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Mechanisms of silencing *TP53* in EBV-related neoplasias

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PREFACE

This study was performed at Molecular Oncology & Viral Pathology Group of the Portuguese Oncology Institute of Porto (IPO Porto).

The results obtained in this study were submitted to publication: M. Malta, J. Ribeiro, C. Oliveira, A. Galaghar, L.P. Afonso, R. Medeiros and H. Sousa. **p53 ACCUMULATION AND EXPRESSION IN EPSTEIN-BARR VIRUS ASSOCIATED EPITHELIAL TUMORS: GASTRIC AND NASOPHARYNGEAL CARCINOMA.** Oncotarget (submitted). In addition, part of this work was presented as a poster at the 17th International Symposium on EBV and associated diseases.

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RESUMO

Introdução: O vírus de Epstein-Barr (EBV) tem sido associado com o desenvolvimento de tumores epiteliais, tais como carcinoma da nasofaringe (NPC) e, mais recentemente, com o carcinoma gástrico (GC). *TP53* é um gene supressor tumoral frequentemente mutado em tumores humanos; no entanto, em neoplasias malignas epiteliais associadas ao EBV as mutações neste gene são raras apesar de ocorrer frequentemente desregulação da via de sinalização da p53. Neste estudo, o nosso objetivo foi caracterizar a acumulação de p53 e a expressão de *TP53* mRNA em tecidos de NPC e carcinoma gástrico associada ao EBV (EBVaGC).

Metodologia: Um estudo retrospetivo foi realizado com 10 NPC, 12 EBVaGC e 31 GC EBV-negativo (EBVnGC) para avaliar a acumulação e expressão de p53. Foram utilizadas secções histológicas a partir de blocos de tecido embebidos em parafina e fixados em formalina (FFPE). A deteção de acumulação de p53 foi realizada por imunohistoquimica (IHC) e a expressão do mRNA do gene *TP53* foi avaliada por qRT-PCR com o GAPDH como mRNA normalizador.

Resultados: IHC demonstrou que a p53 está acumulada em 42/43 GC e nos 10 casos NPC, com mais de 50% dos casos com 50-100% de células com acumulação de p53. Esta elevada taxa de acumulação de p53 foi mais comum nos NPC e EBVaGC do que nos EBVnGC. Os nossos resultados demonstraram uma diferença estatisticamente significativa na acumulação de p53 entre EBVaGC e EBVnGC (p=0,027). Em relação à expressão de *TP53*, nos NPC foi observada a presença de mRNA *TP53*. Além disso, nos GC a análise da expressão do gene *TP53* revelou que o nível de *TP53* mRNA nos casos EBVaGC foi aproximadamente 80% mais baixo ($2^{-\Delta\Delta Ct}$=0,21; p=0,010), quando comparado com EBVnGC, e este resultado foi independente dos subtipos histológicos.

Conclusão: Os nossos resultados demostraram que a acumulação de p53 foi observada em 100% das neoplasias epiteliais associadas ao EBV (NPC e EBVaGC) e em 96,8% dos EBVnGC. Além disso, nossos dados mostraram uma diferença significativa na acumulação de p53 em EBVaGC comparando com EBVnGC, sugerindo que a acumulação de p53 nos carcinomas gástrico é dependente de infeção EBV. A diminuição significativa de *TP53* mRNA nos EBVaGC em comparação com EBVnGC sugere que a carcinogénese viral interfere com a via da p53 e que esta parece ocorrer independentemente da presença de mutações.

ABSTRACT

Background: Epstein-Barr virus (EBV) has been associated with the development of epithelial tumors such as Nasopharyngeal Carcinoma (NPC) and more recently to Gastric Carcinoma (GC). *TP53* is a tumor suppressor gene frequently mutated in human cancers; nevertheless, in EBV-associated epithelial malignancies mutations are uncommon even with frequent deregulation of the p53 pathway. In this study, we aimed to characterize p53 accumulation and *TP53* mRNA expression in NPC and EBV-associated gastric carcinoma (EBVaGC) tissues.

Methods: A retrospective study was performed with 10 NPC, 12 EBVaGC and 31 EBV-negative GC (EBVnGC) cases, in order to evaluate p53 accumulation and *TP53* mRNA expression. Histological sections of each sample were obtained from formalin-fixed paraffin-embedded (FFPE) tissue blocks. The detection of p53 accumulation was performed by immunohistochemistry (IHC) and *TP53* mRNA expression was evaluated by qRT-PCR with GAPDH as normalizer mRNA.

Results: IHC showed that p53 is accumulated in 42/43 GC and all 10 NPC cases, with more than 50% of cases showing 50-100% of cells with p53 accumulation. This high rate of p53 accumulation was more common in NPC and EBVaGC rather than EBVnGC. We found a statistically significant difference in p53 accumulation between EBVaGC and EBVnGC (p=0.027). Regarding the expression of *TP53*, in NPC it was observed the presence of *TP53* mRNA. Furthermore, in GC the *TP53* expression analysis revealed that the levels of *TP53* mRNA in EBVaGC are almost 80% lower ($2^{-\Delta\Delta Ct}$=0.21; p=0.010) when compared with EBVnGC, and these results were independent of the histological subtypes.

Conclusion: Our results showed that p53 accumulation was observed in 100% of EBV-associated epithelial malignancies (NPC and EBVaGC) and in 96.8% of EBVnGC. Furthermore, our data demonstrated a significant difference of p53 accumulation in EBVaGC comparing with EBVnGC, suggesting that accumulation of p53 in gastric cancer is dependent of EBV infection. The significant decrease of *TP53* mRNA in EBVaGC comparing with EBVnGC, suggests that viral carcinogenesis interferes with the p53 pathway and that this seems to occur independently of the presence of mutations.

ABREVIATIONS LIST

B

BARTs - BamHI A rightward transcripts

BER - base excision repair

BL - Burkitt's Lymphoma

BMI - body mass index

BSCC - basaloid squamous cell carcinoma

C

CD - cluster of differentation

CDKs - cyclin-dependent kinases

cDNA - complementary DNA

CIMP - CpG island methylator phenotype

CSF - colony stimulating factor

D

DAB - diaminobenzidina

DDB - DNA damage-binding protein

DNA - deoxyribonucleic acid

E

EBER-ISH - EBER in situ hybridization

EBERs - Epstein-Barr Virus-encoded RNAs

EBNAs - Epstein Barr Nuclear Antigens

EBV - Epstein-Barr virus

EBVaGC - EBV associated gastric carcinoma

EBVnGC - EBV non-associated gastric carcinoma

F

FFPE - formalin-fixed paraffin-embedded

G

GC - gastric carcinoma

H

HCMC - human cytomegalovirus

HDGC - hereditary diffuse gastric cancer

HHV - human herpesvirus

HSV - herpes simplex virus

HL - Hodgkin lymphoma

HLA - human leukocyte antigen

I

IARC - International Agency for Research on Cancer

IHC - immunohistochemistry

IL - interleukin

IM - infectious mononucleosis

K

KSCC - keratinizing squamous cell carcinoma

KSHV - Kaposi’s sarcoma-associated herpesvirus

L

LOH - loss of heterozygosity

M

MHC - major histocompatibility complex

miRNAs - microRNAs

miRs - also know miRNAs

MMR - DNA mismatch repair

mRNA - messenger RNA

N

ncRNAs - noncoding RNAs

NER - nucleotide excision repair

NPC - nasopharyngeal carcinoma

O

ORFs - open reading frames

P

PBS - phosphate-buffered saline

PBS-T - phosphate-buffered saline containing 0.02% Tween 20

PCR - polymerase chain reaction

PTLDs - post-transplantation lymphoproliferative disorders

Q

qPCR - quantitative polymerase chain reaction

R

RNA - ribonucleic acid

ROS - reactive oxygen species

RT - reverse transcription

S

SPSS - statistical package for social sciences

T

TGCA - The Cancer Genome Atlas

U

USP7 - ubiquitin-specific-processing protease 7

UV - ultraviolet

V

VC - variation coefficient

VCA - viral capsid antigen

VZV - varicella zoster virus

W

WHO - World Health Organization

FIGURE LIST

Figure 1. Epstein-Barr virus structure.

