower level of *TP53* mRNA and higher level of p53 protein when compared with non-EBV associated cancers (EBVnGC) (10, 66). Regarding the *PIK3CA* mutation patterns, non-silent *PIK3CA* mutation is found in 80% of EBVaGC. In EBV positive cases the mutations are more dispersed than in EBV negative cases, in which they are located mainly in the kinase domain (10).

In EBVaGC, the post transcriptional regulatory role is carried out by small microRNAs and long coding RNAs (lncRNAs) (67). miRNAs are related with some processes directly linked to carcinogenesis such as oncogenesis, cell adhesion, signal transduction and apoptosis, and the epithelial-to-mesenchymal transition can be induced by the downregulation of host cellular miRNAs caused by EBV latent genes (68). LncRNAs are known to act in poorer prognosis factors such as larger tumors, greater invasion and lower survival rates in the way that these molecules amplify molecular pathways related to these processes (67).

3. p53

3.1. p53 structure and function

TP53 is included in 12q13.1 and encodes for p53, the guardian of the human genome, a 53kDa phosphoprotein responsible for the regulation of hundreds of genes and noncoding RNAs (26, 69). It is composed by two amino-terminal transactivation domains (TADs), a tetramerization domain (TET) and a sequence-specific DNA-binding domain (DBD), essential for the protein activity (70).

It has been described that p53 is able to control multiple biological processes in response to environmental and physiological stress such as ionizing radiation, UV irradiation, hypoxia, oxidative stress and DNA damage (26, 71). The regulated processes can be divided into classical/canonical mechanisms, which have an impact in cancer development, and the emerging p53 functions (70). The classical ones are cell cycle arrest, senescence and apoptosis, all leading to allow DNA repair, essentially by the upregulation of pro-apoptotic and downregulation of pro-survival genes (69, 71). Among the emerging functions DNA metabolism, angiogenesis, cellular differentiation, and the immune response can be highlighted (26). The type of response to the stress depends on the cell type, context, duration and origin of the stimuli and the protein is usually activated by displacement of its negative regulators: mdm2 and murine double minute 4 (MDM4), also known as murine double minute x (MDMX) (70, 72, 73).

3.1.1. Cell cycle arrest

This mechanism consists in a G1 or G2 transient cell cycle arrest in order to allow the DNA to be repaired and the cellular homeostasis reestablished (70). In response to DNA damage, p53 induces the transcription of Cdkn1a, which leads to p21 expression, a cyclin dependent kinase inhibitor (70). p21 then binds to cyclin E/Cdk2 and cyclin D/Cdk4, causing G1 and G2 arrest, respectively. Both of these Cdks inhibition by p21 lead to pRb binding to E2F1, avoiding the transcription of genes related with DNA replication and cell-cycle progress (74). Even though p21 is involved in both phases of arrest, p53 is only essential for the G1 arrest (75).

3.1.2. Senescence

Upon bigger DNA damage, the cell cycle needs to be arrested in a more stable form - senescence (70). This state was seen as irreversible, but some studies showed that with the right stimuli, these cells may reverse to a proliferative state (74, 76). Differently from other p53 classical functions, p53 is essential for both initiation and maintenance of senescence (76). Some of the p53-induced senescence genes are Cdkn1a, Pml, Pai1and E2F7 (70, 76). ROS p53-dependent generation also appears to be a crucial event to senescence regulation (76).

3.1.3. Apoptosis

Even though p53 is not essential to apoptosis, it has the ability to promote apoptosis by transcriptional activation, transcriptional repression and through mitochondrial membrane permeabilization, one of the transactivation-independent functions of p53 (70, 72, 75).

In the transcriptional activation, p53 has several targets, such as Bcl-2 family genes Bax, Puma and Noxa, death receptors, apoptosis execution factors and PTEN (70, 74, 77). Transcriptional repression happens with anti-apoptotic factors, such as Bcl-2 and survinin (75). DNA damage leads to ATM activation, , and consequent p53 and mdm2 phosphorylation, leading to p53 stabilization and mdm2 degradation, thus ultimately increasing p53 levels and inducing apoptosis (77).

Regarding mitochondrial membrane permeabilization, it allows cytochrome c to be released, which connects with the apoptotic protease activating factor 1 (Apaf-1) and the caspase 9 pro-enzyme, forming the “apoptosome” complex. The complex allows caspase 9 activation and consequent activation of caspase 3 pro-enzyme and the latter adheres to other caspases, leading to the caspases pathway that ultimately results in intracellular protein lysis (74, 78).

3.1.4. DNA Repair

DNA repair is also a p53 function through protein-protein interactions (75). Among p53 target genes there are several genes directly related with DNA repair, from different repair mechanisms, such as base excision repair (BER), mismatch repair (MMR), homologous recombination (HR) and non-homologous end-joining (NHEJ) (72, 75). Regarding the involved proteins, p53 regulates, for instance, Gadd45a, damage-specific DNA binding-protein 2 (Ddb2), xerodema

pigmentosum, complementation group C (Xpc) and Fanconi anemia, complementation group C (Fancc) (72).

3.2. p53 and cancer

TP53 is one of the genes with major impact in cancer as it is the most commonly mutated gene in human tumors (79). Its function is usually altered due to loss of heterozygosity, mutation, and more rarely methylation (26, 77).

Missense mutations are detected in about half of the tumor cases, sometimes more than one mutation can be detected in the same tumor, and in around 40% of the GC, while p53 overexpression in 80% of GC (26, 69, 79). These mutations are usually found in the DNA-binding site-specific coding region, where 95% of the mutations occur in the coding region of the sequence-specific DNA-binding domain, blocking its transactivation ability (26, 80). Mutations can also be found in precancerous lesions, indicating that it is an early event in GC development (79). The mutational burden tends to increase with the patient age, since the incidence of *TP53* mutations is lower in younger patients and more common in tumors from the cardia than in the antrum (79).

In some cases p53 mutation by itself is not the major driver to carcinogenesis but the protein that it rises instead (80). For instance, p21, a CDKI, regulation by p53 gives this transcription factor the ability to control cell cycle and induce G1 arrest (81). Its negative regulators elevation can also lead to the function loss (82).

p53 accumulation is observed in nasopharyngeal carcinoma (NPC) and the mutation rate is low, being less than 10% (83). Overexpression and accumulation have also been reported in GC, where the accumulation can be an indicator of *TP53* mutant form (26). A study from our group also found p53 accumulation in EBVaGC but conjugated with wt *TP53*, suggesting a different pattern that can be viral-related (66).

EBV infection of primary cells leads to p53 expression without causing mutations, this occurs due to the activation of DNA damage response signaling pathway (71). Several viral proteins are described as interfering in p53 expression and activity. BZLF1, a protein involved in the latent-lytic infection transition, has been shown to increase p53 and p21 expression, leading to cell cycle arrest, through transient induction during the early stages of viral production, and also p53 degradation at the latest stage of the lytic phase of the virus (81). LMP1 blocks p53-mediated apoptosis and EBNA3C downregulates p53 expression (71). EBV infection also

reflects in p53 phosphorylation since p53 is in a hypo-phosphorylated form in latently-infected cells (81). A study from Szkaradkiewicz A., analyzed gastric cancers with the presence of EBV, comparing with the ones with *H. Pylori*, and based, on the proportion of patients with abnormalities in p53, concluded that the abnormalities in this gene are not related with EBV and observed a low or absent p53 mutation in EBVaGC cases (84).

4. MURINE DOUBLE MINUTE 2

4.1. Mdm2 structure and function

Murine Double Minute 2 protein is the p53 major negative regulator and may be the explanation for some p53-dysregulation mechanism (85, 86). Mdm2 is a E3 ubiquitin ligase nuclear protein, coded by a proto-oncogene in 12q14.3-q15 (87). This protein has been described has being involved in multiple biological mechanisms, such as apoptosis, cell cycle arrest, and tumorigenesis. In all of these cases it can be involved in a p53-dependent or -independent way, in both cases the involved pathways and protein are the ones described previously (88). Mdm2 contains a nuclear export signal (NES), a nuclear localization signal (NLS), nucleolar localization signal (NoLS) and a E3 ubiquitin-ligase-mediating RING-finger domain (89, 90).

MDM2 is regulated by two independent promoters P1 and P2: the P1 promoter is located upstream of the first intron and is responsible for the basal expression; while the P2 promoter is inducible by other proteins and is located in the first intron (90). Each of the promoters lead to a different length transcript since the P1 promoter does not allow the transcription of the exon 2 and the P2 transcript does not include two ORFs. Besides, both of the transcripts, when translated, lead to a full mdm2 protein, since the two first exons do not code for the protein (89).

4.2. Mdm2 and p53 regulation

In normal cases p53 is maintained at low levels mainly by mdm2 regulation, through two main mechanisms that allow mdm2 to inhibit p53 action: by binding to the p53 N-terminal, blocking the interaction between p53 and the transcriptional machinery; and the ubiquitination process (91, 92). Mdm2 ubiquitinates six lysine residues from the C-terminus of p53 through its RING-finger type E3 ubiquitin ligase and marks it to proteasomal degradation or nuclear export (80, 91). Low levels of mdm2 lead to mono-ubiquitination and nuclear export while higher levels promote polyubiquitination and degradation signaling (80, 90).

This process is enhanced by the presence of mdm4/mdmx. Mdmx does not have an E3 ubiquitin ligase activity but contributes both by forming a heterodimer and stabilizing mdm2 ligase activity and by binding to p53 and inhibit its transcriptional activity (89, 93). The mdm2-p53 regulatory feedback process can be impaired by p53 N-terminus phosphorylation upon certain cellular stresses,

such as DNA damage, since it affects negatively the mdm2-p53 interaction and leads to mdm2 auto-ubiquitination and decrease and consequent p53 increase (89, 91).

Activated wt p53 transactivates *MDM2* expression through binding to the P2 promoter, while the mutant one is not capable of that (80, 90). In these cases, the P1 promoter action is enough to maintain p53 levels low if there were no stress stimuli (80).

4.3. Mdm2 and cancer

MDM2 is found overexpressed in about 10% of human cancers, including in some GC cases, being this percentage variable among cancer types, which indicates that this protein can impact tumor formation (82, 91, 93). This overexpression contributes to genetic instability and thus cancer development, besides it is usually mutually exclusive from p53 mutations, suggesting that both alterations have a similar cellular effect (89, 91).

There are several mechanisms by which *MDM2* can be overexpressed such as gene copy number amplification, increased expression of mRNA or the protein itself (91). Regarding translation, a difference between efficiency of the P1 and P2 transcripts has been described. This difference is due to differences in the 5'UTR sequence, since P1 transcripts have a sequence element that inhibits translation, by reducing ribosome loading efficiency through lower RLP26 affinity, while P2 transcripts have elements that enhance translation through the binding protein La antigen recognition of a 27-nucleotide segment (90, 91). Taking into account that the P2 promoter is inducible, this translation increase can be induced by several pathways activity, such as Ras and Myc (89, 91). Another factor that enhances *MDM2* transcription is the presence of a polymorphism, as the SNP309 (89). This SNP is characterized by a T to G change in the 309 nucleotide of P2 promoter that seems to increase the affinity to Sp1 transcription factor leading to *MDM2* higher expression (94).

