CHARACTERIZATION OF EBV-ASSOCIATED GASTRIC CANCERS

Caracterização de carcinomas gástricos associados ao EBV

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by

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RESUMO

A nível mundial, o cancro gástrico (CG) é o sexto mais comum com cerca de 1 milhão de novos casos estimados em 2012. Em Portugal, os dados revelam que o CG é o quinto cancro mais frequente com cerca de 3000 novos casos por ano. Para além disso, o CG tem uma elevada taxa de mortalidade sendo responsável por 1387 mortes nos homens e 898 nas mulheres. Dados recentes têm mostrado que o vírus Epstein-Barr (EBV) é detectado em diferentes subtipos histopatológicos de carcinoma gástrico sendo estes tumores responsáveis por aproximadamente 10% de todos os casos.

O presente estudo pretende caracterizar os carcinomas gástricos associados ao EBV (EBVaGC) através da sua detecção em tecidos tumorais gástricos de 136 pacientes atendidos no Instituto Português de Oncologia do Porto (IPO Porto FG EPE) no ano de 2011. A detecção de EBV foi realizada por hibridização *in situ* (ISH) utilizando uma sonda de ADN complementar para ARNs codificados pelo EBV (EBERs).

Os resultados demonstraram que os carcinomas associados ao EBV representam 6,6% de todos os casos de CG. Analisando a distribuição de EBV entre os diferentes tipos histológicos, observou-se que o EBV estava presente em 6,6% dos tipos intestinais, em 11,1% dos tipos indeterminados e em 100% dos linfoepiteliomas, contudo nenhum caso foi detectado nos carcinomas difusos (p<0,001). A análise de risco, apesar de não ter sido estatisticamente significativa, sugeriu que os pacientes com carcinomas do tipo intestinal (p=0,350; OR=1,98; 95% IC=0,37-10,5) ou indeterminado (p=0,238; OR=2,78; 95% IC=0,55-15,5) apresentam um risco aumentado de ter EBVaGC; enquanto os pacientes com carcinomas difusos (p=0,078; OR=0,14; 95% CI=0,01-2,58) apresentam um risco diminuído de ter EBVaGC. Quanto à localização do tumor, verificou-se que os carcinomas das regiões superiores do estômago apresentam um risco aumentado de ter um EBVaGC (p=0,032; OR=4,68; 95% IC=1,11-19,7).

Em conclusão, a infecção por EBV nos carcinomas gástricos em Portugal é semelhante à de outros países. Por outro lado, as características clínico-patológicas apresentaram diferenças quando comparadas com estudos anteriores, principalmente a ausência de EBV nos carcinomas difusos. Este é o primeiro estudo de caracterização dos EBVaGC em Portugal que reforça a necessidade de mais estudos para esclarecer o papel do EBV como biomarcador preditivo/prognóstico no desenvolvimento do cancro gástrico.

PALAVRAS-CHAVE: Vírus Epstein Barr, Carcinomas gástricos associados ao vírus Epstein Barr, hibridização *in situ*

ABSTRACT

Worldwide, Gastric Cancer (GC) is the sixth most common malignancy with nearly 1 million new cases estimated in 2012. In Portugal, data reveal that GC is the fifth most frequent cancer with about 3000 new cases per year. Moreover GC has still a higher mortality rates being responsible for 1387 deaths in men and 898 in women. Recent data showed that Epstein-Barr virus (EBV) has been detected in different histopathological subtypes of gastric carcinoma and EBV-associated gastric carcinoma (EBVaGC) accounts about 10% of all cases.

This study pretends to characterize EBVaGC in our population through detection of EBV in gastric carcinoma tissues from 136 consecutive patients attended at Portuguese Institute of Oncology of Porto (IPO Porto FG EPE) in the year of 2011. EBV detection was performed by *in situ* hybridization (ISH) targeting EBV-encoded small RNA (EBER-ISH) with an EBER-DNA probe.

The results showed that in our population EBVaGC represent 6.6% of all GC cases. Analyzing the distribution of EBV among the different histological types, we observed that EBV was present in 6.6% of intestinal-types, 11.1% of indeterminate types, 100% of lymphoepithelioma-like carcinomas and there were no positive cases among diffuse types (p<0.001). The risk analysis revealed that, despite there are not statistically significant differences, patients with intestinal (p=0.350; OR= 1.98, 95% CI=0.37-10.5) and indeterminate (p=0.238; OR=2.78, 95% CI=0.55-15.5) GC have an increased risk of having an EBVaGC; while diffuse GC (p=0.078; OR=0.14, 95% CI=0.01-2.58) have a decreased risk of having an EBVaGC. Regarding tumor location, the results demonstrated that patients with tumors in upper regions of stomach have an increased risk to have an EBVaGC (p=0.032; OR=4.68, 95% CI=1.11-19.7).

In conclusion, the EBV infection rate among gastric carcinomas in Portugal is similar to that ascertained in other countries. Conversely, the clinicopathological features showed differences when compared with previous studies, mainly the absence of EBV in diffuse-type gastric carcinomas. This is the first study to characterize EBVaGC in Portugal which reinforce the need of further studies to clarify the role of EBV and to explore its potential value as predictive/prognostic biomarker in gastric cancer development.

KEY-WORDS: Epstein Barr Virus, Epstein Barr virus-associated gastric cancer, *in situ* hybridization

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ABBREVIATIONS

APC: Adenomatous Polyposis Coli

ASR: Age Standardized Rates

BARTS: BamHI A Rightward Transcripts

BL: Burkitt's Lymphoma

CagA: Cytotoxin Associated Antigen A

CagPAI: *Cag* Pathogenicity Island

CISH: Chromogenic *in situ* Hybridization

COX2: Cyclooxygenase 2

CR2: Complement Receptor 2

DAB: 3,3'-Diaminobenzidine

DNA: Deoxyribonucleic Acid

DNMT1: DNA Methyltransferase 1

DDR: DNA Damage Response

EBER: Epstein–Barr Virus-Encoded Small RNAs

EBNA: Epstein–Barr Nuclear Antigen

EBNA-LP: Epstein-Barr virus Nuclear Antigen Leader Protein

EBV: Epstein Barr Virus

EBVaGC: EBV-associated Gastric Cancer

FFPE: Formalin-Fixed Paraffin-Embedded

GC: Gastric Cancer

HBV: Hepatitis B virus

HCV: Hepatitis C virus

HDGC: Hereditary Diffuse Gastric Cancer

HHV-4: Human Herpes Virus 4

HHV-8: Human Herpes Virus 8

HL: Hodgkin's Lymphoma

HPV: Human papilloma virus

HRP: Horseradish Peroxidase

HTLV-1: Human T lymphotropic virus

ISH: *in situ* Hybridization

IHC: Immunohistochemistry

IgA: Immunoglobulin A

LCLs: Lymphoblastoid Cell Lines

LELC: Lymphoepithelioma-Like Carcinoma

LMP: Latent Membrane Proteins

miRNAs: MicroRNAs

mRNA: Messenger RNA

NBs: Nuclear Bodies

NF-κB: Factor Nuclear kappa B

NK: Natural Killer

NPC: Nasopharyngeal Carcinoma

PI3K: Phosphoinositide3-Kinase

PML: Promyelocytic Leukemia

PTEN: Phosphatase and Tensin Homolog

PTLD: Post-Transplant Lymphoproliferative Disorders

RNA: Ribonucleic Acid

TFSS: Translocate the Bacterial Effectors

VacA: Vacuolating Cytotoxin

WHO: World Health Organization

I. INTRODUCTION

1. Virus and Cancer

The first association between a virus and cancer was shown in 1911 when Rous discovered chicken sarcoma and attributed Rous Sarcoma Virus (RSV) as the etiological agent (Rous, 1911). Over the next 100 years, several viruses have shown to be associated with cancer development. In the past 30 years, the viruses have been fundamental instruments in scientific investigation since they allow to explain the mechanisms of carcinogenesis in some types of human tumors (Butel, 2000).

Approximately 12% of all human cancers worldwide may be attributed to viruses (Mesri *et al*., 2014). Both DNA and RNA viruses have been well described as agents responsible to induce either single or multiple tumors (Table 1.1). This variability is supported by characteristic tissue tropism of the given virus (Butel, 2000, Chang *et al*., 2013).

Table 1.1 - Viruses and its related human tumors

Virus	Genome	Human cancers	Cell tropism
EBV	DNA	Burkitt's lymphoma NK-T cell lymphoma Lymphomas in immunosuppressed host Nasopharyngeal carcinoma (NPC) Gastric carcinoma	B-cells, oropharyngeal and gastric epithelial cells
HBV	DNA	Hepatocellular carcinoma	Hepatocytes
HCV	RNA	Hepatocellular carcinoma	Hepatocytes
HHV-8	DNA	Kaposi sarcoma	B-cells
HPV	DNA	Cervical carcinoma	Squamous epithelial cells
HTLV-1	RNA	Adult T-cell lymphoma/leukemia	T cells

Nevertheless, the viral infection is generally not sufficient to induce the development of cancer. Only a minority of infected individuals progress to cancer and it occurs mainly years or even decades after primary infection (Butel and Fan, 2012). Additional factors such as immunosuppression, genetic predisposition, somatic mutations, epigenetic alterations and environmental exposure to carcinogens have demonstrated to be relevant for virus-associated tumorgenesis (Au, 2004, Junjie *et al*., 2012, Li *et al*., 2013).

The role of viruses in cancer has been demonstrated by *in vitro* transformation of cells which has allowed to elucidate functions of viral proteins involved in carcinogenesis both at molecular and biochemical levels (Butel and Fan, 2012).

Human oncogenic viruses rely on persistence in host cells through their replication and immune evasion. Persistent infection can lead to cancer development by acquisition of cancer hallmarks as result of activation of anti-apoptotic and proliferative programs (Figure 1.1). Each cancer hallmark represents a biological consequence of oncogenic alterations by viral oncogenes that include:

1) Host Signaling: Viral proteins can modulate host-signaling mechanisms that regulate cell growth and survival;
2) DNA damage response (DDR): Viral replication induces DDR however host cells acquire genetic instability leading to increase of mutations rate and chromosomal alterations;
3) Chronic inflammatory responses to persistent viral infections: Inflammation leads to oxidative stress by reactive oxygen species generation that promotes acquisition of mutations.

The most cancer-related mutations are found in target genes which either can promote a gain of function of oncogenes or lead to the loss of function of tumor-suppressors genes (Hanahan and Weinberg, 2000).