Figure 2. EBV infection in healthy carriers.

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INTRODUCTION

1. EPSTEIN-BARR VIRUS

1.1HISTORICAL BACKGROUND

The first step towards Epstein-Barr virus (EBV) discovery happened when, in 1958, Denis Burkitt described a geographically restricted tumor occurring among children in Tropical Africa, later named as Burkitt's lymphoma (BL) [1]. Due to the dependence on temperature and humidity of this type of tumor, Burkitt raised the possibility that this was vector-transmitted and may be virus-induced [2]. Burkitt's hypothesis was clarified in 1964, when Anthony Epstein, Yvonne Barr and Bert Achong, using electron microscopy, discovered herpesvirus-like particles in the "Epstein-Barr" cell line derived from a BL biopsy. The virus was then named Epstein-Barr Virus (EBV) [3]. Further studies established this virus as a new member of the human herpesvirus family, although antigenically and biologically different from any of the human herpesviruses known until then [4, 5].

In the late 60s, antibodies against EBV were identified in sera of patients with Burkitt's lymphoma as well as in healthy individuals [6] and in patients with infectious mononucleosis (IM) [7]. Since then, serological studies developed to examine the EBV seropositivity in different cancers revealed that the prevalence of EBV antibodies in patients with primary nasopharyngeal carcinoma (NPC) was higher when compared to the EBV seropositivity found in patients with BL, which increased the interest for the study of NPC [8]. In 1970, zur Hausen and his collaborators showed the presence of EBV in NPC and BL cells by in situ hybridization and EBV was recognized as the first virus to be directly associated with human cancers [9].

1.2EPIDEMIOLOGY

EBV is an ubiquitous pathogen that is harbored by approximately 90% of all adults throughout the world [10]. EBV infection, despite easily spread through saliva and oropharyngeal secretions, is not highly contagious. In infants, saliva on toys and fingers are the main routes of EBV transmission, while in adolescents and adults it is transmitted mainly by kissing [11].

There are two peaks of seroconversion described by literature, one at 1–6 years and the other at 14–20 years [12]. In developing countries, almost all infections occur at an earlier age, with more than 90% of children over the age of 2 years being seropositive. Typically, this seroconversion occurs at a subclinical level, being asymptomatic or associated with nonspecific illness such as low-grade fever or sore throat [13]. In contrast, the developed countries commonly have an increased rate of primary EBV infection at the adolescence or early

adulthood, and this late seroconversion leads to a significant numbers of individuals to become ill and in 30% to 50% to the development of IM [10, 11, 14].

1.3BIOLOGY OF EBV

1.3.1Taxonomy

EBV, known as human herpesvirus 4 (HHV-4), is a member of the family *Herpesviridae*, subfamily *Gammaherpesvirinae*, genus Lymphocryptovirus with a structure indistinguishable from the others human herpesviruses [12].

The Human Herpesvirus family can be further divided in three subfamilies based on biological properties of the viruses such as growth characteristics and cell tropism [15]. The *alpha* subfamily is constituted by neurotropic viruses that primarily infect mucoephitelial cells including herpes simplex virus (HSV) 1 and 2, and varicella zoster virus (VZV) [15]. The viruses of the *gamma* subfamily are EBV and Kaposi's sarcoma-associated herpesvirus (KSHV), both lymphotropic viruses. The *beta* subfamily is characterized by its ability to establish infection in many different types of cells and include human cytomegalovirus (HCMV) and human herpesvirus (HHV) 6 and 7. These eight human herpesvirus have a significant impact among pediatric population.

1.3.2Structure, Genome and Strain Variability

The mature virions of EBV are approximately 150 to 200 nm in diameter and are composed by three layers surrounding the viral genome [16]. EBV genome have a linear, doubled-stranded DNA of ~184 kilobase pairs in length and $100x10^6$ Da of molecular weight [12, 17]. Like other members of the herpesvirus family, EBV DNA is surrounded by an icosahedral nucleocapsid composed by 162 triangular capsomeres, which is enclosed by a protein tegument [12, 18]. The third layer is an irregularly shaped envelope constituted of multiple viral glycoproteins that play an important role in cell tropism, host range and receptor recognition (Figure 1) [12].

Structurally the EBV genome comprises short and long sequence domains (U_S and U_L) alternate with internal tandem repeat regions (IRs) that are flanked by terminal repeat sequences (TRs) [17, 19]. The EBV genome is linear but as soon as the virion reaches the nucleus, after the infection of the cell, it adopts an episomal form which is essential for viral genome replication [19].

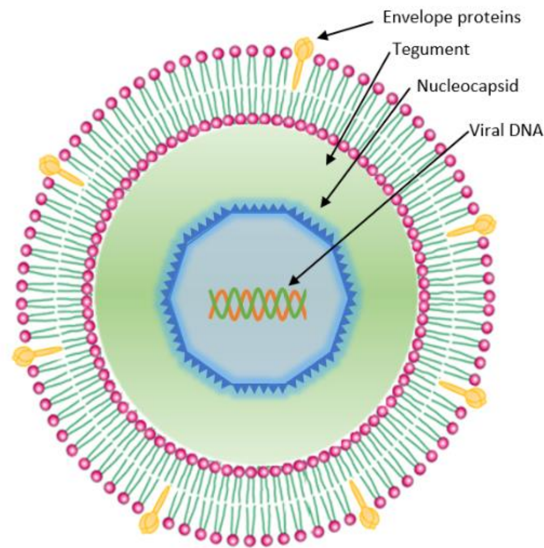

Figure 1. EBV virion structure.

Literature reveals that there are two types of EBV, type 1 and type 2 [12, 16, 20]. They differ at the domains which code for the EBV nuclear antigens (EBNAs) - EBNA2, EBNA3A, EBNA3B, and EBNA3C [21, 22]. The two EBV strains are distinguished by their patterns of restriction endonuclease digestion and biological differences between the two virus types have been reported [16] . *In vitro* studies showed that they differ in their ability to spontaneously enter in lytic cycle as well as different transforming capabilities, with EBV-1 being more efficient at immortalizing B lymphocytes when compared to EBV-2 [12, 20, 21, 23].

Despite the absence of specific geographical restriction, EBV-1 has a predominance of over 95% in the Western hemisphere and Southeast Asia whereas in some regions EBV-2 is more prevalent, including central Africa, Papua New Guinea and Alaska [23, 24]. The association of these EBV subtypes with specific diseases development is not yet clarified [12]; however, EBV-1 appears to predominate in majority of EBV-associated diseases while EBV-2 is principally related with immunocompromised patients [12, 25].