Another mdm2 characteristic that have been suggested to be related with tumorigenesis are the mdm2 isoforms (89, 90). They result from an alternative or aberrant splicing and have been correlated with poor prognosis in high-grade tumors. There are already about 72 mdm2 isoforms described but some of them lack the ability to be translated into protein and many of them lose some domains, such as the p53-binding domain (89, 90).

Upon oncogenic stimuli, mdm2 can be inhibited through several pathways, such as E2F, Ras or Myc, that activate ARF, an mdm2 inhibitor that binds to the E3 ubiquitin ligase domain, blocking its activity. Besides that, ARF also stabilizes p53, having a double action in this pathway (91).

II. AIMS

EBVaGC has been described as having particular features, and recent data point to the accumulation of p53 in the tumor cells without the presence of mutations. The mechanism for this is remains to be explained, and some authors suggest that the p53-regulators may contribute for this accumulation.

In this study we intend to characterize *MDM2* expression and variations in EBV-associated gastric carcinomas. The specific aims of the study are:

1) Characterize *MDM2* variations; and to

2) Evaluate the expression profile of *MDM2*.

III. STUDY I

1. MATERIAL AND METHODS

1.1. Literature search and study selection

A systematic review of literature was performed using the *Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA)* guidelines. The literature search was performed in PubMed and Scopus databases on 22nd January 2019 using the following key words combination: "gastric cancer AND polymorphism AND MDM2". Different combinations of words and MeSH terms were tested and the selected query was the more representative. The literature search was performed independently by two elements of the group without any restriction on time period, sample size or population.

Studies were included if they met the following inclusion criteria: (1) assessment of any *MDM2* polymorphism and risk of gastric cancer; (2) case-control design, and (3) providing genotype frequencies for both cases and controls. Studies were excluded if: 1) other languages than English; 2) duplicated data; 3) other study design rather than case-control (reports of clinical cases, comments, series, reviews and editorials), and 4) insufficient data or data not available. Review studies were checked for their references for other relevant studies. Articles involved with two or more case-control tests or two or more SNPs were regarded as two or more different studies. Case-control studies were the only selected to be included because they provide the necessary data for meta-analysis considering the association with gastric cancer risk.

1.2. Data extraction

Manuscripts were first screened independently by two elements of the group, by analyzing title and abstracts, based on the inclusion/exclusion criteria. Full texts were independently reviewed and data extracted (first author, year of publication, original country, ethnicity of the population studied, genotyping method, histological gastric cancer, numbers for cases and controls of all genotypes). Any disagreement regarding manuscript inclusion or exclusion was mediated by a third group element.

1.3. Statistical analysis

All studies were assessed for Hardy-Weinberg equilibrium (HWE) of genotypes distribution. The collected data was analyzed using Review Manager version 5.3 (The Nordic Cochrane Centre, The Cochrane Collaboration, 2014). The program was used to assess the association between the different genotypes and gastric cancer risk, by calculating odd-ratios (OR) and its confidence intervals (CI). Funnel and forest plots were created to summarize the differences in the studies and their significance, considering the relative weight of each study. A statistical significance level was considered by calculating *p*-value <0.050.

2. RESULTS

2.1. Characteristics of included studies

The study selection flow diagram is presented in Figure 7. The literature search provided 56 manuscripts, 21 of them were duplicated between databases. Of the remaining 35, a total of 21 were excluded by the following reasons: not gastric cancer (n=4), not *MDM2* (n=2), no *MDM2* polymorphism analysis (n=3), reviews (n=4) and meta-analysis (n=8). A total of 14 manuscripts were assessed for full-review, of which three were excluded: one manuscript was not available, one had a duplicated population of another published study and one was not a case-control study. After the revision process, a total of 11 articles were used for data analysis (95-105). The online databases were searched after the review process to identify new manuscripts on the field, on which two new studies are available but none is related specifically to *MDM2* polymorphism and gastric cancer.

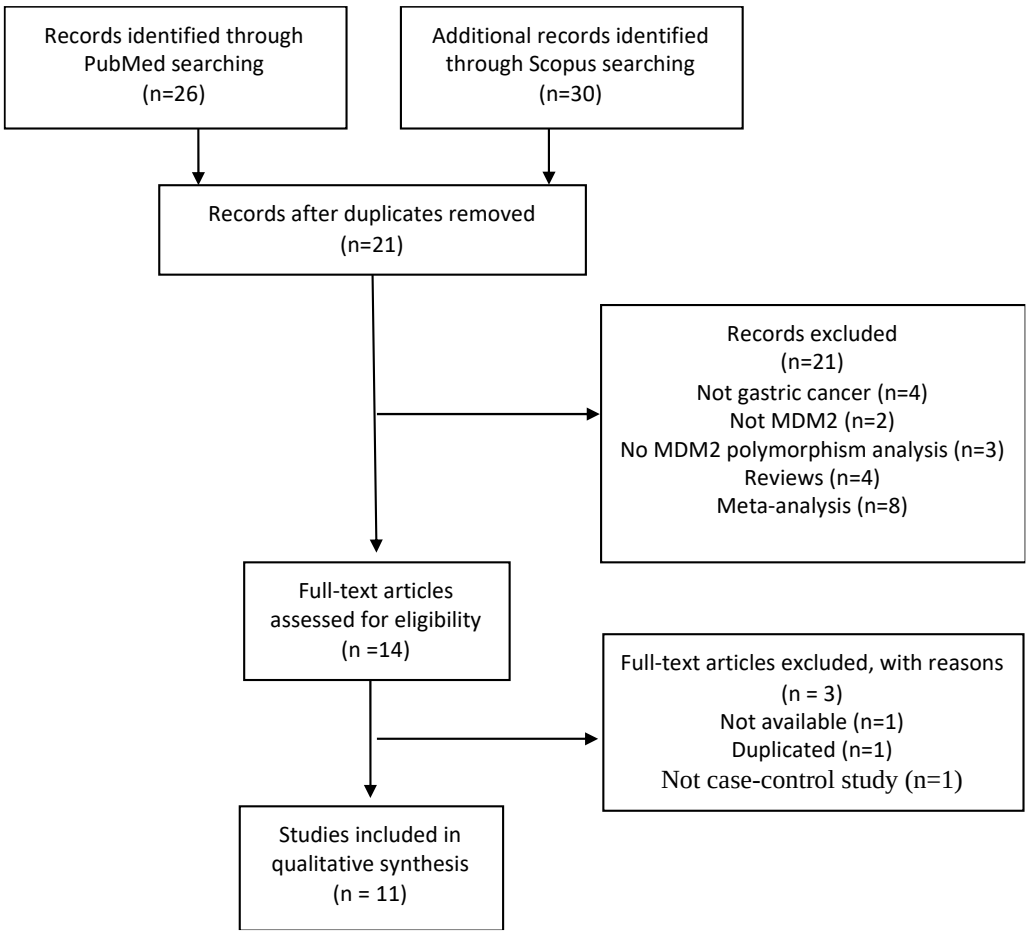


Figure 7 - PRISMA flowchart.

Among the included studies, we observed that 10 studies were performed in Asiatic populations and one from Brazil. Regarding the SNPs studied in association

with gastric cancer, one study analyzed rs937283, one analyzed rs3730485 and nine studies analyzed the rs2279744. Regarding rs2279744, a total of 3003 GC cases and 3676 controls were included in our meta-analysis. Despite these numbers, three studies have less than 200 patients. The characteristics of included studies are described in Table 3. Among the rs2279744, the genotype distributions of four studies (97, 99, 101, 105) were not consistent with HWE (p<0.050) – Table 3.

Table 3 – Descriptive table of the included studies

SNP/Study	**Genotyping Methods**	**Cases Genotypes, n (%)**			**HWE *p***	**Controls Genotypes, n (%)**			**HWE *p***
rs937283		**AA**	**AG**	**GG**		**AA**	**AG**	**GG**	
Chen B, 2018	PCR-RFLP	318 (69.1)	123 (26.7)	19 (4.1)	0.113	600 (75.0)	182 (22.8)	18 (2.2)	0.344
rs3730485		**InsIns**	**InsDel**	**DelDel**		**InsIns**	**InsDel**	**DelDel**	
Cavalcante GC, 2017	Multiplex PCR	61 (50.8)	46 (38.3)	13 (10.8)	0.339	274 (57.7)	168 (35.4)	33 (7.0)	0.301
rs2279744		**TT**	**TG**	**GG**		**TT**	**TG**	**GG**	
Tas A, 2017	PCR-RFLP	4 (6.1)	39 (60.0)	22 (33.8)	**0.016**	10 (14.9)	45 (67.2)	12 (17.9)	**0.005**
Elingarami S, 2015	Real-Time PCR	28 (26.7)	20 (19.0)	57 (57.3)	**<0.001**	75 (63.6)	36 (30.5)	7 (5.9)	0.348
Wu GC, 2015	Real-Time PCR	153 (23.8)	288 (44.9)	201 (31.3)	**0.013**	255 (35.4)	294 (40.8)	171 (23.8)	**<0.001**
Moradi MT, 2013	PCR-RFLP	16 (7.7)	156 (75.0)	36 (17.3)	**<0.001**	60 (30.0)	132 (66.0)	8 (4.0)	**<0.001**
Pan X, 2013	PCR-RFLP	173 (30.1)	260 (45.3)	141 (24.6)	**0.029**	199 (34.7)	296 (51.6)	79 (13.8)	0.060
Wang X, 2009	PCR-RFLP	74 (28.5)	120 (46.1)	66 (25.4)	0.220	82 (31.5)	141 (54.2)	37 (14.2)	0.057
Cho YG, 2008	PCR-RFLP	64 (26.8)	110 (46.0)	65 (27.2)	0.219	61 (20.4)	152 (50.8)	86 (28.8)	0.680
Yang M, 2007	PCR-RFLP	107 (21.4)	250 (50.0)	143 (28.6)	0.907	298 (29.8)	498 (49.8)	204 (20.4)	0.877
Ohmiya N, 2006	PCR-RFLP	98 (23.9)	188 (45.8)	124 (30.2)	0.109	99 (22.6)	241 (55.0)	98 (22.4)	**0.036**

SNP, Single Nucleotide Polymorphism; PCR-RFLP, Polymerase Chain Reaction followed by Restriction Fragment Length Polymorphism; HWE, Hardy-Weinberg Equilibrium; *p*, *p*-value; n, sample size; %, percentage.

2.2. Meta-analysis

Of the three SNPs described in the different studies, only rs2279744 had more than one study with genotyping data. Data analysis was performed considering the G allele as the risk allele into different genetic models: 1) the dominant model (GG + TG *vs* TT), and 2) the recessive model (GG *vs* TT + TG) (106). As shown in the forest plots (Figure 8 and 9), the results of the dominant model analysis showed that GG + GT were significantly associated with increased GC risk

compared with the TT genotype (OR=1.45; 95% CI 1.30-1.62; p<0.001). The combined analysis for the recessive model also showed an increased risk of gastric cancer development for the GG genotype (OR=1.73; 95% CI 1.54-1.94; *p*<0.001).