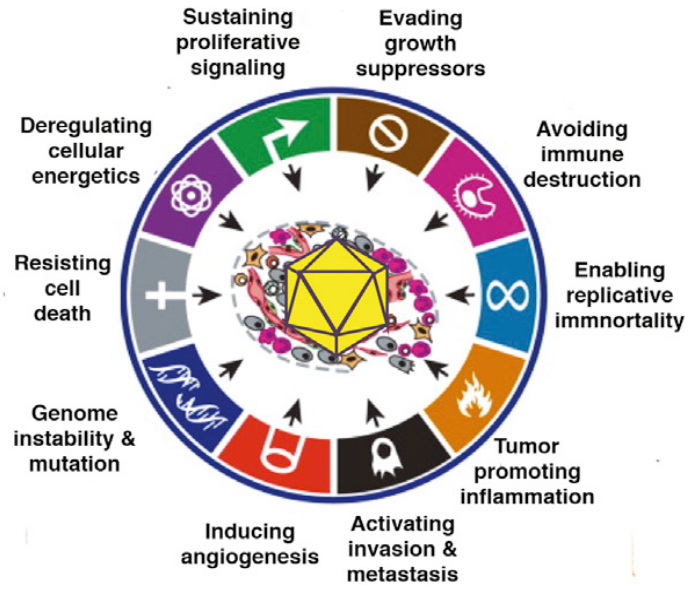

Figure 1.1 – Hallmarks of Cancer by human oncoviruses (Mesri et al., 2014)

2. Epstein Barr Virus (EBV)

2.1 Structure and characteristics

Epstein-Barr virus (EBV), also called Human Herpes Virus 4 (HHV-4), is a member of the *Herpesviridae* family. It is ubiquitous in nature and infects more than 90% of adult population worldwide. Primary infection normally occurs in childhood or early adulthood through salivary contact. Majority of children are asymptomatic, but some adolescents and young adults can develop infectious mononucleosis with harmless clinical manifestations (Grotto *et al*., 2003). As other members of *Herpesviridae* family, EBV is composed of a linear double-stranded DNA genome surrounded by an icosahedral nucleocapsid which encodes about 85 genes. The viral capsid contains 162 capsomeres, a protein tegument and an outer envelope with glycoprotein spikes (Thompson and Kurzrock, 2004).

EBV has been divided into two major types, EBV-1/EBV-A and EBV-2/EBV-B. Worldwide EBV-1/EBV-A is the most frequent type while EBV-2/EBV-B is more characteristic in Africa (Zimber *et al*., 1986). These variants show differences between nuclear antigens EBNA2, 3A, 3B, 3C, although the most sequence variation occurs in EBNA2-encoding gene, with only 50% of similarity (Cancian *et al*., 2011).

EBV exhibits dual tropism infecting both B-lymphocytes and some types of epithelial cells, especially those of nasopharynx. Cell entry is mediated through interaction of at least five envelope viral glycoproteins with host cell surface. The mechanisms of entry are different depending if EBV infects B-cells or epithelial cells (Shannon-Lowe and Rowe, 2014). In B-cells the virus enters via endocytosis (Figure 1.2). An initial interaction occurs between the major glycoprotein of the viral envelope (gp350/220) and B-cell specific complement receptor 2 (CR2 or CD21) (Tanner *et al*., 1987, Shannon-Lowe and Rowe, 2014). However, it has been referred that the entry into the cell is mediated by other viral glycoproteins (gp42, gH, gL and gB) which also interact with the host cell membrane. Gp42 binds to B-cell HLA class II and it is achieved through binding gH/gL complex that modify gp42 conformational form. Moreover, gH and gL glycoproteins are also responsible for the activation and recruitment of gB, another ligand of B-cell receptors (Molesworth *et al*., 2000, Hutt-Fletcher, 2007).

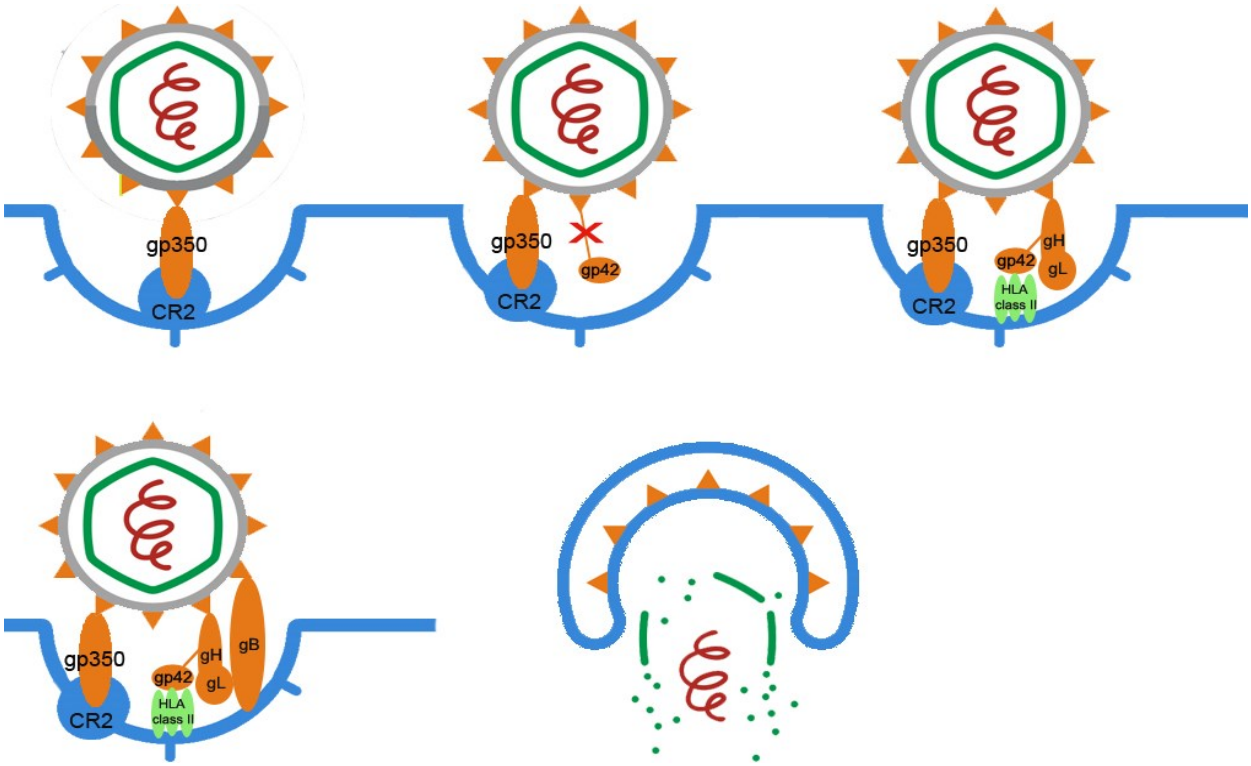

Figure 1.2 - Model of EBV entry into B-lymphocytes. The first interaction occurs between gp350 and CR2. Following this step, gp42 is cleaved to be possible the gp42/gH/GL complex formation. This complex can modify gp42 conformation to a form that is essential for HLA class II binding. Gp42-HLAII interaction is insufficient to establish full fusion. Thus, gH/gL complex is also responsible for activation, recruitment and binding of gB glycoprotein which promotes the fusion between the viral envelope and the B-cell endocytic membrane.

Regarding EBV infection of epithelial cells, some reports have described absence or restriction of CD21 expression and therefore the attachment of EBV in epithelial cells is less efficient (Kim *et al*., 1998, Burgos and Vera-Sempere, 2000, Jiang *et al*., 2008). The absence of CD21 in certain cells has led to study of others possible mechanisms that explain EBV entry (Shannon-Lowe and Rowe, 2014). One of these is explained by the virus coated with immunoglobulin A (IgA) specific to gp350/220 binds to the polymeric immunoglobulin A-receptor from surface membrane (Sixbey and Yao, 1992). Other mechanism is the interaction between gH/gL complexes with specific molecules present in epithelial cells (integrins) such, avb5, avb6 and avb8 (Chesnokova *et al*., 2009). Finally, on polarized epithelial cells, an interaction between a multispanvirus

membrane protein encoded by the BMRF2 openreading frame and integrins has also been demonstrated (Tugizov *et al*., 2003).

2.2 EBV "life" cycle

EBV infection cycle includes a lytic and a latent phase that describes a persistent infection. Latent infection is more advantageous for the virus to persist for long periods since it expresses a limited set of latent genes which can be recognized by the host immune system (Murata *et al*., 2014).

Lytic infection

During lytic infection, EBV expresses nearly 100 proteins, which play an essential role in viral replication, formation of virions as well as modulation of host immune response (Murata *et al*., 2014). Lytic cycle results in the production of infectious viral particles both after initial infection in oropharyngeal cells and reactivation from latently infected cells. Upon induction of lytic program, there are two key EBV immediate-early (IE) lytic genes (BZLF1 and BRLF1) which are expressed. BZLF1 and BRLF1 genes encode transactivators able to activate viral and certain cellular promoters triggering a coordinated cascade of viral gene expression. The early genes (E) of EBV are involved in DNA replication and metabolism whereas late genes (L) expression is associated with viral structural proteins. In contrast with latent cycle, the replication occurs through viral DNA polymerase which is expressed with the other lytic genes (Tsurumi *et al*., 2005).

Latent infection

During latent phase, EBV remains persistently in infected cells and the viral DNA present in the nucleus acquires episomal or integrated form by fusing terminal repeats (TR, repetitive 500-bp structures) at both ends (Reisinger *et al*., 2006). EBV-terminal repeats have a specific structure contributing to evaluation of EBV clones, viral integration and the state of viral activation (Fukayama *et al*., 2008). The replication occurs in synchronization with the host genome (S-phase) and then EBV genome is delivered to daughter cells in mitosis (Murata *et al*., 2014).

Latent infection is characterized by a limited set of viral genes which lead to the expression of six nuclear proteins: EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C,

EBNA leader protein (EBNA-LP), and two latent membrane proteins: LMP1, LMP2. In addition, the non-polyadenylated EBER RNAs and rightward transcripts from the BamHI A region (BARTS) can be also detected (Young and Rickinson, 2004).

The expression of EBV-latent genes can affect cell cycle, apoptosis and immune response contributing to tumor initiation and propagation (Seo *et al*., 2010). According expression pattern (Table 1.2), latency can be classified into three types (I, II, III) suggesting that EBV may affect cell growth by different ways (Lorenzetti *et al*., 2010).

Table 1.2 - Latency pattern of EBV according to latent gene expression and associated malignancies (Lorenzetti *et al*., 2010)

Latency type	EBV-latent gene expression						Associated tumors
	EBERs BART's	EBNA1	LMP1	LMP2A	EBNA2	EBNA3s/EBNA-LP	
I	+	+	-	-	-	-	BL, GC
II	+	+	+	+	-	-	HL, NPC
III	+	+	+	+	+	+	PTLD's

BL: Burkitt's lymphoma; GC: Gastric carcinoma; HL: Hodgkin's lymphoma; NPC: Nasopharyngeal carcinoma; PTLD's: post-transplant lymphoproliferative disorders.

Despite there are three latency patterns, all EBNAs are only expressed in latency III through differential splicing from a large primary mRNA. Complete expression of nuclear antigens is achieved due to initial activation of Wp promoter responsible for expression of EBNA2 and EBNA-LP following Cp activation able to express the other nuclear proteins (EBNA1, 3A, 3B) as well as Wp-initiated transcripts described above. Qp is another promoter that normally is not activated in latency III. However, in latency I and II, it is active to promote only EBNA1 expression (Shannon-Lowe *et al*., 2009).