1.3.3Primary Infection and Lytic Replication

Primary EBV infection occurs in the oropharynx, where the virus infects epithelial cells and almost simultaneously resting B-cells in adjacent lymphoid tissue [26]. Literature has shown that EBV is also capable of infect other cells, including T-cells and natural killer cells, however with a much lower efficiency [20].

The infection of epithelial cells leads to the activation of lytic cycle, wherein replication of the virus occurs and the mature virions are released. The infection of resting B-cells usually results in a latent infection, characterized by the expression of a few of the nearly one hundred proteins coded by EBV genome without viral replication and production of virions [10, 27]. Nevertheless, in B lymphocytes, EBV infection leads to two distinct outcomes depending on the stage of the B cell: 1) when resting B-cells differentiate into memory B cells, EBV establishes a long-term persistency characterized by latency; and 2) when B-cells are activated and differentiated into plasmocytes, that are destined to die, EBV activates lytic cycle as a survival strategy [27]. Lifelong infection of the human host is a result of the synchrony between these two phases of infection, hiding it from the immune system in memory B cells and replicate to produce new virions, which have the capability to infect more host cells or other individuals [27].

EBV attaches to B cells through the binding with different cell surface receptors: while viral envelope gp350 glycoprotein binds to B cell surface molecule CD21, also known as the C3d complement receptor [28]; the viral glycoprotein gp42 interacts with the major histocompatibility complex (MHC) class II molecule serving as a co-receptor for EBV [29]. In epithelial cells, the lack CD21 is compensated by the interaction of EBV BMRF-2 protein with adhesion molecules of cell surface, such as the β1 integrins, and afterwards EBV gH/gL envelope protein is able to triggers fusion via interaction with αvβ 6/8 integrins [16]. The subsequent steps of endocytosis of the virus into vesicles and fusion of the virus with the vesicle membrane leads to the release of the nucleocapsid into the cytoplasm. These nucleocapsid is then dissolved and the EBV genome is transported from the cytoplasm to nucleus, where replication begins through the action of DNA polymerases [16, 20]. Lytic viral replication is accompanied by expression of almost 100 viral proteins and viral lytic gene products can be divided in three temporal classes: immediate-early (IE), early (E) and late (L) [16, 30]. The major immediate-early proteins of EBV are encoded by BZLF1 (also termed Z Epstein–Barr replication activator, ZEBRA, or Zta) and BRLF1 (also known as Rta). BZLF1 and BRLF1 are essentials for the switch from latency to lytic cycle and their presence is the earliest indicator of lytic infection. These two proteins activate transcription of viral early genes [12, 20]. The early genes (also termed early antigens, EA) are a group of viral transcripts composed by around 30 early proteins that have a wide range of functions that include replication, metabolism, and blockade of antigen processing. The early proteins BHRF1 and BALF1 are capable of protect infected cells from apoptosis due to their homology with bcl-2, a cellular protein that inhibits apoptosis; BHRF1 also acts as colony stimulating factor (CSF)-1 receptor, blocking the ability of CSF-1 to enhance secretion of the cytokine, and inhibits cell death in both B-cells and epithelial cells; BALF1 modulate the

effect of BHRF1 in epithelial cells; and BSMLF1 and BMRF1 proteins, which belong to early antigen–diffuse complex, activate expression of other early genes [20].

EBV late lytic genes comprise a family of nucleocapsid proteins, viral glycoproteins and a viral cytokine. Viral capsid antigen (VCA) is the major nucleocapsid protein and its detection is used in the diagnosis of virus infection [12]. EBV glycoproteins include gp350, gp85, gp42, and gp25, all involved in viral infectivity and spread. EBV gp350 is the major viral envelope protein and when binds to CD21 promotes virus attachment to B cell. The trimolecular complex, formed by gp85, gp42, and gp25, is responsible for the virus entry into cells: gp85 is responsible for virus fusion with B-cells and virus absorption by epithelial cells; gp25 works as a viral chaperone to transport gp85 to the cell membrane; and gp42 binds to MHC class II molecules and act as co-receptor for EBV entry in B cells. Nevertheless, gp42 is not necessary for epithelial cells infection because this cells do not have MHC class II molecules [12, 20]. The viral cytokine IL-10, that has 80% similarity with human IL-10 and less activity than its cellular homolog, inhibits interferon gamma secretion and release of IL-12, protecting the virus-infected cells from cytotoxic T-cells, and stimulates growth of B-cells (Figure 2) [20].

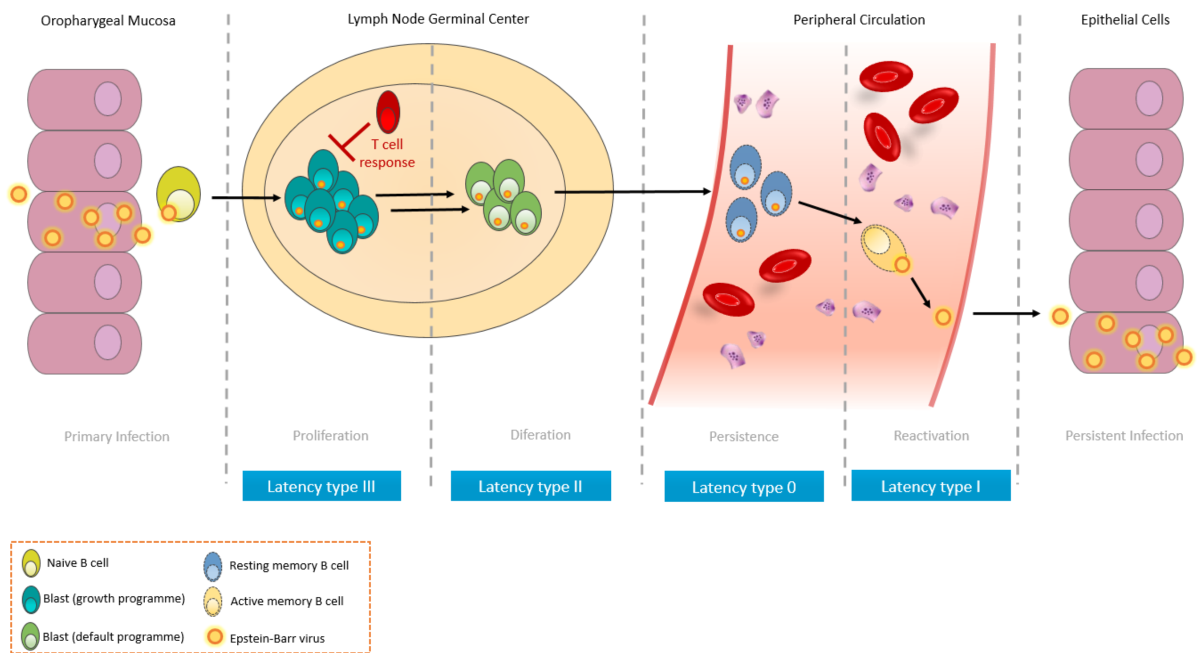

Figure 2. EBV infection in healthy carriers.