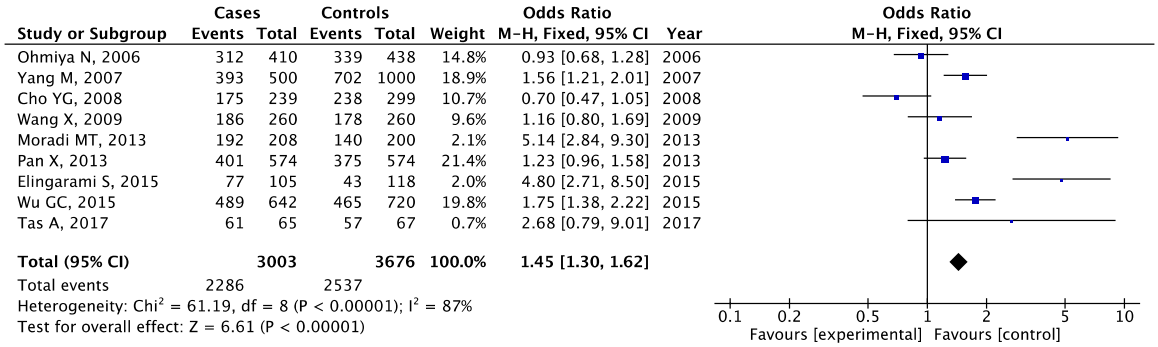


Figure 8 - Forest plot for dominant model analysis (GG + TG *vs* TT).

OR, Odds-Ratio; 95%CI, 95% confidence interval; *p*, *p*-value.

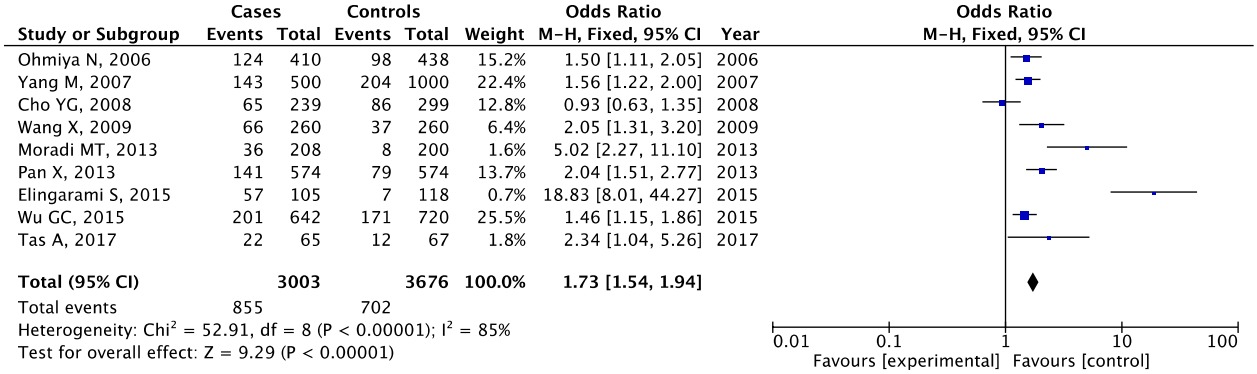


Figure 9 – Forest plot for recessive model (GG *vs* TG + TT).

OR, Odds-Ratio; 95% CI, 95% confidence interval; *p*, *p*-value.

2.3. Test of heterogeneity

Regarding the funnel plot analysis, we observed that some studies deviate from the expected outcome either in the dominant or recessive model analysis (*p*<0.05) – Figure 10. The studies that showed deviation from the expected outcome, according with both genetic models, were Cho *et al.*, Elingarami *et al.* and Moradi MT *et al.* (98, 99, 103). Even though we have not performed a qualitative analysis, the data extraction of all included manuscripts does not disclose any evident study

bias other than the description of GC histological subtypes that is not shown for the majority of studies.

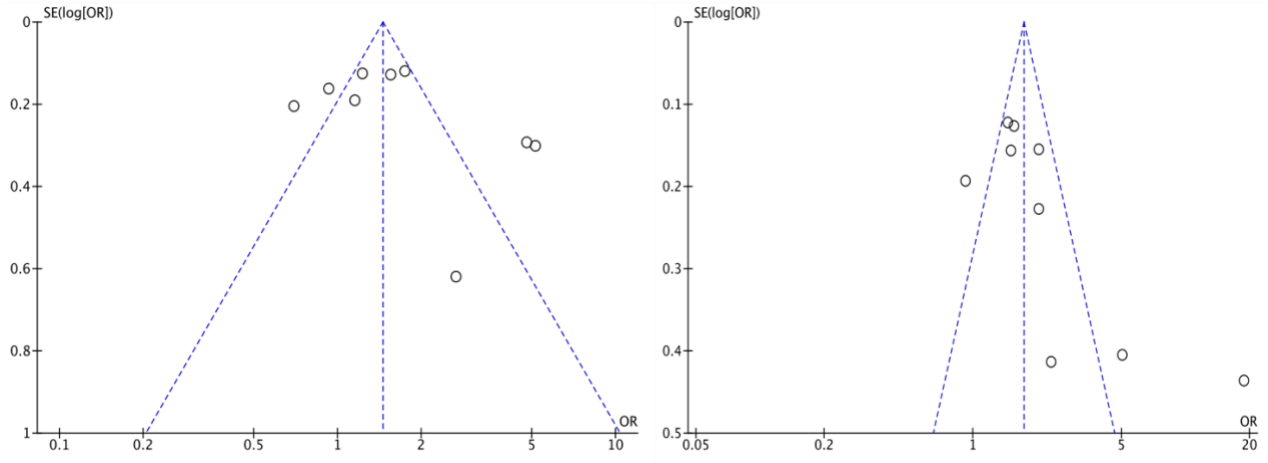


Figure 10 – Funnel Plots. a) Dominant model; b) Recessive Model.

OR, Odds-Ratio.

3. DISCUSSION

MDM2 has been associated with several cancers due to its p53 regulatory functions (85). In exposure to stress, p53 expression increases promoting transcriptional activity that leads to cell cycle arrest, repair and/or apoptosis (91). This p53 increased expression leads to increased mdm2 protein expression since *MDM2* P2 promoter is p53-dependent, establishing an autoregulatory feedback loop (107). Indeed, *MDM2* is considered an oncogene because of its p53 inhibition. In normal cases, mdm2 E3 ubiquitin ligase domain binds the p53 transactivation domain, leading to the inhibition of the transcriptional activities, followed by the promotion of proteasomal degradation and ended by the export of p53 from the cell nucleus, which is crucial for the repression of p53 suppressor functions (91). In addition to p53 regulation, *MDM2* also plays an oncogene function by interfering with other proteins function that participate in pathways from DNA repair, apoptosis, motility and invasion (107). Evidence suggests that *MDM2* amplification has been detected in several cancers and many studies have shown a potential role of genetic polymorphisms in cancer development (87, 107).

Previous studies have shown an association between *MDM2* alterations and gastric cancer (82, 108) and, considering the impact that some genetic polymorphisms have shown in GC development risk, we performed a systematic review in order to clarify what is being studied regarding *MDM2* polymorphism and GC. We are aware of some possible limitations of our meta-analysis, since the majority of studies are from Asian populations and the outcomes may not be applicable worldwide. Furthermore, these populations have higher GC incidences which may explain the special interest for studies of this nature (1).

The NCBI SNP database reveals a total of about 9642 *MDM2* polymorphisms, nevertheless only a few have been studied for their potential functional role. In this systematic review, we identified only three different *MDM2* polymorphisms (rs937283, rs3730485, and rs2279744) that have been studied in association with GC development.

The rs937283 is characterized by an A to G change in the 2164 nucleotide of *MDM2* promoter region that seems to lead to an enhancement of *MDM2* expression (95, 109). This SNP was studied by Chen B, that concluded that this polymorphism significantly increases the risk for GC development in the Chinese population. In this study the G allele was associated with increased risk for GC development either when considering G carriers *vs* AA (OR 1.34; p=0.024) or GG *vs* A carriers (OR 1.87; p=0.061). This polymorphism has been studied in other

types of cancer, such as lung (95), liver (110) and retinoblastoma (111) with similar impact.

Cavalcante *et al*. studied rs3730485, a 40bp deletion in the P1 promotor of *MDM2*, in a Brazilian population (112). This study revealed an increased risk for the development of gastric GC, with the presence of the deletion in both alleles (OR 1.37; p=0.021). This polymorphism has been associated with several types of cancer, such as breast (113, 114), prostate (114), ovarian (115) and hepatocellular carcinoma (116). The literature review provided a total of nine individual case-control studies on *MDM*2 rs2279744 polymorphism and GC risk. This polymorphism, also known as SNP309, is the most studied *MDM2* polymorphism and it is characterized by a T to G change in the 309 nucleotide of P2 promoter of *MDM*2. This genotype change seems to increase the affinity of Sp1 transcription factor, thus increasing the protein expression and subsequently leading to a higher inhibition of p53-dependent pathways (94). This polymorphism has been associated with several cancer types, such as bladder (117), endometrial (118), cervical (119), hepatocellular (116), colorectal (120, 121), among others. The meta-analysis of the included studies revealed a significant association between the G allele and increased risk of gastric cancer development, especially in the homozygous model (OR=1.73; p<0.001). Regarding the dominant model, two studies (103, 105) did not show increased risk for GC development, while in the recessive model this was observed only in one study (103). On the contrary, there was one study that showed a highly increased risk of GC development when considering the recessive model (98). We observed that all studies were performed in Asian populations, most of them Chinese, which is somehow expected taking into account that these populations have highest incidence rates of GC. Regarding the genotyping methods, two studies were performed by real-time PCR (98, 101), while the other seven were performed by PCR-RFLP (97, 99, 100, 102-105). This may have some impact on the genotype distribution as the specificity and sensitivity of assays is not the same. Furthermore, we observed that four studies were not consistent with HWE (p<0.050) (97, 99, 101, 105), and the funnel plot revealed that some of these studies may have bias that are affecting their results. These data increase the need for better characterization of these populations and the development of more studies regarding this SNP and GC development.

This study revealed that three different *MDM2* genetic polymorphisms (rs937282, rs3730485 and rs2279744) have been studied for their association with GC development. The low number of studies, some of which with a smaller number

of cases, and the fact that the populations are all Asian are important remark due to the potential impact in the quality of the data analyzed. Indeed, the qualitative analysis of studies should be performed once there is more significant data. Nevertheless, our study shows that all these *MDM2* polymorphism seem to be associated with gastric cancer development. This meta-analysis shows that rs2279744, which is the most studied polymorphism, seems to be significantly associated with GC development. The fact that the number of studies is low and the studied populations are mainly Asian emphasizes the need for the development of more studies in other populations to corroborate the association of these polymorphism with GC.

IV. STUDY II

1. MATERIALS AND METHODS

1.1. Population and type of study

A retrospective study was performed using 40 patients attended at *Portuguese Oncology Institute of Porto* (IPO-Porto). All cases were histologically confirmed by a pathologist from our institution and categorized according to the Lauren classification systems for each type of cancer. GC cases were selected from a cohort of patients diagnosed with GC in 2011 in our institution (66), including 12 EBV-positive cases and 28 matched (histological type, age and stage of disease) EBV-negative cases. EBV-positive cases were previously characterized using in situ hybridization for the detection of EBV-encoded small RNA (EBER-ISH) (122).