EBNA-1 contributes to maintenance and replication of episomal EBV genome through specific binding to the plasmid origin (OriP). In addition, EBNA-1 interacts with viral promoters contributing to transcriptional regulation of other EBNAs (including EBNA-1 itself) and LMP-1. EBNA-2 and LMP-1 are essential proteins for growth and transformation of both B-cells and epithelial cells. EBNA2 is a protein involved in transactivation of both cellular and viral genes. It up-regulates the expression of certain B-cell antigens such CD21 and CD23 as well as the viral proteins LMP1 and LMP2. LMP-1 induces the expression of cellular adhesion molecules, cytokines, anti-apoptotic

proteins as well as activation of transcription factors that lead to an uncontrolled cell proliferation (Young and Rickinson, 2004, Young *et al*., 2000).

Development of persistent infection

The life cycle of EBV (Figure 1.3) is initiated when the virus infects oropharyx involving squamous epithelial cells and possibly also locally-infiltrating lymphocytes. There, EBV develops a lytic replication with release of infectious viral particles able to spread throughout the lymphoid tissues and peripheral blood lymphocytes. Within B lymphocytes, the virus establishes an expression pattern designed by latency III which is characterized by expression of all six nuclear antigens (EBNA1, 2, 3A, 3B, 3C e -LP) three membrane proteins (LMP1, 2A e 2B) and two small molecules of RNA (EBER1 and 2). When EBV establishes latency III, it is able to promote B-cells transformation into lymphoblastoid cell lines (LCLs) leading to spontaneous proliferation (Odumade *et al*., 2011).

Both lytic and latent infections, the viral antigens induce strong cell-mediated responses in the immunocompetent hosts, involving natural killer (NK) cells and CD4+/CD8 T cells, resting a limited EBV-infected B-cells as long term viral reservoir. However, some of these cells can reach germinal center where down-regulate the expression of growth-transforming proteins thereby escaping immune system recognition and establishing latent state (latency 0) in re-circulating memory B-cell pool. Latency 0 is characterized by restricted viral gene expression with only expression of protein-coding transcripts, (EBERs) and BamA rightward transcript (BART) (Young and Rickinson, 2004).

Occasionally, when immune system is compromised, these resting B lymphocytes can enter the lytic cycle where the virions will infect either new B-cells or epithelial cells (Sousa *et al*., 2011).

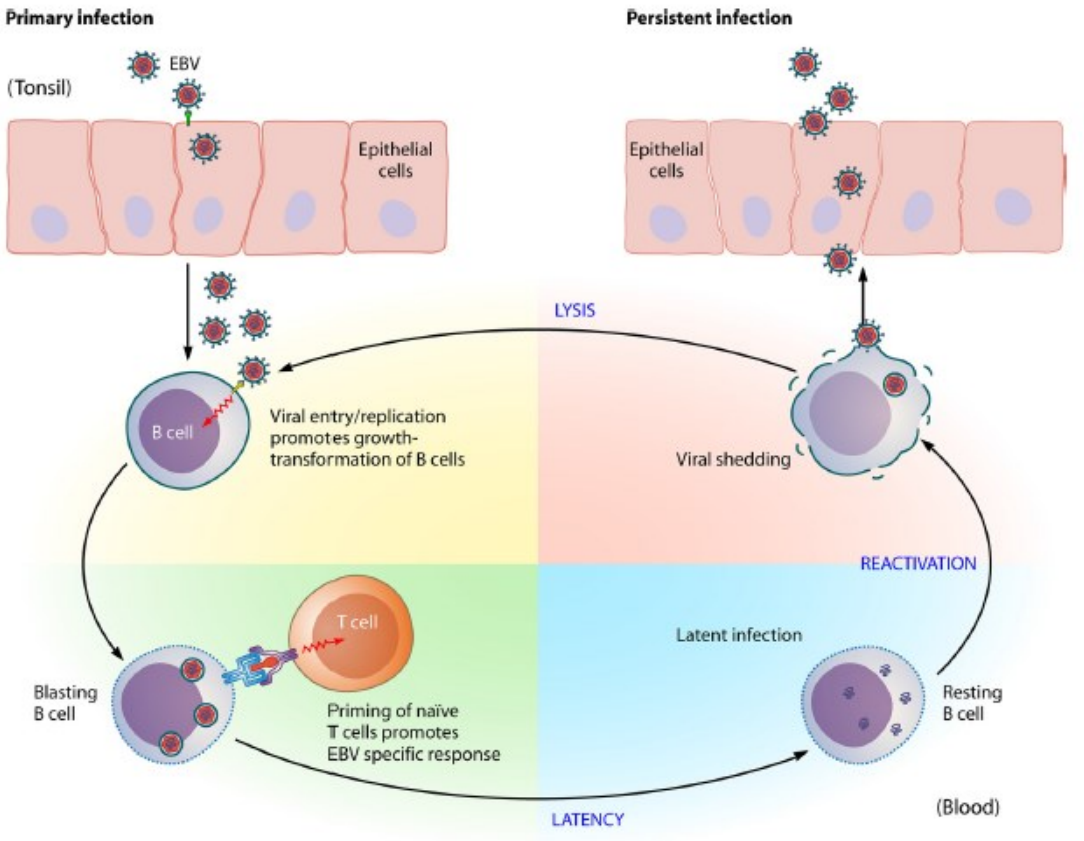

Figure 1.3 - EBV infection in healthy carriers (Odumade et al., 2011)

2.3 EBV-associated diseases

EBV was the first virus related to neoplastic cells infection in humans with a proven role in carcinogenesis. It was discovered in 1964 when virus-like particles were identified in a cell line from Burkitt's lymphoma biopsies through electron microscope (Epstein *et al*., 1964). Nowadays, it is well known that EBV can contribute to the development of several human diseases, including benign and malignant disorders. Infectious mononucleosis is a self-limiting benign and acute illness characterized by high fever, lymphadenopathy and pharyngitis or tonsillitis which occurs more frequently in adulthood and young adults, mainly when immune system is compromised. Oral hairy leukoplakia is another non-malignant disease that results in hyperplasia of epithelial cells in oral mucosa from immunocompromised patients with human immunodeficiency virus infection/acquired immunodeficiency syndrome (HIV/AIDS) or submitted to solid bone marrow transplantation (Tsuchiya, 2002).

Generally, EBV infection is well controlled by immune system. However, immune control can be disrupted leading to prolonged proliferation of EBV-infected cells and their malignant transformation. EBV latent infection has shown strong evidences in carcinogenesis of Burkitt's lymphoma (BL), NK/T cell lymphomas, nasopharyngeal carcinoma (NPC), Hodgkin's lymphoma (HL) and malignant lymphomas in immunocompromised patients such, post-transplant lymphoproliferative disease (PTLD). In contrast, in gastric and breast carcinomas its role in carcinogenesis is not well understood (Grywalska *et al*., 2013).

Recognition of EBV-infected cells in biological tissues is well achieved by detection of EBV-encoded RNAs (EBERs). These molecules are small nopolyadenylated noncoding RNAs which are the most abundant viral transcripts in latently EBV-infected cells. For this reason, EBERs are considered reliable markers when *in situ* hybridization (ISH) is used as detection method (Iwakiri, 2014).

3. Gastric Cancer

3.1 Epidemiology

Gastric cancer (GC) is the sixth most common malignancy in both sexes worldwide with nearly one million new cases estimated in 2012 (952.000 cases). The incidence rates are twice higher in male (8.5%) than in female (4.8%) with about 631.000 and 320.000 new cases per year, respectively. Nevertheless, age standardized rates (ASR) show that carcinoma of stomach is the fourth most frequent in men and the sixth in women. Despite a significant reduction of incidence and mortality rates over the past few decades, GC still remains the third leading cause of death by cancer. Approximately, 8% of cancer-related mortality in world (723.000 deaths) is attributed to tumors of stomach. It is a major health problem with a distinct distribution according to geographical areas, socio-economic conditions and ethnic diversity. More than 70% of total cases occur in developing regions being the Eastern Asia (half cases in China), Eastern Europe and Latin America the areas with the highest age-standardized incidence rates (Figure 1.4). In contrast, the lowest incidence rates are observed in United States, Australia and some North European countries (Ferlay *et al*., 2013).

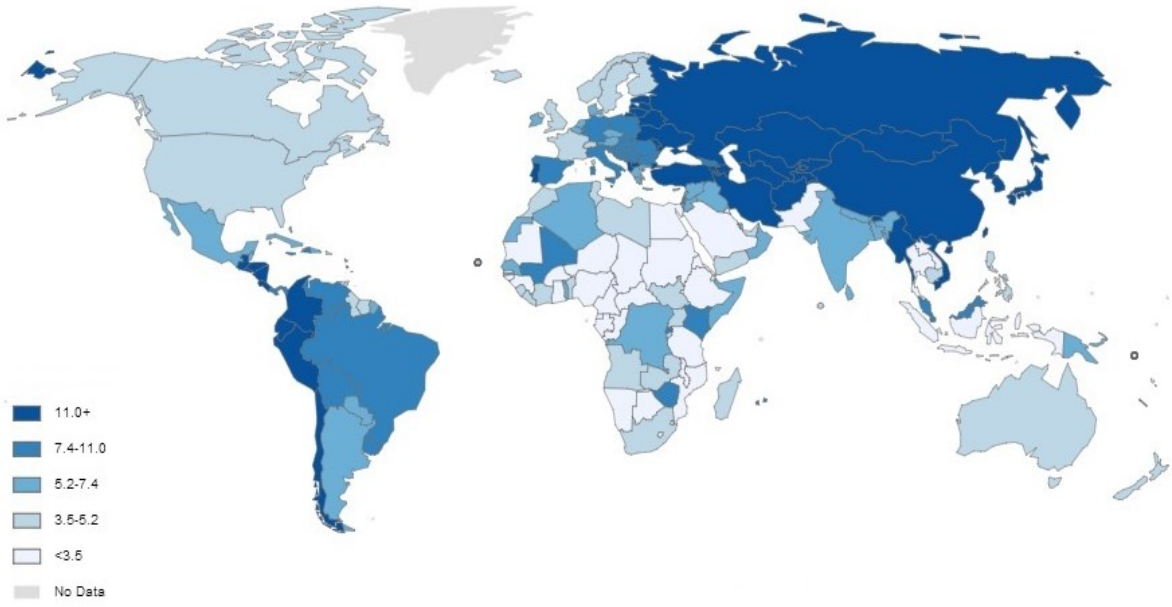

Figure 1.4 - Age-standardized incidence rates (World) per 100.000 habitants of gastric cancer in both sexes (Ferlay *et al*., 2013)

Portugal is one of the European countries with the higher incidence and mortality rates associated to gastric cancer. It represents the fifth most common cancer (Figure 1.5) in both sexes with about 3000 new cases per year. As in other countries, mortality rate is also high (9.5%) being this cancer the fifth most frequent, responsible for 1387 deaths in men and 898 in women (Ferlay *et al*., 2013).