1.3.4Latent infection

As all human herpesvirus, EBV can establish a lifelong latent state of infection, characterized by persistent, non-productive viral infection in which the virus genome is maintained in the nucleus of the infected cell without production of virions [16, 31] . In latently infected B cells, EBV genome normally exist as an episome, although some studies report that, in some cases, virus genome can become integrated within host DNA [32]. Despite this, during latent infection, EBV genome seems to behave as host chromosomal DNA; it is packaged with cellular histones, replicated once in S phase via host DNA polymerase, and divided equally into daughter cells during the mitotic phase [16, 30].

1.3.4.1 Latent Gene Transcripts

In contrast with lytic replication, there is a limited expression of EBV genes during latency. These include six EBV-encoded nuclear antigens (EBNAs) (EBNA1, 2, 3A, 3B, 3C and leader protein (EBNA-LP)), three latent membrane proteins (LPMs) (LMP1, 2A and 2B), EBV-encoded small RNAs (EBERs) (EBER1 and 2) and BamHI A rightward transcripts (BARTs) [12, 16, 33]. Together, EBV latent genes target multiple cellular and signaling pathways, and thus, contributing to carcinogenesis in EBV-associated malignancies [34].

a. EBV-encoded nuclear antigens (EBNAs)

EBNA1 was the first EBV latent protein to be reported and is expressed in both stages of the infection, playing multiple essential roles in latent infection, including replication and mitotic segregation of EBV episomes. EBNA1 contributes for the persistence of viral genome in latent infection and to cell immortalization throughout its function as transactivator of EBV latent genes. EBNA1 is also capable of modify the cellular environment, and thus, contributing to cell survival and proliferation as well as viral persistence [33, 35].

EBNA2 and EBNA-LP are co-expressed shortly after B cell infection and EBNA2 has been considered crucial for EBV-mediated B-cell immortalization by contributing for the transactivating expression of several other viral genes [36]. EBNA-LP is a specific coactivator of EBNA2 and, although not essential for B cells transformation, enhances the immortalization of infected B cells by complementing the effect of EBNA2. Together, EBNA2 and EBNA-LP activate viral and cellular gene transcription for B cells transformation [33, 36].

EBNA3A, EBNA3B and EBNA3C are a family of proteins with a central role in EBV latency in B cells by reprogramming host genes expression and, thus, affecting cell proliferation, survival, differentiation and immune surveillance [37]. EBNA3A and EBNA3C are classified as viral oncoproteins because they target tumor suppressor pathways involved in the proliferation of cells and both are essential for B-cell transformation [37, 38]. In contrast, EBNA3B is completely dispensable for *in vitro* B-cell transformation and could be a virus-encoded tumor suppressor. EBNA3B, contrary to EBNA3A and EBNA3C, upregulates CXCL10, an T cell–chemoattractant, and has a growth inhibitory role [33, 37]. Importantly, in B-cell lymphomas EBNA3B is frequently mutated and its inactivation promotes immune evasion and virus-driven lymphomagenesis [39].

b. Latent membrane proteins (LMPs)

LMP1 is expressed in the majority of EBV-associated malignancies and has a high potential for the deregulation of cellular signal transduction pathways and as a result, target cell proliferation and, simultaneously, subvert cell death programs [40]. LMP1 is also important in regulation of tumor angiogenesis through the global alteration of gene and microRNA expression patterns. In addition, LMP1 has other functions that include cytokine and chemokine induction, immune modulation, cell–cell contact, cell migration, and invasive growth of tumor cells [40, 41].

LMP2 has two isoforms, LMP2A and LMP2B, which differ in their 5' exons, and is expressed in many EBV-associated malignancies [42]. LMP2A mimics cellular signaling pathways of B cells, leading these cells to a state of proliferation and activation, which provides a favorable environment for viral replication [43]. Besides, LMP2A is also capable of induce ubiquitination-dependent proteasomal degradation of cellular proteins. These two counterbalancing mechanisms of LMP2A allow the virus to stay in a latency state without inducing an effective immune response of the host [19, 42]. LMP2B lacks the 19-amino acid N-terminal domain present in LMP2A that is responsible for modulation of cellular signal transduction pathways in B cells [42]. Indeed, LMP2B function in EBV infection is not yet completely understood; however, some studies suggest that it is involved in the regulation of switching from latent to lytic state of EBV infection in B cells through the regulation of LMP2A. LMP2B seems to negatively regulate the function of LMP2A and might be responsible for the inhibition of modification of cellular signaling pathway induced by LMP2A [42, 44, 45].

c. EBV Noncoding RNAs

EBV expresses a large number of viral noncoding RNAs (ncRNAs) during latent infection, including EBV-encoded RNAs (EBERs), BamHI A rightward transcripts (BARTs) and viral microRNAs (miRNAs or miRs) [46, 47].

EBER1 and EBER2 are the most highly expressed EBV RNAs during the latent stage of the infection and are commonly used to detect/identify the presence of EBV in tissues [20]. The role of EBERs in EBV-induced B-cell transformation is not yet fully understood. While initial studies have postulated that they were dispensable, recent reports suggest that EBERs expression increases colony formation and growth, enhances resistance of cells to apoptosis and cytokines, including IL-10, IL-9, IGF1 and IL-6, and modulates innate immune response [33].

BARTs are another class of abundant and stable viral transcripts that are detectable during both lytic and latent EBV infection. These viral noncoding RNAs were first identified in NPC tissues and subsequently in other EBV-associated malignancies. BARTs encode a number of potential open reading frames (ORFs) that include BARF0, RK-BARF0, A73 and RPMS1, and despite protein products of these ORFs have not been detected, *in vitro* studies have suggested their potential role in negative regulation of EBNA2 and modulation of kinase signaling [20, 46].

Viral microRNAs (miRs), recently identified as a form of EBV ncRNA, are small, noncoding RNAs with 21-24 nucleotides in length. Until now, 44 mature EBV miRs were described of which 4 are derived from the BHRF1 cluster and the BART cluster encodes the remaining 40 miRs. Intriguingly, BART miRNAs seem to be predominantly expressed in latently infected epithelial cells whereas BHRF1 miRNAs appear to have high expression levels in B cells undergoing stage III latency [48]. Regarding viral miRs function, the presently available information indicates that EBV uses its miRNAs to inhibit the apoptotic response in infected cell in order to establish latent infection and interferes in the expression of viral genes to mask the infected cell and escape from the immune system. However, the importance of viral miRNAs in EBV life cycle and malignant transformation need to be clarified [46, 49].

Together, EBV latent gene transcripts may contribute to tumorigenesis by targeting several hallmarks of cancer described by *Hanahan and Weinberg* [50] (Figure 3).