This study did not interfere with clinical decisions. Clinicopathological data was collected from individual clinical records and inserted in a database with unique codification. All procedures were approved by the ethical committee of IPO Porto (CES IPO 74/2015).

1.2. Samples

In this study we used tumor tissues samples collected from the institution archives. DNA and RNA were extracted from tumor tissues collected from formalin-fixed paraffin-embedded (FFPE) tissue blocks and samples were used for immunohistochemistry (IHC). RNA extraction was performed using Absolutely RNA FFPE Kit.

1.3. Mdm2 status in tissue

Mdm2 expression in tissues was performed by IHC using a specific monoclonal antibody IF2 (Invitrogen, CA, EUA). Brefly, FFPE tissue samples (3 μm sections) were submitted to deparaffinization/rehydration and, after that, antigen retrieval was performed using Bond TM Epitope Retrieval 2 (Leica Biosystems, Wetzlar, Germany) for 20 minutes. Then, samples were processed in the BOND-III Fully Automated IHC and ISH Stainer (Leica Biosystems, Wetzlar, Germany). IF-2 mouse anti-human mdm2 monoclonal antibody diluted 1:300 (Invitrogen, CA, EUA). Detection of hybrids was achieved by Bond Polymer Refine Detection (Leica Biosystems, Wetzlar, Germany).

Mdm2 expression was defined as positive when at least 10% of neoplastic cells showed nuclear staining; tumors with no staining or less than 10% were classified

as negative. Tumors positive for mdm2 staining were categorized into two categories according to the percentage of positive cells: less and over 50% - Figure 11.

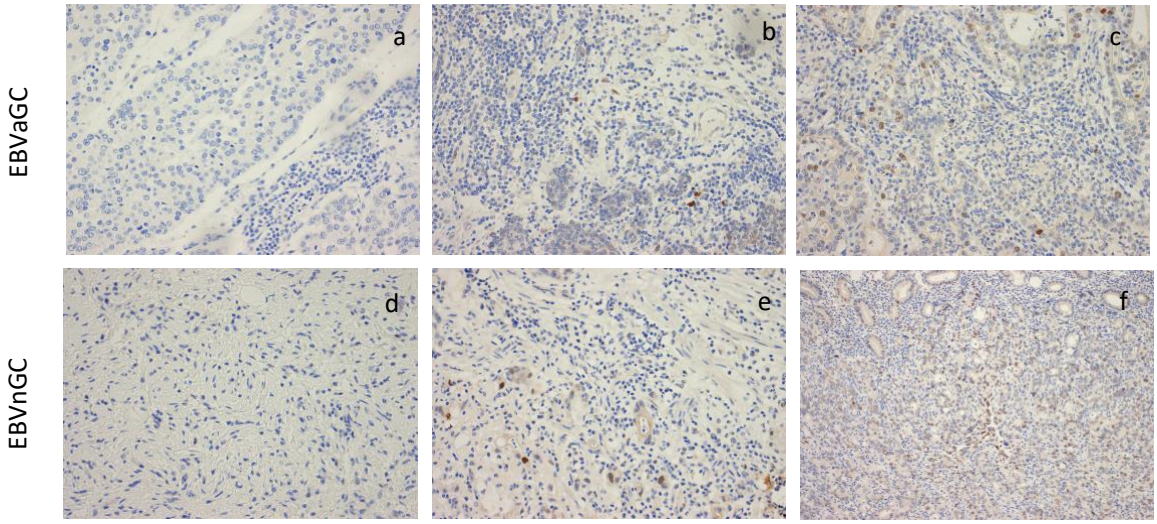


Figure 11 – Examples of immunohistochemistry staining on gastric carcinomas.

Mdm2 (200x): a and d) Negative; b and e) Positive: 10%; c) Positive over 50%. mdm2 (100X): f) Positive over 50%.

1.4. *MDM2* mRNA expression

MDM2 mRNA was analyzed by real-time RT-PCR. RNA was extracted from tumor tissues collected from FFPE using Absolutely RNA FFPE Kit (Agilent Technologies, San Diego CA, USA) and quantified using the NanoDrop™ Lite Spectrophotometer v3.7 (Thermo Fisher Scientific, Waltham MA, USA).

Reverse transcriptase reactions were performed using High-Capacity cDNA Reverse Transcription Kit (PN 4368814; Applied Biosystems, Foster CA, USA) according to the manufacturer's instructions, with a 20μL final volume.

MDM2, Ribosomal Protein L29 (*RLP29*) and Beta-2-Microglobulin (*β2M*) mRNA levels were assessed by two-step real-time polymerase chain reaction (PCR) using hs01066930_m1, hs00988959_gh and hs00187842_m1 *TaqMan Gene Expression Assays* (Applied Biosystems, Foster CA, USA), respectively.

qPCRs were performed in triplicates with a 10μl final volume mixture containing 1X of TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, California USA), 1X RNA Assay (Applied Biosystems, Foster City, California USA), and 20ng of cDNA. Amplification was run in Applied Biosystems Step-One Real

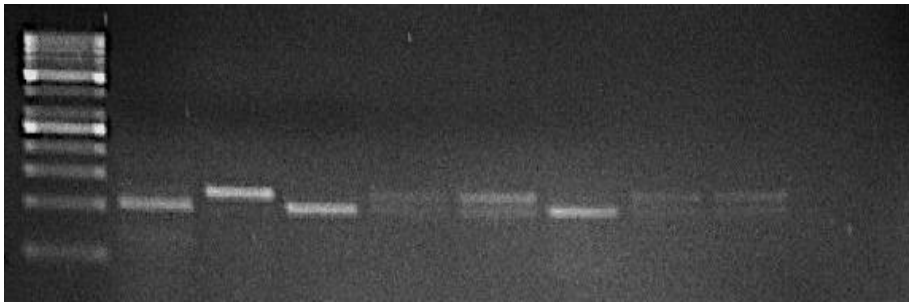
Time PCR System (Applied Biosystems, Foster CA, USA) with the following conditions: 20s at 95°C followed by 45 cycles of 1s at 95°C and 20s at 60°C.

The relative quantification of MDM2 mRNA expression was analyzed using the 2ΔΔCt method (Livak method), in which the Ct from the target RNA (*MDM2*) in both test and control cases are adjusted in relation to the Ct of a normalizer RNA (RPL29 and β2M) resulting in ΔCt. To calculate ΔΔCt, the mean of the EBVnGC ΔCt was subtracted to each sample' ΔCt, in order to normalize the samples. For the comparison between EBVaGC and EBVnGC we calculated $2^{-\Delta\Delta Ct}$ value, which allowed to determine the magnitude of difference of *MDM2* mRNA expression.

1.5. *MDM2* SNP209 polymorphism genotyping

MDM2 SNP309 TG polymorphism was genotyped by PCR according to the protocol described by Sousa *et al*. (123), using the forward primer 5'-GAT TTC GGA CGG CTC TCG CGG C-3' and reverse primer 5' -CAT CCG GAC CTC CCG CGC TG-3'. The PCR reaction was performed in a 50μl solution containing 1X DreamTaq green buffer (Thermo Fisher Scientific, Waltham MA, USA), 0.2mM DNTP's Mix, 1U DreamTaq DNA Polimerase (Thermo Fisher Scientific, Waltham MA, USA), 0.2μM each primer and 2μl of genomic DNA. The amplification followed these conditions: 95°C for 7min for the initial denaturation, followed by 40 cycles of denaturation at 94°C (45s), annealing at 60°C (45s) and extension at 72°C (45s), and a final extension step consisted in 7min at 72°C. Negative controls were used in all reactions by using RNA free water instead of DNA in one case to guarantee the good conditions of the reagents referring to contamination. The amplification success was confirmed by electrophoresis. In a 1.5% agarose gel (w/v) stained with GreenSafe Premium (NZYTech).

The PCR amplification yields a fragment of 121 bp, which was digested for 45min by FastDigest PstI restriction enzyme (Thermo Scientific, CA) at 37°C. Digestion mix was done according to the manufactory instructions. A positive control for the enzymatic digestion was used in all reactions by adding DNA previously genotyped as homozygotic for the G allele. The products of the digestion were evaluated through electrophoresis in a 3% agarose gel (w/v) stained with GreenSafe Premium (NZYTech) - Figure 12. In the presence of the G allele, the DNA is cleaved by the restriction enzyme originating two fragments, with 104 and 17bp, whereas the T allele is not cleaved by the enzyme.



MM 1 2 3 4 5 6 7 8

Figure 12 - PCR-RFLP analysis of *MDM2* SNP309 polymorphism.

Agarose gel (3%, w/v) stained with ethidium bromide: MM - 50bp DNA ladder; lane 1 - Positive control; lane 2 - *MDM2* SNP309 T allele homozygous; lanes 3 and 6 - G allele homozygous; and lanes 4, 5, 7 and 8 - heterozygous.

For confirmation of the genotypes, we have randomly selected 9 samples (3 from each genotype) for sequencing using the Prism Big Dye® Terminator Cycle Sequencing Ready Reaction kit (Life Technologies, Foster City CA, USA) and the automated sequencer ABI Prism® 3730 Genetic Analyzer (Life Technologies, Foster City CA, USA) – Figure 13.

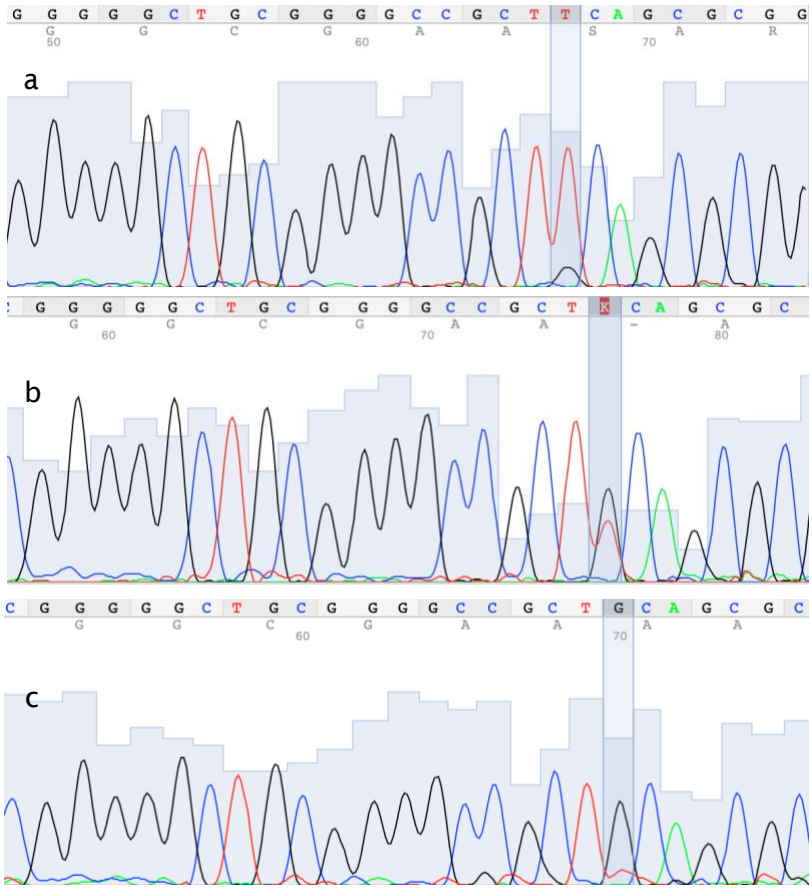


Figure 13 – Genotyping results example for a) TT genotype; b) TG genotype, and; c) GG genotype.