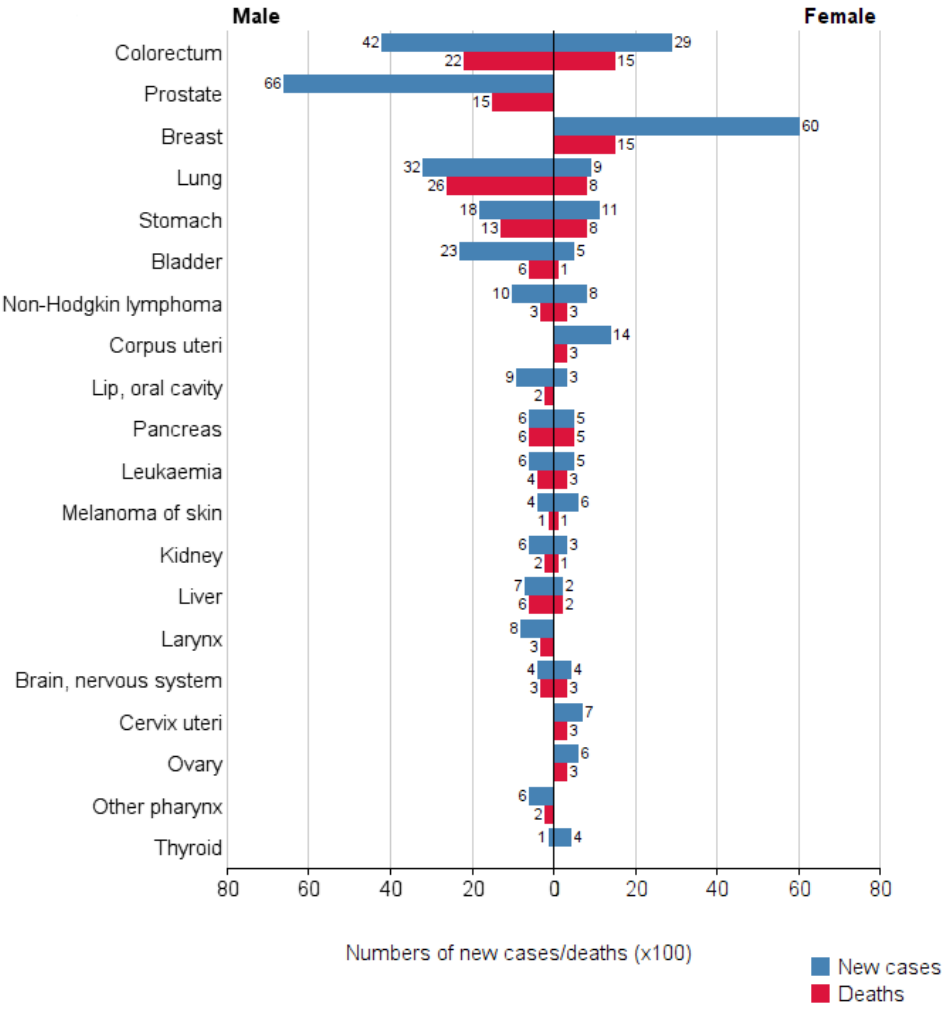

Figure 1.5 - Numbers of new cases/deaths stratified by sex in Portugal (Ferlay *et al*., 2013)

3.2 Classification

Gastric tumors are classified anatomically and histologically. Anatomically, GC is divided into proximal and distal tumors depending on their localization of stomach. Proximal tumors are found in cardia region whereas distal carcinomas are often located

in the antrum/pyloric region. Histologically, tumors of stomach show high heterogeneity at both architectural and cytological level that difficult the establishment of well-defined classification system. Some classifications have been established to classify the histologic pattern of gastric adenocarcinomas: Ming, Carneiro and Goseki, but the most commonly used are those of World Health Organization (WHO) and Lauren (Lauren, 1965, Roy *et al*., 1998, International Agency for Research on Cancer, 2010, Hu *et al*., 2012).

Lauren's classification is an essential system in gastric cancer history that over time have contributed to describe an association with several environmental factors, incidence trends and ethology. According to this classification, the two major histologic subtypes are intestinal and diffuse adenocarcinomas. The other types are classified to indeterminate type, when carcinoma is too undifferentiated and co-exist histological features, or uncommon variants (Lauren, 1965). The relative frequencies are approximately 54% for intestinal type, 32% for the diffuse type and 15% for the indeterminate type. In 2010, WHO referred five subtypes that have been correlated with Lauren's classification as described in table 1.3 (International Agency for Research on Cancer, 2010, Hu *et al*., 2012).

Table 1.3 - Comparison of gastric cancer classifications between Lauren's and WHO classification systems

WHO (2010)	Lauren (1965)
Papillary adenocarcinoma Tubular adenocarcinoma Mucinous adenocarcinoma	Intestinal type
Signet-ring cell carcinoma Poorly cohesive carcinoma	Diffuse type
Mixed carcinoma	Indeterminate
Uncommon variants	-

Lymphoepithelioma-like carcinomas or medullary carcinomas are described by WHO as an uncommon subtype but are not represented in the Lauren's classification. This specific tumor, which is characterized by uniform proliferation of cancer cells

throughout the lymphoid stroma, represents about 4% of all gastric carcinomas and more than approximately 80% of cases have EBV-infected cells (Herath and Chetty, 2008).

3.3 Risk factors

Gastric cancer is a multifactorial disease which has been etiologically associated with several genetics, environmental and lifestyle factors (Figure 1.6). Many causes have been recognized to initiation and progression of gastric carcinomas including modifiable factors (infectious agents, behavioral habits) and non-modifiable factors (age, sex). The risk factors may differ according to tumor location and histologic type whereas others are common for all types (Karimi *et al*., 2014). Some infectious agents and diet have demonstrated to be the most important risk factors to development of gastric cancer. Both *Helicobacter pylori* and diet are considered strong and well-established influences in intestinal-types (Yassibas *et al*., 2012). On the other hand, EBV has been studied for scientific community so that its presence in tumor cells can be definitely understood. Others risk factors such, family history, alcohol and tobacco have been associated to an increased risk of GC. (de Martel *et al*., 2013). Regarding to common features in overall tumors of stomach, male patients with older age are those, which have shown the greatest gastric cancers occurrence (Tural *et al*., 2012).

Figure 1.6 - Genetics, environmental and lifestyle risk factors associated to gastric cancer

Helicobacter pylori

Since 1994, WHO has considered *Helicobacter pylori* as class I carcinogenic due its role in development of gastric cancer. The presence of *H. pylori* in gastric mucosa

contributes to chronic inflammation and atrophy that can promote malignant transformation of the epithelium (Krejs, 2010, Zhu *et al*., 2014). Actually, it is clear that individuals with positive serology tests for *H. pylori* have shown an increased risk to development of GC (Kamangar *et al*., 2006). In contrast, several studies have described that early *Helicobacter pylori* eradication is associated with decreased risk of GC. (Wong *et al*., 2004, Take *et al*., 2005, Shiotani *et al*., 2010). It can reduce inflammation level preventing the progression of precancerous lesions such, gastric atrophy, intestinal metaplasia (IM) and dysplasia (Haziri *et al*., 2010). Furthermore, Fukase *et al*. (2008) provided evidences of a preventive effect of *H. pylori* eradication even in later events of carcinogenesis avoiding tumor recurrence (Fukase *et al*., 2008).

Epstein Barr Virus

During past decades, EBV has been associated to gastric cancer but it is not yet well-known if the presence of virus is a cause or consequence. A small percentage of cases has been related to EBV and it has also shown differential geographical distribution (Sousa *et al*., 2008). Several studies have described the presence of EBV in gastric tumor cells suggesting a role in carcinogenesis. Some of these studies have detected EBV infection exclusively in tumor cells, suggesting a late event in carcinogenesis (Truong *et al*., 2009, Zur Hausen *et al*., 2004). However, others investigations found EBV both in carcinoma cells and precursor lesions. Establishment of EBV in pre-malignant lesions suggests that virus may be initiating factor in early step of gastric carcinogenesis (Arikawa *et al*., 1997, Yanai *et al*., 1997). Despite these scientific evidences, clinicians are still requiring more investigation to clarify the mechanisms that may lead to initiation or promotion of EBV-associated gastric cancer.

Hereditary cancer

Genetic predisposition for gastric cancer is found in 10-15% of human population, however only 1-3% of these cases progresses to hereditary malignant diseases. Hereditary diffuse gastric cancer (HDGC) syndrome is the most common that leads to development of diffuse gastric adenocarcinoma cancer (Sereno *et al*., 2011). HDGC is autosomal dominant disease caused by germ line mutation in CDH1 gene that results in loss of expression of the cell adhesion protein (E-cadherin). Functionally E-cadherin is responsible for the maintenance of normal tissue morphology and cellular

differentiation. For this reason, CDH1 is considered a tumor suppressor gene and, when inactivating mutations occur, it contributes to the loss of cell adhesion, proliferation, invasion and metastasis (Fitzgerald and Caldas, 2004).

Diet

Epidemiological studies from different populations have shown a strong association between diet and gastric cancer, mainly in intestinal-type carcinomas (Fay *et al*., 1994, Bastos *et al*., 2010, Lunet *et al*., 2007). An adequate intake of fresh fruits and vegetables rich in polyphenols and vitamins may prevent GC. Their antioxidant effects inhibit formation of *N*-nitroso compounds (NOC) which are potential human carcinogens (Hernandez-Ramirez *et al*., 2009). In contrast, poor dietary habits and high salt consumption have associated with an increased risk. Moreover, in some populations smoked/cured meats or fish, pickled vegetables and chili peppers have established as a risk factor to occurrence of gastric cancer (Yassibas *et al*., 2012, Barad *et al*., 2014)

Others

Several studies have demonstrated that alcohol, tobacco and occupational exposures to nitrosamines and inorganic dusts are also risk factors of GC (Moy *et al*., 2010, Santibanez *et al*., 2012).

4. Gastric carcinogenesis

The gastrointestinal tract has a great exposure to injury by infections and dietary toxins. Cellular metaplasia due to chronic inflammation, injury and repair are the most accepted processes in gastric carcinogenesis. However, additional mutations can be acquired conferring advantage to development of malignant phenotype.

4.1 *Helicobacter pylori*-associated GC

Helicobacter pylori infection is the leading cause of gastric cancer worldwide, mainly in well differentiated tumors, such as intestinal types (Wang *et al*., 2014b). The carcinogenic model, which has been generally accepted, is based on development of precancerous lesions with potential progression to malignant disease. Intestinal-type gastric carcinomas are the most common type and its carcinogenesis includes a sequential steps where initially occurs a chronic active non-atrophic gastritis followed by multifocal atrophy, intestinal metaplasia, dysplasia and finally invasive carcinoma (Correa and Houghton, 2007).

Upon infection, *H. pylori* is responsible to activate multiple intracellular pathways in cells from gastric epithelium. This mechanism affects cellular signaling triggering an increased inflammatory cytokine production, altered apoptosis rate, epithelial cell proliferation and differentiation and consequently a malignant transformation. *H. pylori* strains that contain the *cag* pathogenicity island (CagPAI) induce direct effects by its virulence factors, such as the cytotoxin-associated antigen A (CagA), vacuolating cytotoxin (VacA) and other CagPAI-encoded proteins. However, indirect effects by inducing of gastritis and atrophy also play a critical role in initiating and progression of tumor. Inflammation leads to production of carcinogenic compounds as well as the hypermethylation of tumor suppressor genes (Ding *et al*., 2010).