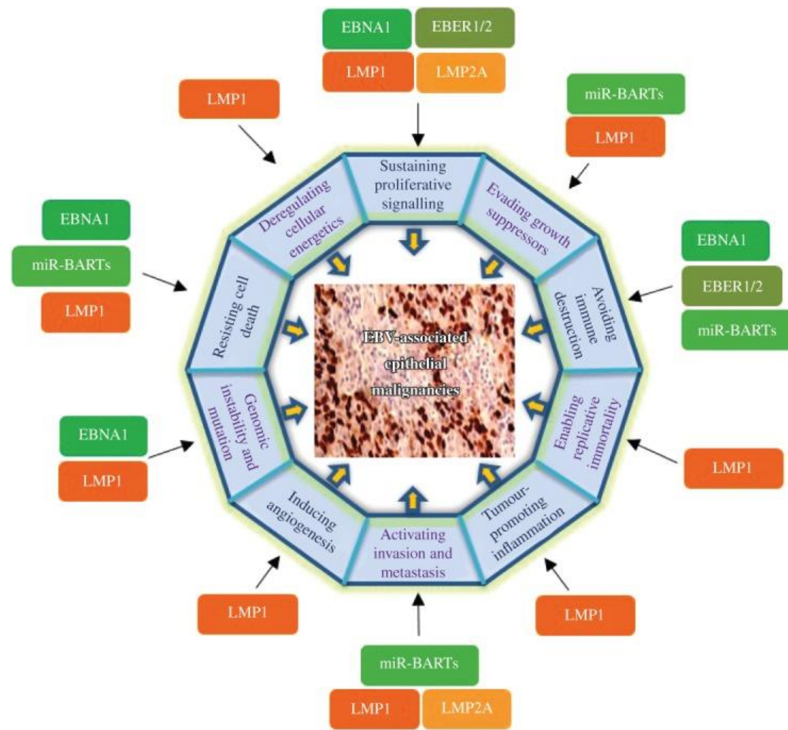

Figure 3. EBV latent genes target cancer hallmarks of epithelial malignancies (*Tsao et al. 2015).*

1.3.5Latency Patterns

Literature described the existence of four different latency programs for EBV: Latency I, II, III, and 0 [51, 52]. The latency programs differ in their pattern of expression of latent viral transcripts and have been associated with different neoplasias [16] (Table 1): Latency I, frequently found in Burkitt's Lymphoma, is characterized by EBNA1 and EBERs expression [53]; Latency II has been associated with nasopharyngeal carcinoma and Hodgkin's lymphoma and in addition to EBNA1 and EBERs, LMP1, LMP2 are also expressed [16, 54]; the full panel of viral latent gene products is expressed in Latency III and is found in immunocompromised individuals and during acute infectious mononucleosis [16]; and Latency 0 is characterized by no viral genes expression and has been described in quiescent, memory B cells [51, 52].

Interestingly, all latency patterns can occur in B cells and are dependent on the B-cell stage [55]. Typically, after infecting naïve B cells EBV enters in type III latency, characterized by the expression of all latent viral genes, leading to B cell proliferation and resulting in the transformation of naïve B cells in proliferating blasts [19]. Later, as B-cells differentiate into latently infected memory B-cell, EBV proteins expression becomes restricted to latency II, with less viral proteins being expressed [55]. In memory B-cells the virus enters in latent persistence phase characterized by no expression of viral proteins - latency 0 [56]. In this latency 0, the host immune system is not capable of detect EBV and the latently infected memory cells circulate in the peripheral blood. When memory B cells divides, EBV enters in type I latency, with a restrictive expression of latent genes, allowing only the replication of EBV genome synchronized with memory B cell replication [55]. This process of latency patterns change according to B-cell stage is illustrated in Figure 2.

Table 1. EBV latency programs.

	EBNA1	*EBNA2*	*EBNA3*	*LMP1*	*LMP2*	*EBERs*
Latency I	+	-	-	-	-	+
Latency II	+	-	-	+	+	+
Latency III	+	+	+	+	+	+
Latency 0	-	-	-	-	-	-

EBNA, Epstein–Barr virus nuclear antigen; LMP, Latent membrane protein; EBERs, Epstein–Barr virus-encoded small RNAs.

2.EBV-ASSOCIATED MALIGNANCIES

EBV infection has been associated with both benign and malignant disorders [34, 57] and can be divided in two groups, those that occur in immunosuppressed individuals versus those that occur in immunocompetent individuals [58].

In immunosuppressed individuals, post-transplantation lymphoproliferative disorders (PTLDs) are the main chronic disease that arises from EBV infection, with reactivation occurring in about 10% of transplant recipients,[10].

EBV has been also associated with some lymphoproliferative disorders in immunocompetent individuals, such as Burkitt lymphoma and Hodgkin lymphoma [19].

Burkitt lymphoma (BL) can be divided in endemic or sporadic variants [59]. Endemic-BL occurs frequently in children living in equatorial regions of Africa, Papua and New Guinea and over 95% are associated with EBV infection. In contrast, sporadic BL has a weak association with EBV (only 15 to 30% of cases are EBV-associated) and occurs in young adults with no specific geographic distribution [19, 60].

Hodgkin lymphoma (HL) has been divided into classical HL, which accounts for about 95% of all cases, and nodular lymphocyte predominant HL. EBV infection is associated with about 40% of classical HL cases [61].

In addition to lymphoproliferative disorders, EBV has been linked to epithelial malignancies that include nasopharyngeal carcinoma and a subset of gastric cancers [19, 58]. The next two chapters will focus mainly in these two EBV-associated epithelial carcinomas.

2.1NASOPHARYNGEAL CARCINOMA

2.1.1EPIDEMIOLOGY

Nasopharyngeal carcinoma (NPC) is considered a rare type of cancer, accounting only for 0.6% of all cancers [62]. According to Globocan, in 2012 occurred 86.700 new cases and approximately 50.800 NPC-related deaths worldwide [63].The incidence and mortality rates of this neoplasia differ depending on the economic resources of the countries, with economically less developed countries having about 11 times more cases and 14 times more deaths per year, when compared to more developed regions (Figure 4) [63, 64]. The highest incidence and mortality rates of NPC are registered in South-Eastern Asia, which represents more than the double when compared to any other area worldwide [63, 64]. In this region, NPC 2 the sixth most common cancer among males [64]. In contrast, in more developed regions, namely in America and Europe, the incidence of NPC is considerably lower [65] Regardless of the geographical area, NPC is more frequent in males than females with 2 to 3 times higher incidence rates in males than in females (Figure 5) [64].

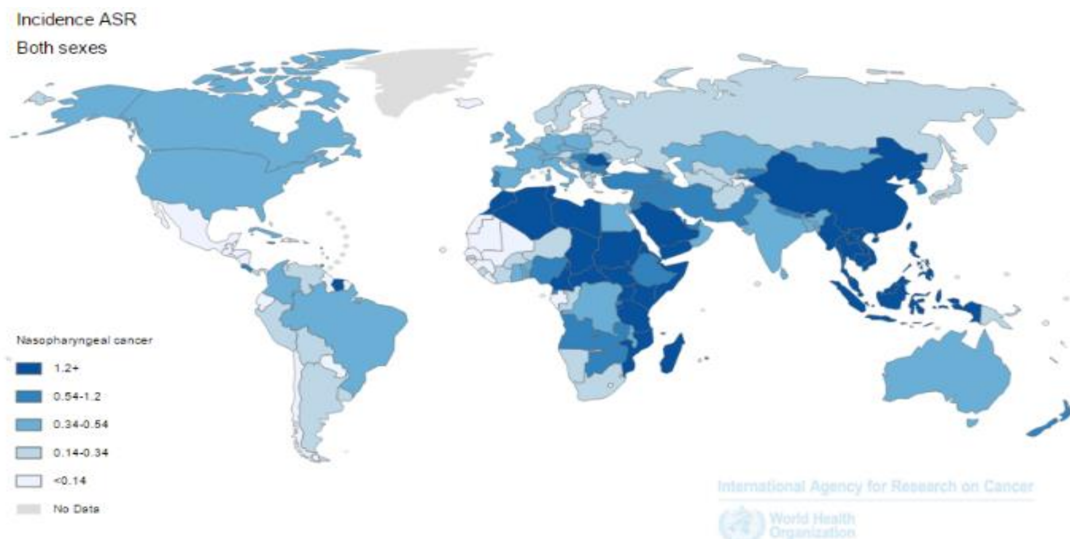

Figure 4. Nasopharyngeal carcinoma incidence worldwide, both sexes, all ages (*Globocan 2012*).