1.6. Statistical Analysis

Statistical analysis was performed using the computer software IBM SPSS statistics for Macintosh, version 26.0 (IBM Corp, Armonk, NY, USA). Chi-Square ($\chi 2$) or Fisher exact- test were used considering a 5% level of significance. Odds Ratio was calculated has a association measurement between the categoric variables with a 95% confidence interval. The graphs were elaborated using GraphPad Prism Software for Macintosh, version 8.0 (San Diego, CA).

2. RESULTS

2.1. Characterization of population

Clinicopathological characteristics of patients from EBVaGC and EBVnGC groups are presented in Table 4. A total of 40 patients (26 males and 14 females) with mean age of 68 were included in this study; the majority were submitted to gastrectomy (n =39), 24 of them were total resections and 15 partial resections, and the remaining patient was submitted to esophagogastrostomy. Regarding the tumor localization in stomach: five (12.5%) were found in proximal site of the stomach; nine (22.5%) in the corpus; and 26 (65.0%) in a distal site. These patients were selected in a previous study. Tumors were classified according to Lauren's classification system (1965) and the most common histological type was intestinal adenocarcinoma (n=15, 37.5%), followed by indeterminate adenocarcinoma (n=13, 32.5%), diffuse adenocarcinoma (n = 9, 22.5%) and the remaining three cases were evaluated as others (n= 3, 7.5%). Regarding the tumors characteristics, the most frequent invasion pattern was infiltrative (55.0% *vs* 27.5% expansive); most of the cases were poorly differentiated (54.5% *vs* 45.5% well or moderate differentiated); lymphovascular invasion was very frequent (63.2%). In what concerns of tumor staging, the majority of cases (45.0%) were at stage III, 30% at stage II, 20% at stage I and 5% in stage IV.

EBVaGC patients included nine males and three females with median age of 68.5 years old - Table 4. Half of the cases were intestinal type and the rest of the cases were evenly distributed by the other histological types. Regarding tumor localization, the cases were five in both distal and corpus and two cases were in the proximal area.

EBVnGC patients included 17 males and 11 females with mean age of 65 years old - Table 4. A total of 11 cases were of indeterminated type and the rest were evenly distributed by the other histological types. Regarding tumor localization, most of the cases were at a distal site (75.0%) and the rest of the cases were evenly distributed by corpus and the proximal site.

Table 4 – Patients clinicopathological characterization

	EBVaGC	EBVnGC
Gender, n=40		
Male, n (%)	9 (75.0%)	17 (60.7%)
Female, n (%)	3 (25.0%)	11 (29.3%)
Age		
Median (Range), years	68.5 (52- 82)	65 (40- 79)
Histology WHO, n=40		
Mixed adenocarcinoma, n (%)	2 (16.7%)	11 (39.3%)
Tubular adenocarcinoma, n (%)	6 (50.0%)	9 (32.1%)
Poorly cohesive carcinoma, n (%)	1 (8.3%)	8 (28.6%)
Carcinoma with lymphoid stroma, n (%)	2 (16.7%)	-
Adenosquamous carcinoma, n (%)	1 (8.3%)	-
Tumor Localization, n=40		
Proximal, n (%)	2 (25.0%)	3 (10.7%)
Corpus, n (%)	5 (41.7%)	4 (14.3%)
Distal, n (%)	5 (41.7%)	21 (75.0%)
Lauren Classification, n=40		
Intestinal, n (%)	6 (50.0%)	9 (32.1%)
Diffuse, n (%)	1 (8.3%)	8 (28.6%)
Indeterminate, n (%)	2 (16.7%)	11 (39.3%)
Outros, n (%)	3 (7.5%)	0
Invasion Pattern, n=39		
Expansive, n (%)	8 (66.7%)	7 (25.9%)
Infiltrative, n (%)	4 (33.3%)	20 (74.1%)
Global Stage, n=40		
Ia, n (%)	1 (8.3%)	4 (14.3%)
Ib, n (%)	2 (16.7%)	1 (3.6%)
IIa, n (%)	2 (16.7%)	3 (10.7%)
IIb, n (%)	1 (8.3%)	6 (21.4%)
IIIa, n (%)	5 (41.7%)	3 (10.7%)
IIIb, n (%)	1 (8.3%)	6 (21.4%)
IIIc, n (%)	-	3 (10.7%)
IV, n (%)	-	2 (7.1%)

EBVaGC, EBV-associated gastric carcinoma; EBVnGC, EBV non-associated gastric carcinoma.

2.2. Mdm2 status in tissue

The results from the IHC analysis are shown in Table 5 and 6. mdm2 positive case for expression was defined as positive when more than 10% of neoplastic cells showed nuclear staining.

Only 39 cases were possible to analyze, of which 15 showed mdm2 accumulation (nine in <50% cells; six in ≥50% cells) while 24 cases did not (including seven cases positive in 1-5% of cells) - Table 5.

Overall, mdm2 was positive in 5 (41,7%) of the EBVaGC and 10 (37.0%) of the EBVnGC cases (*p*=1.000; OR 1.21; 95% CI 0.30-4.87). No significant differences was observed when considering the different histological subtypes for EBVnGC– Table 5. Regarding the percentage of positive cells, we observed that, in EBVaGC cases, four cases had over 50% of cells expressing mdm2; while, for EBVnGC, eight cases had less than 50% of positive (*p*=0.089; OR=16; 95% CI 1.09-234) – Table 6. When EBVnGC where subdivided by histology, we observed a significant difference from EBVaGC when comparing with EBVnGC of diffuse type (*p*=0.048).

Table 5 – Distribution of cases with MDM2 accumulation

	Negative	Positive	p-value	OR, 95% CI
EBVaGC, n=12	7 (58.3%)	5 (41.7%)		
EBVnGC, n=27	17 (63.0%)	10 (37.0%)	1.000	1.21 (0.30-4.87)
Diffuse, n=7	2 (28.6%)	5 (71.4%)	0.350	0.29 (0.04-2.11)
Intestinal, n=9	8 (88.9%)	1 (11.1%)	0.178	5.74 (0.53-61.4)
Indeterminate, n=11	7 (63.6%)	4 (36.4%)	1.000	1.25 (0.23-6.75)

Table 6 – Distribution of cells with MDM2 accumulation

	<50%	≥50%	p-value	OR, 95% CI
EBVaGC, n=5	1 (20.0%)	4 (80.0%)		
EBVnGC, n=10	8 (80.0%)	2 (20.0%)	0.089	16.0 (1.09-234)
Diffuse, n=5	5 (100%)	----	0.048	*
Intestinal, n=1	----	1 (100%)	1.00	*
Indeterminate, n=4	3 (75.0%)	1 (25.0%)	0.206	12.0 (0.51-280)

* Not computable

We have also compared mdm2 expression with p53 accumulation in EBVaGC cases, which is provided by a previous study (66). All of the 12 cases had positive p53 IHC and mdm2 IHC information – Table 7 and Figure 14.

Table 7 – p53 and mdm2 expression in cases and characteristics

Patient	Gender	Age	Localization	Invasion pattern	IHC p53	IHC MDM2
1	Male	80	Distal	Expansive	≥50%	≥50%
2	Male	55	Corpus	Infiltrative	≥50%	Negative
3	Male	64	Corpus	Expansive	≥50%	Negative
4	Male	69	Corpus	Expansive	≥50%	Negative
5	Male	52	Proximal	Expansive	≥50%	Negative
6	Male	75	Distal	Expansive	≥50%	Negative
7	Male	80	Distal	Expansive	≥50%	Negative
8	Female	65	Distal	Infiltrative	≥50%	≥50%
9	Male	75	Corpus	Expansive	≥50%	<50%
10	Female	68	Corpus	Expansive	<50%	Negative
11	Female	66	Distal	Infiltrative	<50%	≥50%
12	Male	82	Proximal	Infiltrative	<50%	≥50%

EBVaGC, EBV-associated gastric carcinoma.

Among these cases, we identified three samples that had p53 expression in less than 50% of the cells and nine in more than 50%. When comparing mdm2 protein expression in tissue in these two groups of p53 expression, there are no significant difference (p=0.310). However, we observed that, among the cases with mdm2 expression, there are more cases expressing high p53.

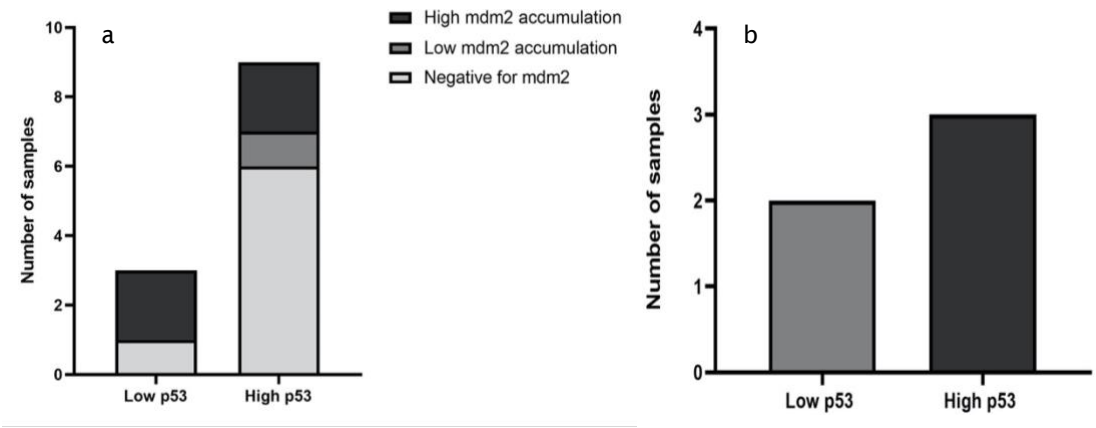


Figure 14 – Percentage of cells with a) mdm2 accumulation, in the cases p53 accumulation; b) p53 accumulation, in the cases mdm2.

2.3. *MDM2* mRNA expression

MDM2, β2M and RPL29 mRNA levels were evaluated in all cases and one EBVnGC was excluded from this analysis due to the lack of β2M amplification. *MDM2* expression was detected in 19 cases, five of them EBV positive. When analyzing *MDM2* expression in EBVaGC, we observed a non-significant increase when compared with EBVnGC (*p*=0.1099) - Figure15.

Further analysis subdividing EBVnGC by histological classification revealed that EBVaGC *MDM2* mRNA is significantly overexpressed when compared with the intestinal subtype (*p*=0.0343) and also tends to be over-expressed when compared with the mix (*p*=0.0570) - Figure 15. Regarding the diffuse type, *MDM2* is overall not expressed.