H. pylori CagA is the major oncogenic factor incorporated into host cells able to disrupt epithelial cell functions. CagA as well as peptidoglycan are delivered into host cells through translocate the bacterial effectors (TFSS) of type IV secretion system. It confers an activation of multiple oncogenic pathways (Figure 1.7) in epithelial cells that includes NF-κB, activator protein-1, PI3K, signal transducers and activators of transcription 3, Wnt/β-catenin and cyclooxygenase 2. Persistent deregulation of these pathways triggers an important molecular mechanism toward the *H. pylori*-induced

carcinogenesis (Chang *et al*., 2004, Franco *et al*., 2005, Bronte-Tinkew *et al*., 2009, Nagy *et al*., 2009).

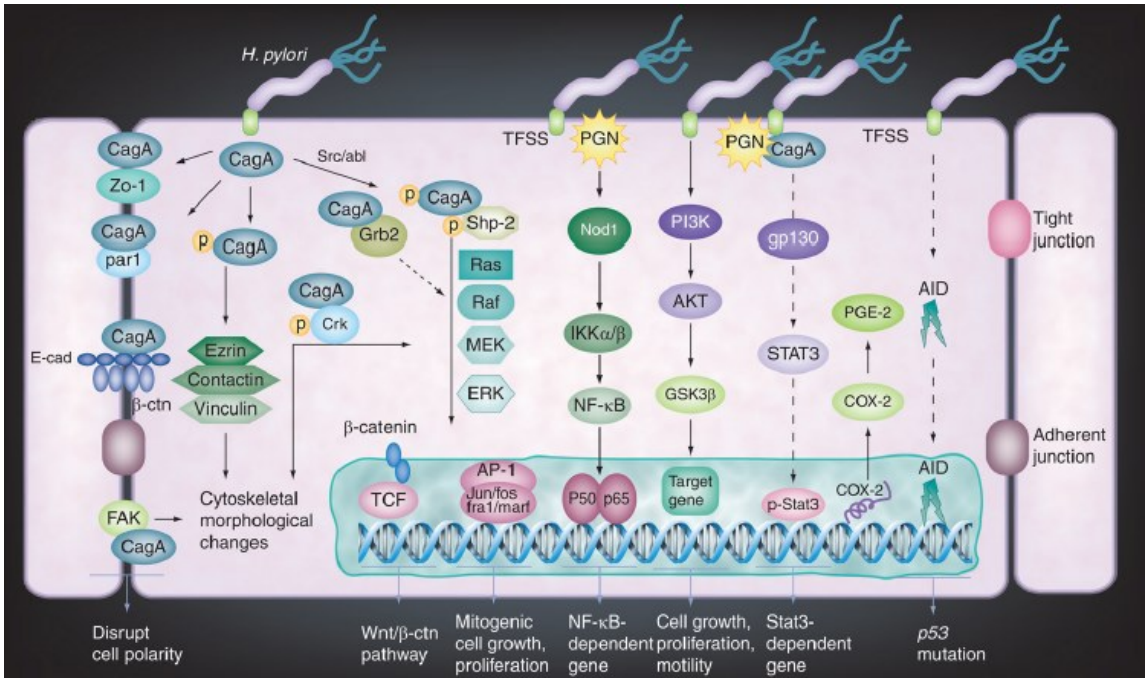

Figure 1.7 - *H. pylori*-induced host cell response and oncogenic signaling in gastric epithelial cells

H. pylori is less virulent when it does not contain the *cag* pathogenicity island (CagPAI), however these strains are also associated to development of cancer. Inflammatory response plays an essential role in tissue cells transformation due to an increased oxidative stress caused to release of reactive species from immune cells. The aberrant promoter methylation of tumor-related genes has been also considered (Matsusaka *et al*., 2014).

Furthermore, the establishment of *H. pylori* bacteria in gastric mucosa contributes to an increased pH of stomach. As consequence, anaerobic bacteria colonize stomach and produce active redutases which promote the transformation of nitrates from diet into nitrite (endogenous nitrosation). Nitrites are reactive molecules able to origin carcinogenic N-nitroso compounds that contribute to the accumulation of DNA damage (Hernandez-Ramirez *et al*., 2009).

4.2 EBV-associated GC

Gastric adenocarcinomas are the most common tumor of stomach and EBV has been found in approximately of 2-20% that varies across the globe (Lee *et al*., 2009). Generally, EBV-associated gastric cancer (EBVaGC) occurs frequently in the upper part of the stomach (cardia and body region). It also shows a male predominance with relatively younger age when compared with EBV-negative gastric cancers (van Beek *et al*., 2004, Truong *et al*., 2009).

The role of EBV in gastric carcinomas is still little known, however monoclonal proliferations and the presence of EBV in all tumor cells have suggested that EBV may act in pathogenesis of these tumors (Seo *et al*., 2010). Several studies have indicated that restricted pattern of viral gene expression categorizes EBVaGC as latency I. However, in some cases, EBV has acquired a modulated latency I because LMP-2A is also found in EBV-infected cells (Luo, *et al*., 2005).

Literature has shown that, DNA methylation of tumor-related genes, suppressor tumor genes silencing by viral miRNAs, loss of p53 activity and induction of growth cell factors by EBERs may be a contribute to carcinogenesis mechanism (Sivachandran *et al*., 2012, Iizasa *et al*., 2012, Hino *et al*., 2009).

DNA Hypermethylation

Aberrant methylation is clearly more frequent in EBVaGC than in EBV negative tumors (Iizasa *et al*., 2012). After EBV infection, methylation of viral promoters has been recognized as host defense mechanism. However, this mechanism does not only occur in viral genes but also in host genome (Uozaki and Fukayama, 2008). CpG-island methylation at promoter regions of tumor-related genes is clearly an epigenetic abnormality in cancer. In comparison with EBV negative tumors, EBVaGC have shown higher promoter hypermethylation able to silence gene expression of various tumor suppressor genes such, p16, p14, TP73, APC (Geddert *et al*., 2011).

The molecular mechanism of DNA hypermethylation observed in host genome is not completely understood. As viral factor of EBV, it has been described that LMP2A induces the phosphorylation of STAT3, triggering DNA methyltransferase 1 (DNMT1) transcription (Iizasa *et al*., 2012). Recently data reported that DNMT1 is responsible for CpG island methylation in promoter region of PTEN, a suppressor tumor gene (Hino *et*

al., 2009). Several studies have suggested that hypermethylation promoted by EBV infection contributes significantly to gastric cancer development. However, further studies are necessary to elucidate the role of EBV in molecular mechanism (Iizasa *et al*., 2012).

Loss p53 activity

Latency I is the most common pattern which is found in EBVaGC and EBNA1 is the only viral nuclear protein expressed. Recent data have shown that EBNA1 contributes to decreasing promyelocytic leukemia (PML) nuclear bodies (NBs) which play important roles in apoptosis, tumor suppression, DNA repair as well as transcriptional regulation. In EBV-GC cells, it was found decreased levels of PML NBs with loss of p53 activity. It suggests that EBNA1 may affect cell cycle contributing to cell survival and proliferation (Sivachandran *et al*., 2012).

Viral miRNAs

miRNAs are endogenous small RNAs that play an important role in regulation of cellular differentiation, proliferation and apoptosis. Several miRNAs and non-coding RNAs, called "oncomirs" have been related with some types of cancer. These molecules act as oncogenes because they are responsible for degradation of various tumor suppressor genes. On the other hand, miRNAs are found in normal tissues as tumor suppressors when they target oncogenes (Nana-Sinkam and Croce, 2011). The levels of these molecules were found deregulated in different types of cancer, including EBVaGC. Down-regulation of the miR-200 family (host miRNAs) has been reported as common event in EBVaGC. It is associated to reduction of E-cadherin expression and loss of cell adhesion suggesting that these mechanisms may be involved in gastric carcinogenesis (Shinozaki *et al*., 2010). Several authors have also studied the association of viral microRNAs and gastric cancer. Actually, miR-BART5, a EBV-encoded miRNA, has shown to play a role in repression of cellular proteins (member of Bcl-2 family) promoting an uncontrolled proliferation (Choy *et al*., 2008).

EBERs

EBERs have been linked to the malignant phenotypes in various EBV-infected cells. Previous studies demonstrated that transfection of EBERs genes into EBV-negative cells promotes growth cell in culture, tumor formation in immunocompromised mice and resistance to apoptosis (Yamamoto *et al*., 2000, Komano *et al*., 1999). Additionally, others investigations have described that EBERs promote the growth and proliferation of some epithelial cells. EBERs appear to induce insulin-like growth factor-1 (IGF1) which acts as an autocrine growth factor for NPC and GC cells. High levels of IGF1 were expressed in EBV-positive but not in EBV-negative NPC or GC tissues, suggesting that EBERs may contribute to epithelial carcinogenesis via the induction of IGF1 expression (Iwakiri *et al*., 2003, Iwakiri *et al*., 2005).

II. AIMS

Despite the high incidence of gastric cancer in Portugal, there are no studies describing EBV-associated gastric carcinomas in our population. Therefore, the aims of this study were:

- To determinate the prevalence of EBV infection in patients with gastric carcinomas in Portugal

- To characterize EBV-associated gastric carcinomas in Portugal

III. METHODOLOGY

1. Type of study

Retrospective cross-sectorial study

2. Population

The population of this study consists in 136 patients with confirmed diagnosis of gastric cancer attended at Portuguese Institute of Oncology of Porto (IPO Porto FG EPE) in the year of 2011. Inclusion criteria: 1) patients diagnosed with gastric cancer; 2) submitted to gastrectomy (total or partial), endoscopic mucosal resection or other surgical procedure; 3) with representative tumor blocks for adequate evaluation of EBV presence.

All tumors were submitted to histological examination by an experimented pathologist from our institution and classified according to the WHO and Lauren's classification (International Agency for Research on Cancer, 2010, Lauren, 1965).

This study did not interfere with the routine procedures. Clinicopathological data was collected from individual clinical records and inserted on a database with unique codification. All procedures were submitted to approval of the IPO Porto FG EPE Ethical Committee.

3. Selection and Processing

Gastric carcinoma tissues were collected from gastrectomy (total or partial), endoscopic mucosal resection and other surgical procedures. Histological sections (3 μm slides) were obtained from formalin-fixed paraffin-embedded (FFPE) tissue blocks.

4. EBV detection

Epstein-Barr virus was identified by *in situ* hybridization (ISH) technique which detects EBV-encoded small RNA (EBER) in FFPE tissue blocks. Hybridization results in duplex formation of EBER sequences in the test material with the fluorescein/biotin-labeled DNA probes. This complex is indirectly detected using antibodies or streptavidin molecules that have high affinity to fluorescein and biotin, respectively. Then, a secondary enzyme-conjugated antibody is also used. The enzymatic reaction using a chromogenic substrate leads to the formation of a color precipitate that can be visualized by light microscopy.