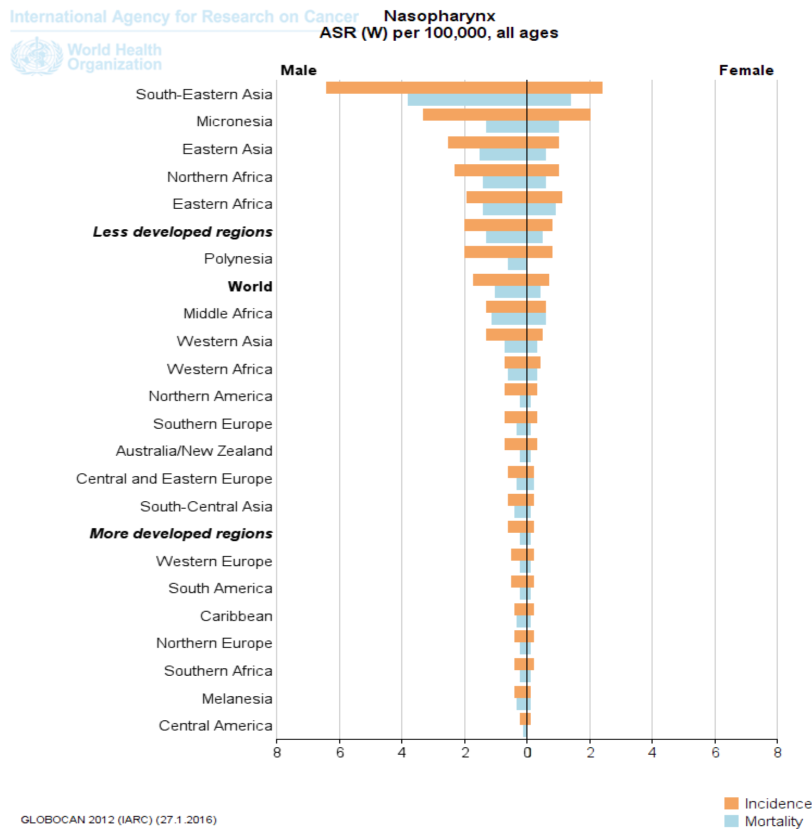

Figure 5. Nasopharyngeal carcinoma incidence worldwide divided by sexes, all ages (*Globocan 2012*).

2.1.2PATHOLOGY

NPC has origin in epithelial cells from the nasopharynx surface and presents different degrees of differentiation [66]. In the 2005 World Health Organization (WHO) classification, NPC is divided into three categories: keratinizing squamous cell carcinoma (KSCC), basaloid squamous cell carcinoma (BSCC) and nonkeratinizing carcinoma, which is subdivided into differentiated and undifferentiated nonkeratinizing carcinomas [67].

KSCC types are commonly diagnosed in non-endemic areas, such as USA and Japan [67-69] and its association with EBV infection varies between populations [70-72]. Basaloid squamous cell carcinoma is uncommon in both endemic and non-endemic areas, and there is very few data reporting EBV infection in this subtype of NPC [67]. Nonkeratinizing carcinoma is the most frequent histological type in endemic regions, representing >85% of all NPC cases [67] and it is invariably associated with EBV infection (~100%) [68].

2.1.3ETIOLOGY AND RISK FACTORS

NPC carcinogenesis has been associated with several etiological factors, including host genetics, environmental exposures and EBV infection [73].

Several studies reported consistent evidence for association of genetic polymorphisms in some genes with NPC development, including immune-related HLA Class I genes [74], DNA repair gene *RAD51L1* [75] and cell cycle control genes *MDM2* [76] and *TP53* [77]. However, the small size of most studies and the lack of attempts to replicate the experiments have limited the progress in understanding the genetics of NPC [78]. In fact, search for genes conferring susceptibility for NPC development have focused on the human leukocyte antigen *(*HLA*)* genes [73]. While some HLA alleles, specially HLA-A2-B46 and HLA-A2-B1, have been associated with 2- to 3-fold increased risk of NPC development in Asian populations, others like HLA-A11-A2 and HLA-A11-B13 seem to represent a decreased risk of 30% to 50% in Caucasians and Chinese, respectively [79].

Large-scale epidemiological studies have proposed associations between several dietary and social practices with an increased risk of nasopharyngeal carcinoma [80, 81]. Salt-preserved fish consumption, which is a dietary base in the most NPC-endemic populations, has been reported with a strong association with risk of NPC development with studies revealing a relative risk for NPC development between 1.38 and 7.50 [81]. Other preserved foods, including meats, eggs, fruits, and vegetables have also been considered [79, 81]. In contrast to preserved foods, frequent intake of fresh fruits and vegetables, particularly during childhood, has been associated with 30% to 50% decrease in risk of NPC [81, 82]. Although the mechanisms by which fruits and vegetables are a protective factors have not been thoroughly investigated, it seems that a diet lacking anti-oxidants could lead to the accumulation of reactive oxygen species (ROS), which may overwhelm the antioxidant defense system resulting in DNA damages and mutations [81, 83, 84].

Cigarette smoking has been consensually established as a risk factor for NPC and studies showed a 2- to 6-fold increase in the risk of developing NPC [79, 85] . Studies conducted in endemic and non-endemic areas reported a significant association between cigarette smoking and KSCC, but with little effect on nonkeratinizing cases [86-88]. Contrarily to salt-preserved foods, the patterns of association of tobacco smoking with NPC are dependent on the population [79, 81]. In addition, the association between alcohol consumption and NPC development is not clearly established and the great majority of studies have shown no significant association between alcohol consumption and the risk for NPC development. However, a meta-analysis revealed an increase of 33% in risk of NPC when the category of

the highest alcohol consumption is compared with the group of minimal alcohol intake [89]. Other risk factors as use of herbal products (herbal medicines; teas and soups) and occupational exposure to formaldehyde and other chemicals or irritants are reported has having some association but results are inconsistent [79, 81].

EBV infection has been the most intensively studied etiological agent and the evidences strongly implicate this virus as a causative factor for NPC development [90]. However, EBV is recognized as a necessary but non-sufficient condition to induce malignant transformation in nasopharynx epithelial cells [91]. This is corroborated by the fact that >90% of all adults worldwide are EBV seropositive and only a minority develops NPC carcinoma [92]. Hence, the literature reinforces that EBV may trigger the cancer development in cells that have been affected by other carcinogenic agents [68, 93].

2.1.4EBV AND NPC

Infection with EBV has been consistently associated to NPC development by several different studies that report elevated anti-EBV antibody titters, free EBV DNA in bloodstream at diagnosis and monoclonal proliferation of tumor cells EBV-positive [94]. Indeed, studies have shown that, almost all non-keratinizing tumors contain monoclonal EBV genomes [54].