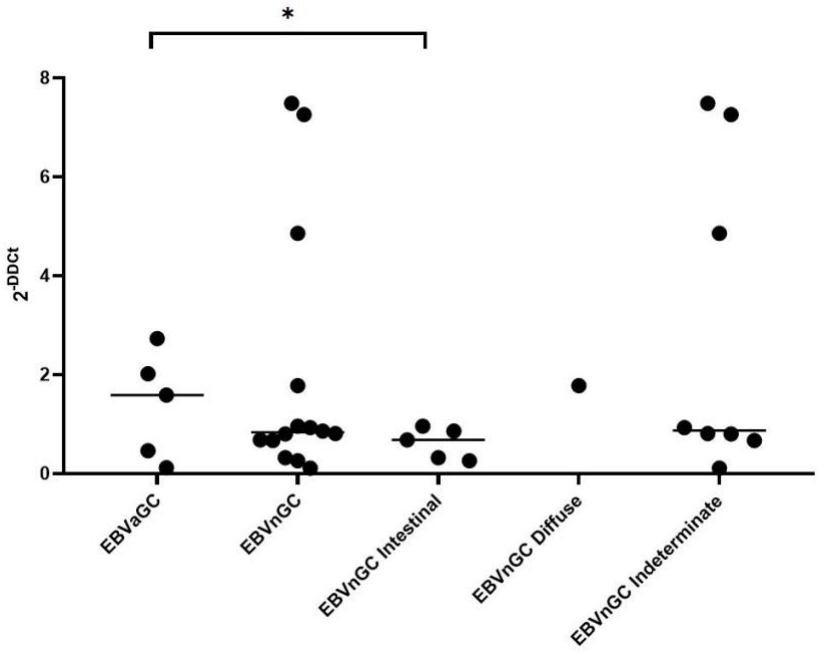


Figure 15 – *MDM2* relative expression in all GC subgroups.

*p=0.0343.

We also compared the mRNA expression levels with the IHC results, which demonstrated that the there are about 50% of cases with detectable RNA expression in the cases positive and negative staining for mdm2, either considering the overall cases or when we separated the cases into EBV positive and negative – Figure 16.

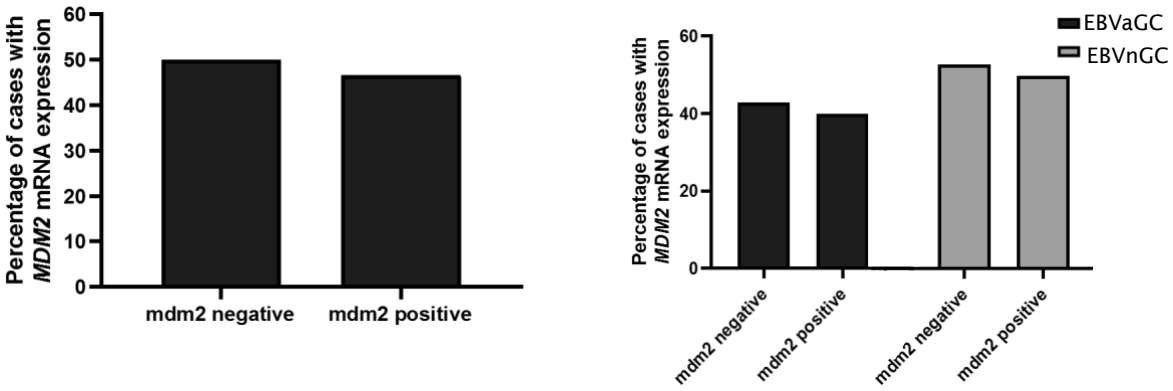


Figure 16 – Number of cases with detectable mRNA expression, according with IHC positiveness in a) overall cases and; b)EBV positive and negative cases.

2.4. *MDM2* SNP 309 genotypes

MDM2 SNP309 genotyping results are shown in Table 8. The SNP genotype was assessed in all the samples except one EBVnGC case that was excluded from the study due to lack of DNA quality.

SNP309 genotype analysis showed that more than half of EBVaGC cases presented a nucleotide change (7 in 12; 58.3%), all the TG genotype. In EBVnGC, 15 cases showed a nucleotide change, 13 (48.1%) presented the TG genotype and 2 (7.4%) the GG. The SNP309 presence showed no significant difference in allele frequency among EBV positive and negative groups (p=0.577), not even between EBVaGC and EBVnGC subtypes – Table 8.

Table 8 – Descriptive table of *MDM2* SNP 309 genotyping

	TT n (%)	TG n (%)	GG n (%)	p-value*	OR (95% CI)*
EBVaGC, n=12	5 (41.7%)	7 (58.3%)	----		
EBVnGC, n=27	12 (44.4%)	13 (48.1%)	2 (7.4%)	0.577	1.12 (0.28-4.43)
Diffuse, n=7	3 (42.9%)	2 (28.6%)	2 (28.6%)	1.000	1.05 (0.16-6.92)
Intestinal, n=9	4 (44.4%)	5 (55.6%)	----	1.000	1.12 (0.20-6.42)
Indeterminate, n=11	5 (45.5%)	6 (4.4%)	----	0.593	1.17 (0.20-6.08)

*Considering TT *vs* G-carrier as the risk model. OR associated with EBVaGC

When we crossed the reference between *MDM2* mRNA expression and SNP309 genotyping we obtained some interesting data. There were no GG cases with *MDM2* mRNA expression. At a global level, both genotypes were associated with similar mRNA expression but, when we divided the cases into EBV positive and negative, the EBVnGC maintained this pattern, while the EBVaGC showed slightly lower expression associated with the TG genotype – Figure 17 and 18.

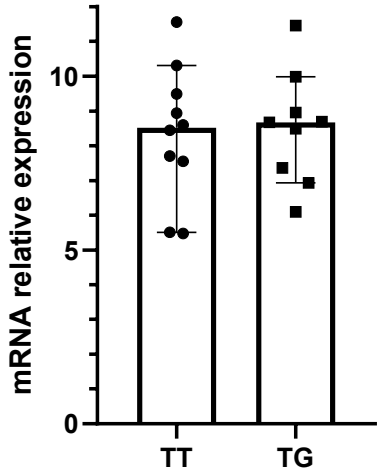


Figure 17 – *MDM2* mRNA levels, according with SNP genotype, in all cases.

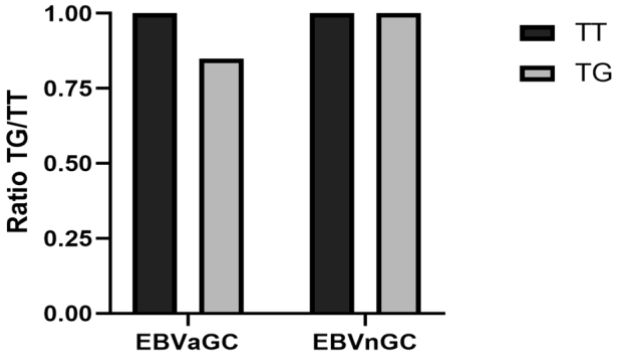


Figure 18 - MDM2 mRNA levels, according with SNP genotype and EBV status.

3. DISCUSSION

MDM2 has been described as a major regulator of p53 (85). Mdm2 downregulates p53 by binding and physically inhibiting p53 transcription factor activity, and by promoting its nuclear export and degradation due to mono- and polyubiquitination, respectively (92). Therefore, *MDM2* has also been considered an oncogene, since this interaction with p53 can lead to cancer development (91).

The regulation of p53 levels may be explained by the activity of *MDM2* and several mechanisms of *MDM2* dysregulation may have a significant impact. Alterations in the p53-mdm2 pathway may lead to mdm2 stabilization and thus mdm2 accumulation (91). In order to assess that, we investigated this protein expression in terms of mRNA and protein expression in tissue and also the presence of the SNP309 polymorphism which has been associated with increased expression of *MDM2* (94).

In our study, the assessment of mdm2 accumulation in tissue was performed by IHC for mdm2 using the IF2 monoclonal antibody, that recognizes this mdm2 and its isoforms, in the nuclei. In our series, we found that mdm2 was detected in both EBVaGC and EBVnGC cases, but not in all of them. The assay used in our experiment binds in the junction of the exon 2 and 3, where several polymorphisms are described, and therefore any mutation or polymorphism that affect amino acid sequence may have impact on the ability to detect mdm2.

Results showed mdm2 accumulation in 41.7% of the EBVaGC cases comparing to 37.0% in the EBVnGC, without statistically significant differences even when comparing the different histological subtypes of EBVnGC. If we considered the percentage of positive cells for mdm2 expression, EBVaGC were more positive comparing to EBVnGC, despite not statistically significant (p=0.089). The statistical analysis shows a clear tendency reflected on the confidence interval and results could be improved by increasing the sample number of the groups. When considering the EBVnGC histological subtypes, a significant difference was observed when comparing EBVaGC with EBVnGC of diffuse type (p=0.048). Previous studies have shown that different histological subtypes may have different levels of mdm2 upregulation (108), and this should be studied better in the future.

We have combined the information from mdm2 with p53 expression, which was accessed previously (66), in the 12 EBVaGC cases. Results showed no significant difference in the mdm2 expression among the two groups of p53 expression

(<50% and ≥50%) (p=0.310). However our data showed that cases expressing mdm2 had more frequently p53 expression in ≥50% of cells. This result is interesting since we know that mdm2 regulates p53 by linking to it and promoting its mono-ubiquitination and degradation through the proteasome. The high expression of *MDM2* may be explained by the higher necessity of degradation of p53, which is also highly expressed in these cases. Previous studies have shown *MDM2* accumulation simultaneously with wt p53 in other tumor types, namely colorectal cancer and oral squamous cell carcinoma, and that has been associated with bad prognosis, in contrast to what is expected for EBVaGC (124, 125). A possible explanation for this mutual accumulation may be the presence of the proteins in a complex, possibly stabilized by viral interference. Further studies should be used to clarify the interaction between these two proteins in these cases.

To assess if this mdm2 accumulation pattern was due to *MDM2* increased transcription, we investigated *MDM2* mRNA. Overall, results show no significant differences between the mRNA levels of EBVaGC versus EBVnGC (p=0.110). The analysis revealed the presence of three "outliers" from the same histological subtype, and therefore we performed a statistical analysis according to the EBVnGC histological subtype. In this analysis we observed that *MDM2* mRNA levels were significantly increased in EBVaGC comparing with EBVnGC intestinal type (p=0.034), and tend to an increase when compared with EBVnGC of indeterminated subtype (p=0.057). The comparison between the percentage of cases with mRNA expression among cases positive and negative for IHC staining showed about 50% in all of the groups, indicating that mRNA levels do not seem to be responsible for protein accumulation.