The ISH was performed using two protocols **1)** *Bond™ Ready-to-use ISH EBER Probe and Anti-Fluorescein Antibody* (Leica, Newcastle upon Tyne, UK) associated to detection system *Ultra Vision Large Volume Detection System Anti-Polyvalent, HRP* (THERMO SCIENTIFIC, Fremont, USA) and **2**) *ZytoFast EBV Probe (Biotin-labeled)* associated to *ZytoFast CISH implementation kit HRP-AEC* (ZytoVision, Bremerhaven, Germany).

Initially, all samples were performed using the first protocol described above which is considered the gold standard method of clinical diagnosis of EBV-associated tumors. Then, 10% of negative samples randomly selected and all positive cases were retested using ZytoFast Kit (ZytoVision, Bremerhaven, Germany).

4.1 EBV-ISH detection: protocol 1

Sample preparation: The samples were dewaxed in xylene for 2 x 3 minutes. After dewaxing, slides were sequentially hydrated in 100% v/v ethanol for 2 x 3 minutes, 96% v/v ethanol for 3 minutes and distilled water for 2 x 3 minutes. Proteolytic treatment was performed by addition of 15 mM proteinase K and incubation at 37ºC during 30 minutes. Finished the incubation time, the slides were immersed in distilled water for 2 x 3 minutes and then dehydrated in 96% v/v ethanol followed 100% v/v ethanol for 3 minutes to facilitate air drying.

Hybridization: Hybridization results in duplex formation of sequence present in EBV-infected cells (EBERs) and specific probe. *The Bond™ Ready-to-use ISH EBER Probe* (Leica, Newcastle upon Tyne, UK) was used with a volume of 20 µl for each slide. Slides were covered with coverslip and incubated at 37ºC for 2 hours.

Blocking: Endogenous peroxidase activity and nonspecific antibody binding were blocked by incubating the slides in 3% hydrogen peroxide (H_2O_2) solution and UV block from *UltraVision Large Volume Detection System Anti-Polyvalent, HRP* (THERMO SCIENTIFIC, Fremont, USA), respectively. Each solution was incubated for 10 minutes at room temperature and washing was performed with TBS, 0.1% v/v Trinton X-100 (TBS-T) 2x 5 minutes.

Immunohistochemistry (IHC): In first step, IHC for EBERs detection was performed with *Bond ™ Anti-Fluorescein Antibody* (Leica, Newcastle upon Tyne, UK) diluted 1:150 in TBS, 3% m/v BSA, 0.1% v/v Trinton X-100 with incubation at room

temperature for 30 minutes. After washing 2 x 3 minutes with TBS, *UltraVision Large Volume Detection System Anti-Polyvalent, HRP* (THERMO SCIENTIFIC, Fremont, USA) was used for revelation of hybrids. Biotinylated Goat Anti-Polyvalent Antibody (THERMO SCIENTIFIC, Fremont, USA) was addicted with incubation at room temperature for 10 minutes. The next step was washing with TBS-T 2 x 5 minutes following the addition of Streptavidin Peroxidase (THERMO SCIENTIFIC, Fremont, USA) with incubation for 10 minutes at room temperature. Streptavidin shows high affinity with several secondary antibody-conjugated biotin molecules providing a good revelation signal. Detection of hybrids is achieved by enzymatic reaction using a specific substrate to peroxidase. *ImmPACTTM DAB, Peroxidase Substrate* (VECTOR, Burlingame, CA USA) was used during 4 minutes at room temperature and diluted 3:100. The final washing was performed with distillated water 2 x 5 minutes.

Final preparation: Mayer's hemalum solution (Millipore, Darmstadt, Germany) was used as counterstain for 10-20 seconds, depending of dye's use. After coloration, slides were washed in running water for 5 minutes and the following step was sequential dehydration in 70% v/v ethanol for 2 x 4 minutes, 96% v/v ethanol for 2 x 4 minutes, 100% v/v ethanol for 2 x 4 minutes and xylene for 2 x 4 minutes. Mounting was performed with Microscopy Entellan (MERCK, Darmstadt, Germany).

4.2 EBV-ISH detection: protocol 2

Sample preparation: The samples were dewaxed in xylene for 2 x 5 minutes. After dewaxing, slides were sequentially hydrated in 100% v/v ethanol for 2 x 5 minutes, 96% v/v ethanol for 2 x 5 minutes and 70% v/v for 2 x 5 minutes. Proteolytic treatment was performed by addition of pepsin solution and incubation at 37ºC for 20-30 minutes. Finished the incubation time, the slides were immersed in distilled water.

Denaturation and hybridization: Hybridization step consists in addition of EBV probe that hybridizes to abundantly expressed Epstein virus encoded-RNA (EBERs) transcripts which are localized in the nucleus of latently infected cells. *ZytoFast EBV Probe (Biotin-labeled)* (ZytoVision, Bremerhaven, Germany) was used with a volume of 10 µl for each slide. Slides were covered with coverslip and then they were denatured at 75ºC for 5 minutes on a hot plate. After denaturation, slides were incubated at 55ºC for 60 minutes

Post-hybridization and detection: Finished hybridization step, coverslips were removed by submerging in 1x Wash Buffer TBS at room temperature for 5 minutes. Two more washes were performed with 1x Wash Buffer TBS for 5 minutes. Then, it was used *ZytoFast CISH implementation kit HRP-AEC* (ZytoVision, Bremerhaven, Germany) and HRP-streptavidin was applied (3-4 drops) to the slides and incubated for 30 minutes at 37ºC. After washing with 1x Wash Buffer TBS 2 x 2 minutes at room temperature, the detection of hybrids was achieved by enzymatic reaction using a specific substrate to peroxidase. AEC substrate was used for 4 minutes at room temperature diluted 3:100. The final washing was performed with distillated water 2 x 5 minutes.

Final preparation: Mayer's hemalum solution (Millipore, Darmstadt, Germany) was used as counterstain for 10-20 seconds, depending of dye's use. After coloration, slides were washed in running water for 5 minutes and following step was sequential dehydration in 70% v/v ethanol for 2 x 4 minutes, 96% v/v ethanol for 2 x 4 minutes, 100% v/v ethanol for 2 x 4 minutes and xylene for 2 x 4 minutes. Mounting was performed with Microscopy Entellan new (MERCK, Darmstadt, Germany) to obtain permanent slides.

5. Quality control

As referred above, ZytoFast Kit was used to confirm the results obtained from *Bond^{TM} Ready-to-use Probe and Anti-Fluorescein Antibody*. The internal positive and negative controls provided by kits were also used. The internal positive control consists of poly-dT oligonucleotides targeting the poly (A) tails of mRNAs. A strong hybridization signals within the nuclei of cells verify the integrity of cellular mRNA in specimens. The internal negative control consists of a set of random sequence oligonucleotides with GC contents of 40-70% without known consensus to any naturally occurring sequences. These probes should not result in positive staining signals and are to be used to assess the unspecific background staining with specimens. As positive control for EBV detection were used EBV-positive FFPE tissues from nasopharyngeal carcinoma.

6. Statistical analysis

Statistical analysis was performed using the computer software IBM SPSS statistics for Macintosh, version 20.0 (IBM Corp, Armonk, NY, USA). Chi-Square (χ^2) or Fisher Exact-test was used to compare categorical variables, with a significance level of 5%. The *Odds Ratio* (OR) was computed with its 95% confidence interval as a measure of the association between variables of interest.

IV. RESULTS

1. Comparison of EBV ISH protocols

Detection of EBV for all samples was performed using **protocol 1** and 10% of negative samples and all positive cases were retested using **protocol 2**. The results were completely concordant (100%). Both kits showed remarkable positivity however the first kit allowed better interpretation due to use of hematoxylin as contrast dye.

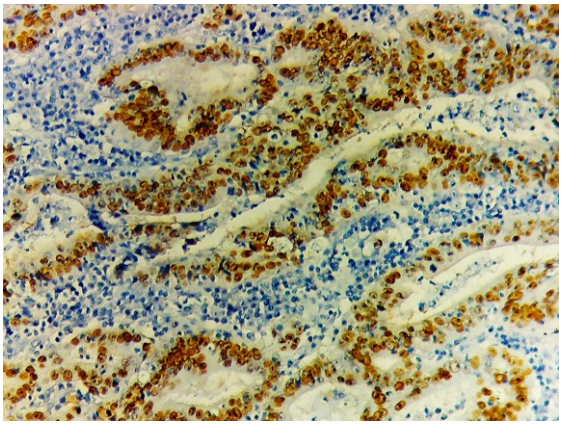

Figure 4.1 - Histological section from gastric carcinoma. Positive result for EBV using protocol 1 (160x).

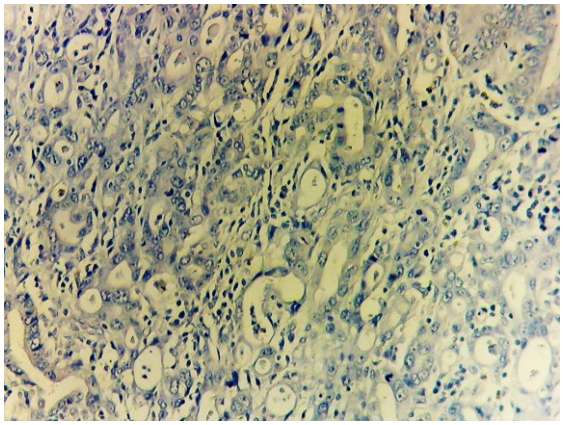

Figure 4.2 - Histological section from gastric carcinoma. Negative result for EBV using protocol 1 (160x).

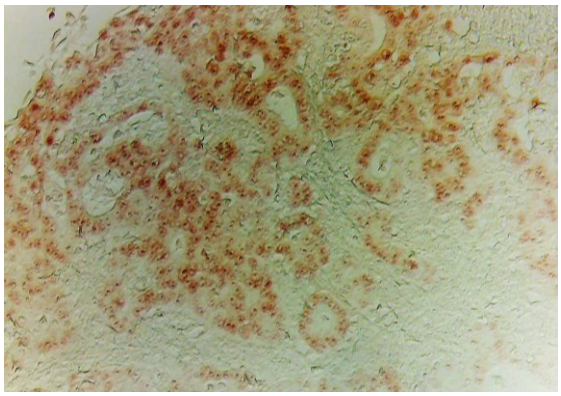

Figure 4.3 - Histological section from gastric carcinoma. Positive result for EBV using protocol 2 (160x).

Figure 4.4 - Histological section from gastric carcinoma. Negative result for EBV using protocol 2 (160x).

2. Characterization of the study population

In this study, 136 cases were included, 82 male patients and 54 female with mean age of 64±12.0 (median age 65; range 34-91) (Figure 4.5). The clinicopathological characteristics of all patients are shown in table 4.1.

Figure 4.5 - Graph illustrating the age distribution of population

The majority of patients were submitted to gastrectomy (n=131), wherein 76 were total resections and 55 partial resections. Moreover, in this group of patients, 3 patients were submitted to mucosectomy and the other 2 to different surgical approaches (Table 4.1).