Although the carcinogenesis mechanism associated to EBV infection in NPC is not fully understood, the accumulated evidence suggests that viral infection occurs before clonal expansion of malignant cells. EBV genome is detected in NPC tumor cells as well as in high-grade pre-invasive lesions (severe dysplasia and carcinoma) [95]. Nevertheless, in low-grade dysplastic lesions and normal nasopharyngeal epithelium, EBV genome is not detected and the most frequent modification found is the loss of heterozygosity (LOH) in both 3p and 9p chromosomes [96, 97]. The identification of genetic changes in pre-malignant lesions when EBV is not detected in the cells has led to the proposal of a multi-step model for the pathogenesis of NPC - Figure 6 [98].

Allelic losses of chromosomes 3p and 9p, which lead to inactivation of tumor suppressor genes, are probably the first step of NPC development and might be the result of exposure to environmental carcinogens, such as tobacco and salt-preserved fish [95, 99, 100]. Interestingly, chromosomes 3p/9p allelic losses in the normal nasopharyngeal epithelium is much more frequent in populations at high risk for NPC development (82.6%) than in the low-risk populations (20%) [101]. These findings suggest that as a result of this genetic changes, low-grade pre-invasive lesions become susceptible to EBV infection which will then be trigged to proliferate leading to NPC development [98, 100]. This hypothesis is supported by *in vitro* data

that showed that EBV infection of epithelial cells requires an altered, undifferentiated cellular environment [78].

As soon as the cells become infected by EBV, the virus express EBERs and the latent proteins LMP1, LMP2 and EBNA1, characteristic of EBV latency II pattern [54]. These EBV proteins interact with the host proteins in order to provide mechanisms of growth and survival to the cells.

EBNA1 is expressed in all NPC cells and has an essential role in maintaining the EBV genome in the tumors cells [102]. Additionally, EBNA1 also interferes with cellular pathways that control cell proliferation, survival, and DNA repair [103]. For example, EBNA1 may protect cells from apoptosis through its interaction with p53 binding domain of USP7 and could also contribute to the increase of genetic instability in NPC cells through the disruption of promyelocytic nuclear bodies, important for DNA repair [104, 105].

LMP2A is expressed in more than 98% of all NPC cases, while expression of LMP2B appeared lower [106]. LMP2A interferes in different cellular signaling networks, affecting growth transformation, differentiation, survival and migration [102]. For example, LMP2A lead to beta-catenin stabilization, the central oncoprotein of Wnt signaling, inappropriately activating the Wnt pathway and thus contributing to survival and growth of malignant cells [107].

LMP1 is expressed in around 70% of all NPC cases, still its expression varies among different studies [108]. Independent of the frequency of expression, a very low level of LMP1 expression in cells is sufficient to induce growth and apoptosis resistance as well as enhance cell motility and invasion [108]. For example, LMP1 upregulates bcl-2, a protein involved in cell death regulation, and cooperates with this host protein to induce epithelial cell transformation [109]. Furthermore, a recent publication indicates that LMP1 also cooperates with a catalytic subunit of the human telomerase to immortalize primary nasopharyngeal epithelial cell cultures [110].

In the last stages of NPC development, LMP1 and LMP2 cooperate to promote aggressive growth and invasive properties of cells and additional genetic and epigenetic changes occur, ultimately, to confer the tumor cells the ability to metastasize [52, 102].

Figure 6 summarizes the steps towards nasopharyngeal carcinogenesis in which EBV infection has an important role in the dysregulation of multiple signaling pathways.

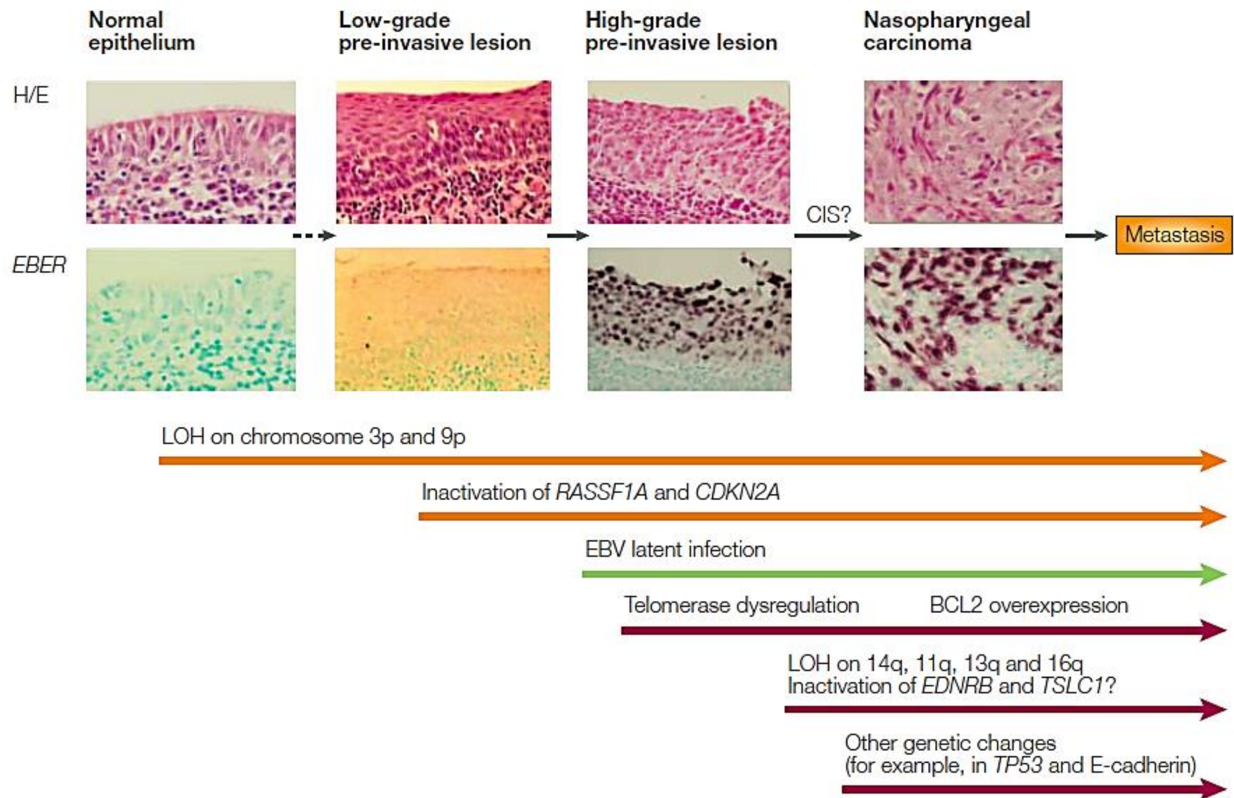

Figure 6. Role of Epstein–Barr virus in the pathogenesis of nasopharyngeal carcinoma (*Young et al. 2004)*

2.2GACTRIC CARCINOMA

2.2.1EPIDEMIOLOGY

Worldwide, gastric cancer (GC) is the fifth most common diagnosed cancer with an estimated 952.000 new cases and approximately 723.000 deaths in 2012, accounting for 6.8% of all cancers and being the third leading cause of cancer death in both sexes [63]. In Portugal, each year 1834 new cases have been diagnosed with gastric cancer, of which 1387 died from the disease, making GC the fifth most common cancer and the fourth most common cause of cancer death [63].