We have also aimed to describe if the most common *MDM2* polymorphism, SNP309, is involved in the expression of *MDM2* in these cancers. Our results show that in EBVaGC, 5 cases were TT and 7 cases were TG; and among the EBVnGC, 12 cases were TT, 13 were TG and 2 were the GG genotype. The GG genotype was not present in the EBVaGC samples, which can be explained by the reduced sample size and its prevalence in our population. Upon statistical analysis we observed that there is no significative difference in allele frequency between the two groups (p=0.577) nor between EBVnGC histological subtypes. When crossing the information of SNP309 genotype and *MDM2* mRNA expression, we observed that globally, both genotype seemed to be associated with similar mRNA expression. This result was rather confusing, since literature describes this change from T to

G in the 309 nucleotide of P2 promoter as being associated with higher affinity to SP1 transcription factor leading to *MDM2* higher expression (94). The differences observed in the results may be due to the fact that we were only analyzing tumor tissue and not normal tissue, which may have implications in the SNP309 impact. Nevertheless, when we performed the analysis in the different groups, we observed that the EBVaGC TT genotype had increased expression when comparing to TG, while the EBVnGC showed similar expression in both genotypes, which can imply viral interference. With our results, we can conclude that there seems to be an increased expression of *MDM2* in EBVaGC, which reflects in a higher number of cases with higher proportion of positive cells. This *MDM2* overexpression is correlated with genotype TG at SNP309, which may, or not, be due to increased transactivation of Sp1 or another transcription factor, induced by viral protein or miRNA.

The results also point that the mdm2 increase is not reflected directly in p53 decrease, which indicates that there is something not understood in the p53-mdm2 interaction. EBNA-LP, is a viral protein described as being able to target p14 ARF, which binds to mdm2 and inhibits its degradation of p53, nevertheless, this protein is not described as being expressed in EBVaGC. Thus, the lack of this protein in these cases may possibly lead to increased difficulty in p53 degradation in EBV associated cancers.

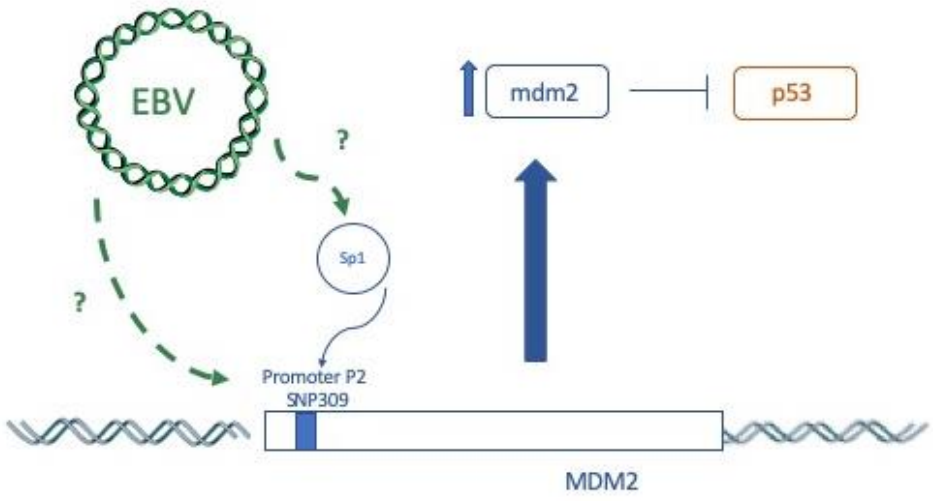


Figure 19 – Representative scheme of MDM2 regulation in EBVaGC.

V. GENERAL DISCUSSION AND FUTURE PERSPECTIVES

The p53-pathway and mdm2-p53 interaction have been described for several years as important steps in carcinogenesis. This was the first study describing *MDM2* in EBVaGC and we obtained some interesting results.

We can conclude that there is an increased expression of *MDM2* in EBVaGC, which may be explained by an increase of transcriptional activity within the *MDM*2 promoter. There is no study showing what happens in these specific cancers types, and we may theorize that a viral protein or miRNA may contribute for this activation and overexpression, leading to p53 mono-ubiquitination and suppression. Nevertheless, this mdm2 increase does not reflect in a p53 decrease, which indicates that there is something in the p53-mdm2 balance promoting accumulation of both proteins, and the imbalance of the regulation mechanism of p53-mdm2. In order to better understand this distinctive expression of the p53 pathway proteins, further studies are needed, such as the assessment of proteins ubiquitination and phosphorylation and if p53 and mdm2 are indeed forming a complex in these cases.

Our study also points for the potential impact of specific SNPs in gastric cancer development, and it seems to be important to increase the number of studies analyzing the different SNPs (especially considering SNP309) in different populations and also in different histological/molecular subtypes of gastric cancer.

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APPENDIX I:

Ethic's committee authorization

CENTRO DE INVESTIGAÇÃO

IPOPORTO
INSTITUTO PORTUGUÊS DE ONCOLOGIA DO PORTO FG, EPE

Exmo. Sr.
Doutor Hugo Sousa
Coordenador do Grupo de Oncologia
Molecular e Patologia Viral do CI-IPOP

Porto, 13 de Junho de 2014

Assunto: Aprovação de Projecto de Investigação (CES 80/14)

Caro Doutor Hugo Sousa,

Venho desta forma informar que está aprovado o projecto de investigação "**EBV-associated gastric cancer: epidemiological and molecular characterization of viral carcinogenesis**" a desenvolver no Grupo de Oncologia Molecular e Patologia Viral do Centro de Investigação do IPO-Porto, no âmbito do doutoramento da Mestre Joana Patrícia Costa Ribeiro.

Mais informo que a Comissão de Ética deu parecer positivo em relação a este projecto de investigação (ver cópia anexa).

Com os melhores cumprimentos,

Dr. Manuel Teixeira
Director do Serviço
N.º Mec.: 2107

Prof. Doutor Manuel Teixeira
Director do Centro de investigação do IPO-Porto

Serviço de Genética, Instituto Português de Oncologia do Porto Francisco Gentil,
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http://sites.google.com/site/ipoportoresearchcenter/

Parecer CES IPO: reav80/014
Assunto: Estudo de Investigação ***EBV-ASSOTIATED GASTRIC CANCER: EPIDEMIOLOGICAL AND MOLECULAR CHARACTERIZATION OF VIRAL CERCINOGENESIS***
Investigadora: **Dr.ª Joana Patrícia Costa Ribeiro**
Data: 08 de Maio de 2014

PARECER

Após receção dos elementos solicitados, é parecer desta CES não existir impedimento de natureza ética ao desenvolvimento do referido estudo de investigação.

Dr. Artur Lima Bastos
Presidente da CES do IPO Porto EPE

APPENDIX II:

Poster IJUP 2019

Murine Double Minute 2 characterization in Epstein-Barr Virus associated Gastric Cancers

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Aim

The aim of this study is to characterize MDM2 expression and variations in EBV-associated gastric carcinomas (EBVaGC).

The specific aims of the study are: 1) to characterize MDM2 variations; 2) evaluate the expression profile of MDM2; and 3) to investigate the correlation of MDM2 with EBVaGC characteristics (viral strains, viral activity, p53 protein expression).

Introduction

Literature shows that viruses are responsible for about 15-25% of all cancers[1], being Epstein-Barr Virus (EBV), which is easily spread through saliva[2] and due to that present in more than 90% of the world population[3], one of the most significant. EBV was the first virus to be directly associated with human cancers and is considerated a Group-I carcinogen by the International Agency for Research on Cancer (IARC) since 1997[4].

Gastric cancer (GC) is the 5th most common cancer, with the 3rd higher mortality rate worldwide in both genders. In Portugal, are predicted 2 885 new cases and 2 275 deaths due to gastric cancer, only last year.[5] A recently published classification on gastric cancer, from The Cancer Genome Atlas group, describe four distinctive subtypes, being tumors positive for Epstein–Barr Virus one of them[6], that is defined by monoclonal proliferation of carcinoma cells with latent EBV infection[7].

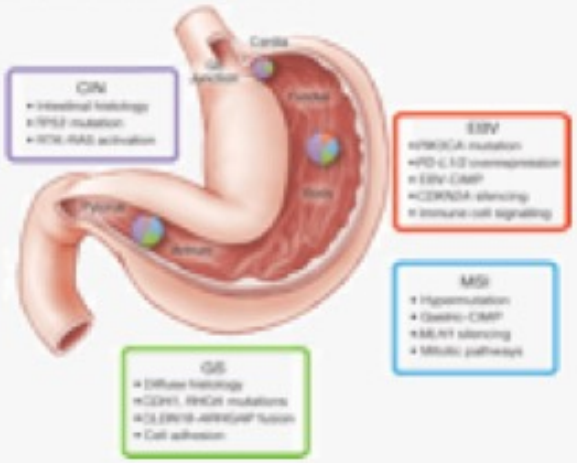

Figure 1 – Essencial features of gastric cancer subtypes, according to the Cancer Genome Atlas Research Network, 20146.

Recent reports, including one from our group[8], shows that EBVaGC corresponds to 10% of all GC[9] and is characterized by a distinct viral latency profile[10]. EBVaGC has some distinctive features in terms of genome alterations, among them no p53 mutations[11]. In a recent study from our group, we have shown that EBVaGC present a lower level of TP53 mRNA and higher level of p53 protein when compared with non-EBV associated cancers[12].

This pattern of p53 expression is not well understood and some authors refer that the Murine Double Minute 2 (MDM2) protein, p53 major negative regulator[13], may be the explanation[14]. In normal cases, MDM2 binds to p53, leading to the inhibition of the transcriptional activities, followed by the promotion of proteasomal degradation and ended by the export of p53 from the cell nucleus[15] (Figure 2). Therefore, p53 protein high levels could be explained by some anomaly in MDM2 protein, leading to lack of degradation.

Figure 2 – Representative scheme of p53 regulation by MDM2 (Nag S. 2013).

Materials and Methods

Sample selection

In this study, we will use tumor tissues samples collected from the institution archives from 39 patients with Gastric Cancer: 9 EBV-positive and 30 matched EBV-negative cases. Cases were selected from previous studies from our group[11]. This study is part of a project approved by the ethical committee of IPO Porto (CES IPO 74/2015).

Tissue samples will be used for both immunohistochemistry and nucleic acid analysis. DNA and RNA were extracted from tumor tissues collected from formalin-fixed paraffin-embedded (FFPE) tissue blocks.

MDMD 2 SNP209 polymorphism genotyping

The polymorphism will be genotyped by polymerase chain reaction (PCR) according to the protocol described by Sousa et al 2011. The PCR amplification results in a 121bp fragment, which was digested for 45min by FastDigest PstI restriction enzyme (Thermo Scientific, CA) at 37°C and were evaluated through electrophoresis (Figure 3). In the presence of the G allele, the amplicon is cleaved by the restriction enzyme originating two fragments, with 104 and 17 bp, whereas the T allele is not cleaved by the enzyme.

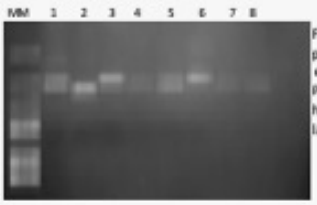

Figure 3 - PCR-RFLP analysis of MDM2 SNP309 polymorphism. Agarose gel (3%, w/v) stained with ethidium bromide: MM - 50bp DNA ladder; lane 1 - Positive control; lane 2 - MDM2 SNP309 T allele homozygous; lanes 3 and 6 - G allele homozygous; and lanes 4, 5, 7 and 8 - heterozygous.