Regarding the tumor localization in stomach, 92 carcinomas were present in distal region (antrum, pyloric, antro/pyloric or body/antro regions) 25 were from body and 19 from proximal regions (cardia, fundus, cardia/fundus, body/cardia or body/fundus regions) (Table 4.1).

All tumors were classified according to WHO (2010) and Lauren's classifications. Regarding the WHO system, it was possible verify that the most common tumor was tubular adenocarcinoma (n=72) followed by poorly cohesive carcinoma (n=40), mixed carcinoma (n=18), mucinous adenocarcinoma (n=3) and papillary adenocarcinoma (n=1). According to Lauren, the classification revealed 76 intestinal-type carcinomas whereas diffuse and indeterminate types represented 40 and 18 of all gastric tumors, respectively. It was also found 2 carcinomas with lymphoid stroma, which are classified as uncommon variant by WHO and is included in others in Lauren's classification (Table 4.1).

Table 4.1 - Characterization of study population

Variables	
Gender, n (%)	
Male	82 (60.3)
Female	54 (39.7)
Age, (64 ± 12,0)	
Surgical procedure type, n (%)	
Gastrectomy	131 (96.3)
Total	76 (55.9)
Partial	55 (40.4)
Mucosal resection	3 (2.2)
Others	2 (1.5)
Tumor localization, n (%)	
Proximal	19 (14.0)
Body	25 (18.4)
Distal	92 (67.6)
Histology, n (%)	
WHO (2010)	
Papillary adenocarcinoma	1 (0.7)
Tubular adenocarcinoma	72 (52.9)
Mucinous adenocarcinoma	3 (2.2)
Poorly cohesive carcinoma and signet-ring cell carcinoma	40 (29.4)
Mixed carcinoma	18 (13.2)
Carcinomas with lymphoid stroma	2 (1.5)
Lauren (1965)	
Intestinal types	76 (55.9)
Diffuse types	40 (29.4)
Indeterminate types	18 (13.2)
Others (lymphoepithelioma-like carcinoma)	2 (1.5)
Invasion pattern, n (%)	
Infiltrative	64 (47.1)
Expansive	51 (37.5)
Mixed	2 (1.5)
Global stage, n (%)	
IA	21 (15.4)
IB	15 (11.0)
IIA	18 (13.2)
IIB	13 (9.6)
IIIA	19 (14.0)
IIIB	20 (14.7)
IIIC	23 (16.9)
IV	3 (2.2)

Regarding tumor anatomic location of the stomach, the intestinal-type was the most frequent in any of the regions of stomach; the diffuse carcinomas were restricted to proximal and body regions with a percentage of 20% and 38%, respectively; the indeterminate types showed a similar distribution throughout the stomach with a percentage of 11% in proximal, 12% in body and 14% in distal region. The remaining cases, which represent lymphoepithelioma-like carcinomas, were localized in body (4%) and proximal region (5%) (Figure 4.6).

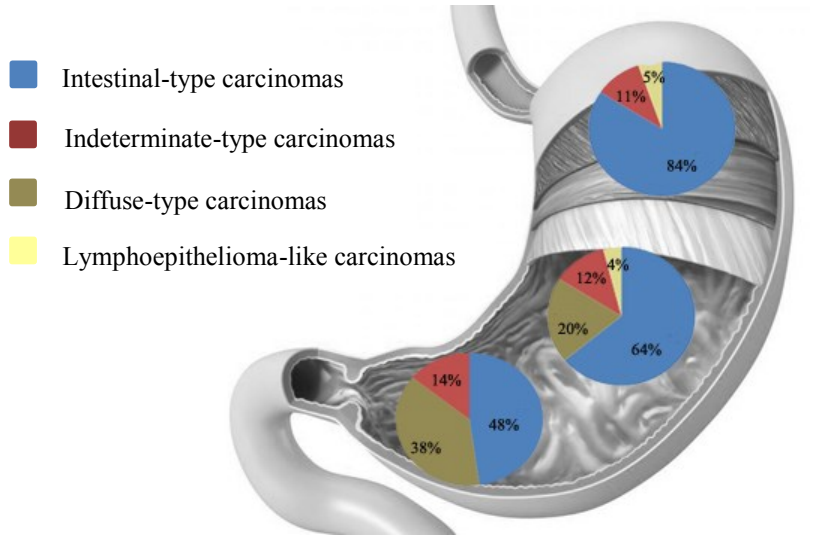

Figure 4.6 - Distribution of histological types according to their location in the stomach

Relatively to the invasion pattern, there was similar distribution for infiltrative (n=64) and expansive (n=51) types. The mixed pattern was found in 2 samples and the information for remaining cases (n= 19) was not accessible.

Regarding TNM staging, the data was available for only 132/136 and it showed homogeneous distribution (Table 4.1). The TNM classification of malignant tumors is a cancer staging notation system for all solid tumors that gives codes to describe the stage of patient's cancer. The TNM combinations correspond to one of five stages: 0, I, II, III or IV as described in Appendix I (Washington, 2010).

3. EBV in GC

In our population, we have found 9/136 (6.6%) EBV-positive tumors. The majority of positive cases were found in males (8.5%) and with age >65 years old (8.6%) (Table 5). However, there were no statistically significant differences comparing the patient's age (p=0.345) and gender (p=0.267). Considering tumor localization of stomach (proximal, body and distal), EBV was more often found in proximal and body regions (10.5% and 16.0%, respectively) while it was rare in distal regions (3.3%) (Figure 4.7). Nevertheless, the distribution showed no significant differences (p=0.058) (Table 4.2).

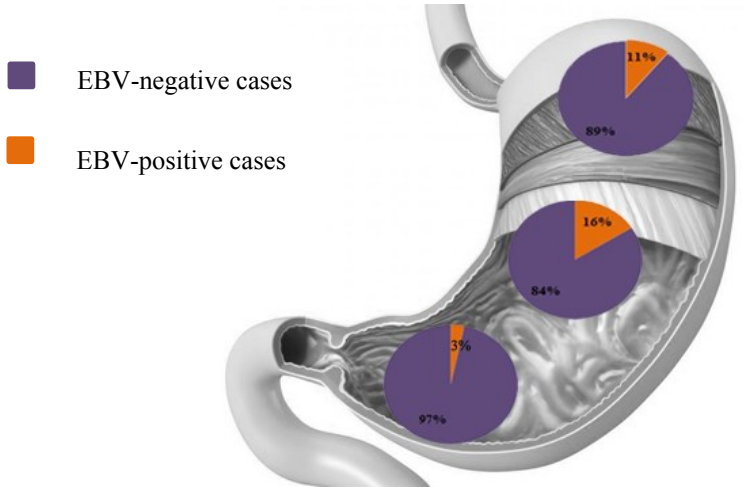

Figure 4.7 - Distribution of EBV according to its location in the stomach

Comparing the distribution of EBV in the different histological types, we observed statistically significant differences either using the WHO or Lauren classifications (p<0.001). According to WHO classification, EBV was detected in 6.9% of tubular adenocarcinomas, 11.1% of mixed carcinomas and 100% of carcinomas with lymphoid stroma. Regarding the Lauren's classification, it was found that 6.6% of intestinal-type carcinomas, 11.1% of indeterminate-type carcinomas and 100% of lymphoepithelioma-like carcinomas were EBV positive. There were no EBV-positive cases identified as diffuse-type carcinomas (Table 4.2).

Regarding invasion pattern, it was observed that tumors with expansive patterns have a higher prevalence of EBV when compared with infiltrative patterns (11.8% *vs* 4.7%, respectively), however these results have no significant differences (p=0.338) (Table

4.2). As also shown in table 4.2, there were no statistically significant differences among clinical stages (p=0.426).

Table 4.2 - Description of EBV frequency in gastric carcinomas

	Negative n (%)	**Positive n (%)**	***p***
Gender, n (%)			
Male	75 (91.5)	7 (8.5)	0.267
Female	52 (96.3)	2 (3.7)	
Age, (64 ± 12,043)			
< 65	63 (95.5)	3 (4,5)	0.345
≥65	64 (91.4)	6 (8.6)	
Tumor localization, n (%)			
Proximal	17 (89.5)	2 (10.5)	0.058
Body	21 (84.0)	4 (16.0)	
Distal	89 (96.7)	3 (3.3)	
Histology, n (%)			
WHO (2010)			**< 0.001**
Papillary adenocarcinoma	1 (100)	-	
Tubular adenocarcinoma	67(93,1)	5 (6.9)	
Mucinous adenocarcinoma	3 (100)	-	
Poorly cohesive carcinoma	40 (100)	-	
Mixed carcinoma	16 (88.9)	2 (11.1)	
Carcinoma with lymphoid stroma	-	2 (100)	
Lauren (1965)			**< 0.001**
Intestinal types	71 (93,4)	5 (6.6)	
Diffuse types	40 (100)	-	
Indeterminate types	16 (88.9)	2 (11.1)	
Others (lymphoepithelioma-like carcinoma)	-	2 (100)	
Invasion pattern, n (%)			
Infiltrative	61 (95.3)	3 (4.7)	0.338
Expansive	45 (88.2)	6 (11.8)	
Mixed	2 (100)	-	
Global stage, n (%)			
IA	20 (95.2)	1 (4.8)	0.426
IB	13 (86.7)	2 (13.3)	
IIA	16 (88.9)	2 (11.1)	
IIB	13 (100)	-	
IIIA	16 (84.2)	3 (15.8)	
IIIB	19 (95.0)	1 (5.0)	
IIIC	23 (100)	-	
IV	3 (100)	-	

4. Risk analysis of EBV-GC associations

Considering the data from EBV-GC characterization, *Odds Ratio* (OR) of EBV-associated GC was calculated according to the different clinicopathological variables (Table 4.3).

The statistical analysis revealed that EBV was predominantly found in the upper regions of the stomach (p=0.032; OR=4.68, 95% CI 1.11-19.7). These results demonstrated that, in our population, patients with tumors in body and proximal regions have a 4.7-fold increased risk to have an EBVaGC (Table 4.3).

Table 4.3 – Risk analysis of clinicopathological variables in EBVaGC

	OR	**95%, CI**	***P***
Gender, n (%)			
Female *vs* **Male**	2.4	0.48-12.1	0.267
Age, (64 ± 12,043)			
< 65 *vs* **≥ 65**	1.9	0.47-8.2	0.345
Tumor localization, n (%)			
Proximal/Body *vs* **Distal**	4.6	1.11-19.7	**0.032**
Histology, n (%) (Lauren (1965)			
Intestinal-types	1.98	0.37-10.5	0.350
Diffuse-types	0.14	0.01-2.58	0.078
Indeterminate-types	2.78	0.50-15.5	0.238
Global stage, n (%)			
Stages I/II *vs.* **Stages II/III**	0.81	0.21-3.17	0.519

The analysis also revealed no significant differences regarding gender (p=0.267) and age (p=0.345). Nevertheless, the estimated OR for male gender was 2.4 (95% CI=0.49-12.1) and for patients ≥65 years old was of 1.9 (95% CI=0.47-8.2) (Table 4.3).