MDM2 mRNA expression

MDM2 and GAPDH (reference gene) mRNA levels will be assessed by two-step real-time PCR. Reverse transcriptase reactions will be performed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster CA, USA) according to the manufacturer's instructions. Reactions will be performed in duplicates with a 10 µl final volume mixture containing 1X of TaqMan® Universal PCR Master Mix (Applied Bio- systems, Foster City, California USA), 1X RNA Assay (Applied Biosystems, Foster City, California USA), and 20-100 ng of cDNA. Amplification will be performed in Applied Biosystems Step-One Real Time PCR System (Applied Biosystems, Foster CA, USA). The relative quantification of MDM2 mRNA expression was analyzed using the 2DDCt method (Livak method).

MDM2 expression in tissues.

The protein levels in the tissue will be assessed by IHC using a specific monoclonal antibody (pe: IF2, Thermo Fisher Scientific, California) (Figure 4). MdM2 expression will be defined as positive when more than 5% of neoplastic cells showed MDM2 nuclear staining.

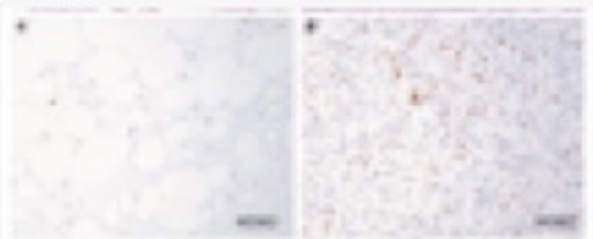

Figure 4 – Representative image of IHC staining MDM2 with the IF2 in dedifferentiated liposarcomas. Adapted from Horvai, AE, et al, 2009.

Expected Results

Finished the project I hope to understand if the p53 deregulation is related directly with MDM2, assuming that the two protein levels observed are related, or if the accumulation is due to the role of another protein in the pathway.

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APPENDIX III:

Poster ESCV 2019

MDM2 REGULATION OF P53 IN EPSTEIN-BARR VIRUS ASSOCIATED GASTRIC CANCER (PRELIMINAR STUDY)

22ND ESCV EUROPEAN SOCIETY FOR CLINICAL VIROLOGY
11-14 September 2019 Copenhagen - Denmark

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Introduction

The Cancer Genome Atlas (TGCA) group describes four distinctive Gastric Cancer (GC) subtypes, including a group of tumors positive for Epstein–Barr Virus (EBVaGC)[1]. These tumors are characterized by monoclonal proliferation of carcinoma cells with latent EBV infection[2] and corresponds to 10% of all gastric cancers[3].

EBVaGC has some distinctive features in terms of genome alterations [4]. In a recent study from our group, we have showed that EBVaGC has no p53 mutations and presents a lower level of TP53 mRNA but a higher level of p53 protein accumulation, when compared with non-EBV associated cancers (EBVnGC)[5]. This pattern of p53 expression is not well understood and some authors refer that the Murine Double Minute 2 (MDM2) protein, p53 major negative regulator[6], may contribute for this p53-regulation[7].

Materials and Methods

Sample selection

In this study, we used tumor tissues samples collected from IPO-Porto archives from 40 patients with Gastric Cancer: 12 EBV-positive (EBVaGC) and 28 matched EBV-negative (EBVnGC) cases. Cases were selected from previous studies from our group[5].

MDM2 expression in tissues

The protein levels in the tissue were assessed by IHC using the antibody IF-2 mouse antihuman MDM2 monoclonal antibody diluted 1:300 (Invitrogen, CA, EUA). Analysis of the results was performed by a pathologist of our institution and MDM2 expression was defined as positive when more than 10% of neoplastic cells showed MDM2 nuclear staining, according with Günther T. *et al* 2000[8].

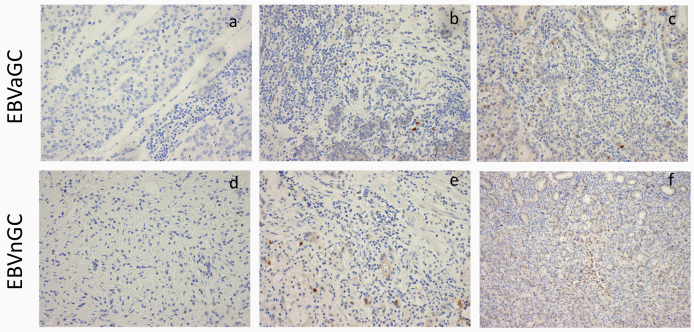

Figure 1 - Examples of immunohistochemistry staining on gastric carcinomas. MDM2 (200x): a and d) Negative; b and e) Positive: 10%; c) Postitive over 50%. MDM2 (100X): f) Positive over 50%.

MDM2 mRNA expression

MDM2 (hs01066930_m1) mRNA levels were assessed by two-step real-time PCR, with a 10µl final volume mixture containing 1X TaqMan Universal PCR Master Mix, 1X RNA assay (TaqMan Gene Expression Assay, Applied Biosystems, Foster CA, USA) and 20 ng of cDNA. RPL29 and β2M (hs00988959_gh and hs0018742_m1, respectively) were used as reference genes for standardization of analysis. Amplifications were performed in Applied Biosystems Step-One Real Time PCR System (Applied Biosystems, Foster CA, USA). The relative quantification of MDM2 mRNA expression was analyzed using the 2DDCt method (Livak method).

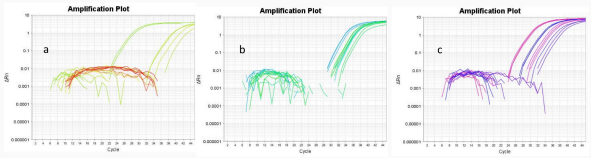

Figure 2 - RT-PCR curve for a) MDM2; b) β2M; and c) RPL29.

MDM2 SNP209 genotyping

The polymorphism was genotyped by polymerase chain reaction (PCR) using primers forward 5'-GAT TTC GGA CGG CTC TCG CGG C-3' and reverse 5' -CAT CCG GAC CTC CCG CGC TG-3'.

The PCR amplification results in a 121bp fragment, which was digested with FastDigest *PstI* restriction enzyme (Thermo Scientific, CA). In the presence of the G allele, the amplicon is cleaved by the restriction enzyme originating two fragments, with 104 and 17bp.

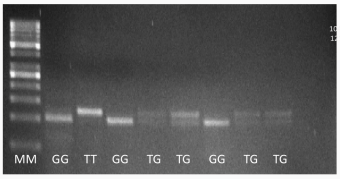

Figure 3 - PCR-RFLP analysis of MDM2 SNP309. Agarose gel (3%, w/v) stained with ethidium bromide.

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Aim of the study

The aim of this study is to characterize MDM2 expression and variations in EBV-associated gastric carcinomas (EBVaGC).

The specific aims of the study are to:

1) evaluate the expression profile of MDM2 (mRNA and protein);
2) characterize the MDM2 SNP309 polymorphism;
3) investigate the correlation of MDM2 with EBVaGC characteristics

Results

MDM2 expression

IHC for MDM2 showed its accumulation in 16 cases, 5 of them EBVaGC – Table1. The majority EBVaGC cases were negative (58.3%) while EBVnGC showed MDM2 accumulation in 59.0% of the cases. A statistical difference regarding MDM2 expression in tissue was not observed in any of the groups.

Table 1 – Distribution of cases, regarding MDM2 expression in tissue

	Negative	Positive	p-value	<50%	>=50%	p-value
EBVaGC, n (%)	7 (58.3%)	5 (41.7%)		1 (20.0%)	4 (80.0%)	
EBVnGC, n (%)	17 (63.0%)	10 (37.0%)	0.851	8 (80.0%	2 (20.0%)	0.089
Diffuse, n (%)	2 (28.6%)	5 (71.4%)	0.350	5 (100%)	----	0.048
Intestinal, n (%)	8 (88.9%)	1 (11.1%)	0.325	----	1 (100%)	1.000
Indeterminate, n (%)	7 (63.6%)	4 (36.4%)	1.000	3 (75.0%)	1 (25.0%)	0.206

MDM2 mRNA expression

qRT-PCR results revealed no significative difference between EBVaGC and EBVnGC cases regarding MDM2 mRNA expression.

When we divided the EBVnGC into histological subtypes, a significant increased MDM2 mRNA in EBVaGC comparing to EBVnGC indeterminate type (p=0.057) and decreased when comparing with EBVnGC intestinal type (p=0.034) – Figure 4.

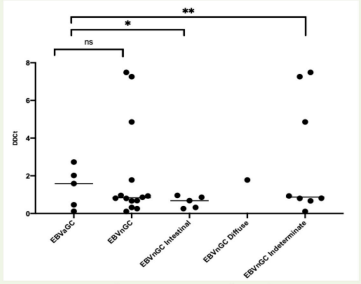

Figure 4 – MDM2 relative expression in all GC subgroups. *p=0.034; **p=0.057.

MDM2 SNP309

SN309 genotype analysis showed no significant difference in allele frequency among EBV positive and negative groups (*p*=0.577), neither when considering the different subtypes – Table 2.

Table 2 – Descriptive table of SNP 309 genotyping

	TT	TG	GG	p-value*	OR, 95% CI*
EBVaGC, n (%)	5 (41.7%)	7 (58.3%)	0		
EBVnGC, n (%)	12 (44.4%)	13 (48.1%)	2 (7.4%)	0.577	1.12 (0.28-4.43)
Diffuse, n (%)	3 (42.9%)	2 (28.6%)	2 (28.6%)	1.000	1.05 (0.16-6.92)
Intestinal, n (%)	4 (44.4%)	5 (55.6%)	0	1.000	1.12 (0.20-6.42)
Indeterminate, n (%)	5 (45.5%)	6 (54.5%)	0	0.593	1.17 (0.20-6.08)

*Considering G-carrier as the risk model. OR associated with EBVaGC

At a global level, TG genotype tends to be associated with a higher mRNA expression but when we divided the cases into EBV positive and negative, the EBVnGC maintained this pattern while the EBVaGC showed higher expression associated with the TT genotype, while comparing with the TG cases – Figure 5.

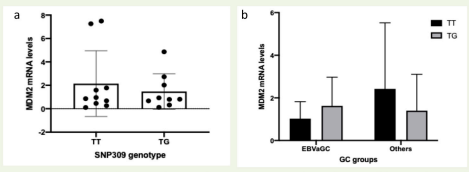

Figure 5 – MDM2 mRNA levels, according with SNP genotype: a) in all cases; b) according with EBV status.

Conclusion

In normal cases, MDM2 binds to p53, leading to the inhibition of the transcriptional activities, followed by the promotion of proteasomal degradation and ended by the export of p53 from the cell nucleus[9]. Therefore, p53 protein high levels could be explained by some anomaly in MDM2 protein, leading to lack of degradation.

In our study, we observed that EBVaGC has a different pattern of expression regarding the SNP309, possibly indicating some interference in the Sp1 transcription factor. Even though this alteration, the MDM2 expression seems to be similar in EBVaGC and EBVnGC regarding both mRNA and protein accumulation in the tissue, indicating that the previously mentioned alteration is not noticed at a global level.