Regarding histological classification, we have performed analysis considering the Lauren's classification. The estimated OR of EBVaGC in intestinal carcinomas was 1.98 (p=0.350; 95% CI=0.37-10.5) and 2.78 in indeterminate carcinomas (p=0.238; 95% CI=0.55-15.5) while for diffuse carcinomas was 0.14 (p=0.078; 95% CI=0.01-2.58). Despite no statistical significant differences, the results point that patients with

intestinal or indeterminate carcinomas are more prone to develop an EBVaGC, rather than diffuse types (Table 4.3).

Regarding the global stage of patients, there was no clear evidence of the relationship between the presence of EBV and clinical stage. The estimated OR for I/II stages *vs.* III/IV stages was 0.81 (95% CI=0.21-3.17) and no statistically significant differences were found (p=0.519) (Table 4.3).

V. DISCUSSION

Gastric carcinoma is a serious public health problem worldwide with high rates of mortality. Over past the 30 years, there have been described a new subset of gastric cancer, EBVaGC. In fact, about 10% of all GC have been associated with EBV infection however, the role of EBV in gastric carcinogenesis remains unclear (Iizasa *et al*., 2012). Recently, two studies suggested a new classification based on molecular features of gastric tumors and in these classifications arise a new four subtypes of gastric cancers: tumors positive for Epstein–Barr Virus, microsatellite unstable tumours (MSI), genomically stable tumors (GS) and tumors with chromosomal instability (CIN). In EBV-positive tumors have been often found PIK3CA, ARID1A and BCOR mutations and rare TP53 mutations (Cancer Genome Atlas Research, 2014, Wang *et al*., 2014a). In addition, these tumors also showed aberrant methylation of multiple genes involved in several molecular pathways of gastric carcinogenesis when compared with EBV-negative tumors (Kang *et al*., 2002).

This cross-sectorial study is the first report characterizing the EBVaGC in Portuguese population. In general, our population showed male predominance accounting 60.3% of all GC cases and the mean age was of 64 years old suggesting that gastric cancer appear more often in older individuals. These finding are in agreement with literature which also has described the occurrence of gastric carcinomas in male and older patients (Barad *et al*., 2014, Tural *et al*., 2012)

This study with patients from the North region of Portugal showed that the prevalence of EBV in gastric tumors is of 6.6%. These findings are in agreement with previous studies, which have described a prevalence ranging 2-20% depending on geographical regions. Moreover, as EBVaGCs account for a small percentage of all gastric cancers, its prevalence is not linear and is influenced by the incidence of GC. In fact, studies have demonstrated that high EBV-positive rate has been found in low-incidence area and low EBV-positive rate has been found in a high gastric-cancer incidence area. Sousa, *et al*. (2008) in a systematic review demonstrated that North of America (region with low prevalence in GC) has shown an association between EBV and GC of 12.9%; conversely, in regions with a high risk for GC (Asia), it was demonstrated that EBVaGCs accounted only 7.99% of all gastric cancers.

The same relationship is verified in our study since we observed a low prevalence of EBVaGC in Portugal (6.6%), a considered country with high incidence of GC. In

addition, it has been shown that EBVaGC rates in Asian countries are lower (7.99%) than in European countries (8.75%) and American countries (11.9%). Moreover, it has been also described variability in different countries of the same region. Yoshiwara *et al*. (2005) reported low EBV-positive rate in Peru (3.9%) while Corvalan *et al*. (2001) showed a prevalence of 16.8% in Chile. Many etiological factors contribute to gastric cancer development and their frequencies in each region influence appearance of EBVaGC.

Regarding the tumor location, we observed that in our population there was a high predominance of gastric tumors in the distal region (67.6%). Curiously, this is the anatomic location with lower prevalence of EBV (3.3%). As previously reported, the presence of EBV has been mostly associated to body, and cardia region of stomach (Herrera-Goepfert *et al*., 2005, Galetsky *et al*., 1997). Hence, our results which also showed a higher prevalence of EBVaGc in proximal regions, may explain the lower prevalence of EBVaGC in own population.

Histology-specific analysis of EBVaGC using Lauren's classification has shown controversial data. Chang et. al. (2001) and Corvalan *et al*. (2001) demonstrated a strong EBV association with diffuse types, however Yoshiwara *et al*. (2005) described an equal proportion between intestinal and diffuse types. In our population, EBVaGC was only found in intestinal-types and indeterminate types without any case reported to diffuse-type. These results may be explained by a predominance of intestinal-type carcinomas in our population. Furthermore, according to our data diffuse carcinomas were more common in a region of stomach (distal region) wherein EBV-positive cases have been described as less frequent. The hereditary carcinomas are normally associated to *CDH1* germline truncating mutation in cases with family history for diffuse gastric carcinoma. It could explain the low prevalence of EBV infection in diffuse carcinomas. However, controversially, a recent meta-analysis showed that CDH1 *g*ermline mutation rates are higher in low-risk areas for GC than in high-risk areas like Portugal (Corso *et al*., 2012). Regarding the lymphoepithelioma-like carcinomas (LLCs) it was observed that all samples showed positivity for EBV. These findings are in agreement with literature which has described that more than 80% of LLCs are associated with EBV infection. Despite the low frequency of LLCs (about 4% of all gastric carcinomas), the pathologists should distinguish this subset of gastric cancer because it has been

demonstrated that patients have a better prognosis when compared with other types of gastric cancer (Bittar *et al*., 2013).

Considering other risk factors for GC development, we found that male predominance is also a strong characteristic of EBVaGC. This result suggests that EBVaGC risk may be associated with lifestyle and occupational factors, which are more common among males, such as tobacco, alcohol consumption, salty food intake and wood dust and/or iron exposure (Akiba *et al.,* 2008). Regarding age distribution of patients with EBVaGC, it is yet little understood. In most of studies, as this study, age dependence is not evident. On the other hand, some reports have suggested high frequency of EBVaGC in younger individuals (Lee *et al*., 2009). In addition, a study from China showed that age distribution may differ with histological type of gastric cancers. The estimated incidence of diffuse-type gastric carcinomas appeared to have a much younger peak of incidence when compared with intestinal-types (Qiu *et al*., 2013). In present study EBV-positive cases were observed in patients with equal or over than 65 years old suggesting that their predominance of intestinal-types in our population may contribute to EBVaGC in older patients.

Research has established that EBVaGC represents a distinct entity of GC, which is characterized not only by unique genomic aberrations, but also by specific clinicopathological features. Since hypermethylation has been implicated in EBVaGC, enzymes which regulate this epigenetic modification have been considered attractive targets for pharmacological intervention. Current epigenetic therapies using DNA methyltransferase (DNMT) inhibitors have shown to contribute to re-expression of genes that are involved in the control of cell-cycle progression, differentiation and/or apoptosis. Meng *et al*. demonstrated that treatment with 5-Aza-dC (DNMT inhibitor) is able to reactivate the expression of P16, hMLH1 and MGMT genes which are involved on the induction of apoptosis. Despite epigenetic therapy shows advantages in sensitization of tumor cells, several problems must be considered. Methylation is a reversible process that may occur after cessation of drug therapy leading to re-silencing of tumor-related genes. Moreover, there are conflicting evidences in literature that suggest effect of epigenetic therapies in non-tumor cells such increases mutation frequency, causes chromosomal rearrangements and decreases fertility in mice (Yau *et al*., 2014). Therefore, the characterization of EBVaGC is of great clinical interest and the investment in the comprehension of EBVaGC should be further stimulated.

VI. CONCLUSION

Epstein-Barr virus-associated gastric carcinomas (EBVaGC) display distinct clinical and genetic features from EBV-negative tumors. However, geographical differences in its incidence suggest that there might be some variations in epidemiological and environmental factors, such as ethnicity, age and/or dietary habits which contribute to permissive environment and genetic susceptibility for EBV association with GC.

In our study, we demonstrated that EBVaGC represent 6.6% of all GC cases in a population from the north region of Portugal. We also found that EBVaGC is only observed in intestinal and indeterminate types and it is not observed in diffuse types. Moreover EBVaGC is more frequently found in tumors with proximal location in the stomach.

This is the first study showing the characteristics of EBVaGC in Portugal and further studies are required to confirm these evidences (especially with more cases and also from different regions of Portugal) and to identify other unique features of these cancers that lead to better clinical approach.

VII. FUTURE WORK

Considering the low prevalence of EBVaGC in our study, its association with only indeterminate and intestinal types and its preferential location in the proximal and body regions of the stomach, it is important to increase the number of cases to comprove these evidences.

It is also important to analyze molecular characteristics of EBVaGC such as ARID1A, PIK3CA, TP53 mutations that have been recently associated to the EBV carcinogenesis of gastric cells; as well as aberrant methylation patterns caused by EBV infection that might be the key of EBVaGC carcinogenesis. Hence, the hypermethylation pattern should be confirmed in EBV-positive cases and then evaluated the promoter methylation of some tumor-related genes.

Furthermore, it is also important to verify if there is an association between the presence of EBV and colonization of *Helicobacter pylori* in gastric mucosa and analyze characteristics of EBVaGC that could be useful for better clinical approaches such as viral activity, viral strains and other epigenetic modifications.

VIII. APPENDIX

Appendix I – Highlights of the 7th edition of the AJCC gastric cancer staging system

Table I - Anatomic stage/prognostic groups, gastric cancer (Washington, 2010)

Stage 0	Tis	N0	M0
Stage IA	T1	N0	M0
Stage IB	T2	N0	M0
	T1	N1	M0
Stage IIA	T3	N0	M0
	T2	N1	M0
	T1	N2	M0
Stage IIB	T4a	N0	M0
	T3	N1	M0
	T2	N2	M0
	T1	N3	M0
Stage IIIA	T4a	N1	M0
	T3	N2	M0
	T2	N3	M0
Stage IIIB	T4b	N0 or N1	M0
	T4a	N2	M0
	T3	N3	M0
Stage IIIC	T4b	N2 or N3	M0
	T4a	N3	M0
Stage IV	Any T	Any N	M1

Table II – TNM category definitions, gastric cancer (Washington, 2010)

T0	No evidence of primary tumor
Tis	Carcinoma in situ: intraepithelial tumor without invasion of the lamina propria
T1	Tumor invades lamina propria, muscularis mucosae, or submucosa
T1a	Tumor invades lamina propria or muscularis mucosae
T1b	Tumor invades submucosa
T2	Tumor invades muscularis propria
T3	Tumor penetrates subserosal connective tissue without invasion of visceral peritoneum or adjacent structures
T4	Tumor invades serosa (visceral peritoneum) or adjacent structures
T4a	Tumor invades serosa (visceral peritoneum)
T4b	Tumor invades adjacent structures
Regional lymph nodes (N)	
NX	Regional lymph node(s) cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in 1-2 regional lymph nodes
N2	Metastasis in 3-6 regional lymph nodes
N3	Metastasis in seven or more regional lymph nodes
N3a	Metastasis in 7-15 regional lymph nodes
N3b	Metastasis in 16 or more regional lymph nodes
Distant metastasis (M)	
M0	No distant metastasis
M1	Distant metastasis

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