Incidence rates of gastric cancer are two fold higher in men than in women and vary widely across the world (Figure 7 and 8). The highest incidence rates are registered in Eastern Asia and Central/Eastern Europe, with almost 60% of all cases occurring in China, Japan and Korea. Conversely, Northern America and Africa have the lowest incidence rates [63, 111] (Figure 7). Regional variations in gastric carcinoma incidence are, in part, the reflection of differences in dietary patterns, salt intake, food storage and prevalence of Helicobacter pylori infection, which are the etiological risk factors for GC [64, 111].

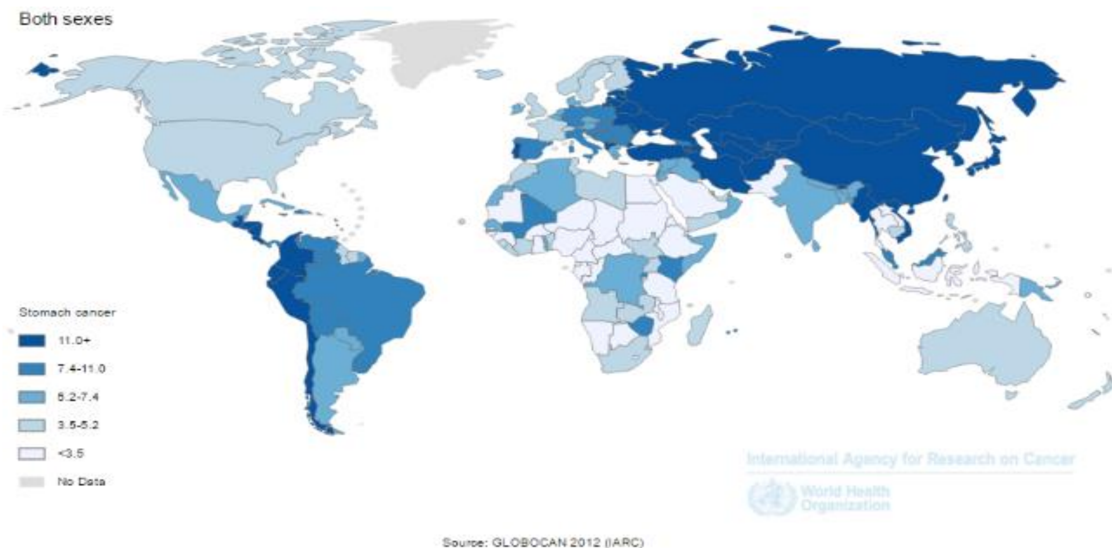

Figure 7. Gastric carcinoma incidence worldwide, both sexes, all ages (*Globocan 2012*).

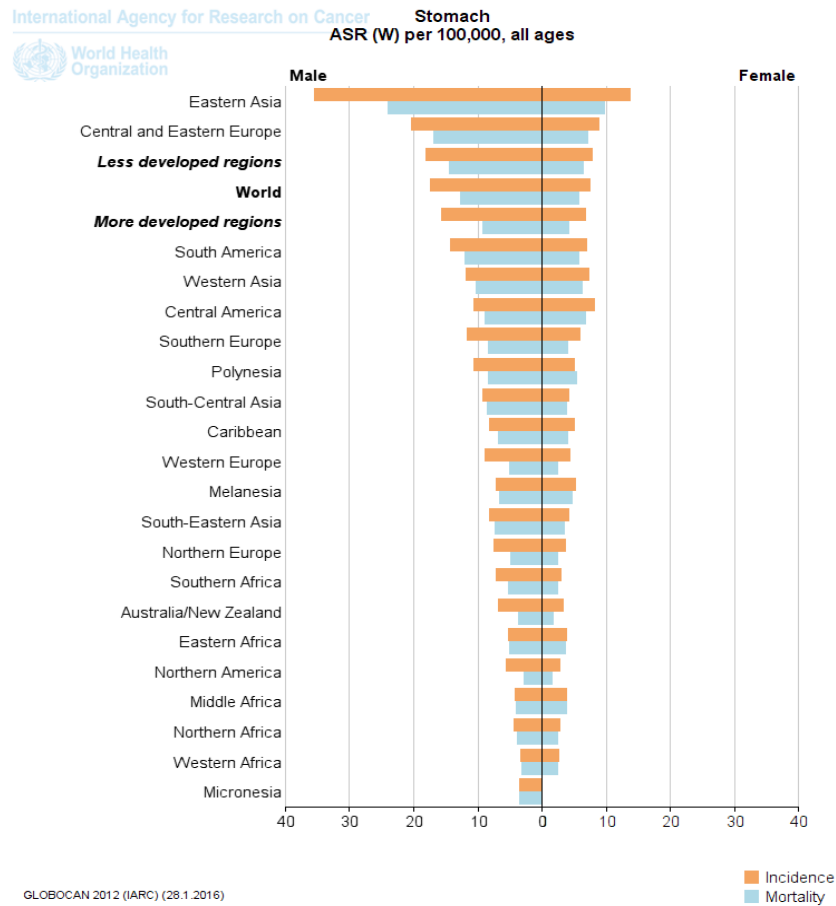

Figure 8. Gastric carcinoma incidence worldwide divided by sexes, all ages (*Globocan 2012*).

2.2.2PATHOLOGY

The diagnosis of gastric adenocarcinomas requires histopathologic assessment however, tumors of stomach demonstrates marked heterogeneity at both architectural and cytologic level that difficult the establishment of a well-defined classification system [112, 113]. Of all stomach cancers, around 90% are adenocarcinomas and the remaining 10% are due to Non-Hodgkin's lymphomas and leiomyosarcomas [114].

Several classification systems have been proposed to describe gastric cancer based on the microscopic appearance of tumors, including Ming, Carneiro, Grundmann and Goseki classifications [115-118]. Nowadays, Lauren and World Health Organization (WHO) systems of classification are commonly used by pathologists (Table 2) [119]. Despite the different classification systems describing gastric adenocarcinomas, there is no consensus concerning

which is the best system of classification combining prognosis and high practicality in clinical diagnosis [119].

Since 1965, Lauren's classification has been used to subdivide gastric adenocarcinomas in two major categories: intestinal type (or well differentiated) and diffuse type (or undifferentiated), plus indeterminate type [120]. These two major subtypes have different clinical and pathological characteristics: the diffuse type has equal gender distribution and occurs in all age groups, occurs in the corpus or entire stomach and has a greater tendency to invade the gastric wall and to metastasize, leading to more rapid disease progression and worst prognosis; contrarily, the intestinal type occurs predominantly in males and older persons, predominates in the antrum and incisura of the stomach and has better prognosis [114, 121]. The 2010 WHO classification subdivides gastric adenocarcinomas in four major groups: tubular, papillary, mucinous and poorly cohesive (including signet ring cell carcinoma), plus uncommon histologic variants [118]. In this new classification, Lauren's intestinal type is branched in tubular and papillary adenocarcinomas and Lauren's diffuse type is divided in mucinous adenocarcinoma and poorly cohesive carcinoma [108].

Table 2. Comparison of Lauren's and WHO classification systems. Adapted from [112].

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

2.2.3ETIOLOGY AND RISK FACTORS

Gastric cancer risk factors can be divided in three major groups: infectious agents, dietary/lifestyle influences and genetic component [122].

H. pylori infection affects around 50% of world population and has been classified by WHO as a class I carcinogen for the development of non-cardia gastric adenocarcinoma [123]. For this subtype of gastric cancer, it is estimated that 89%of all cases are attributable to *H. pylori* infection and that this infection is responsible for a twofold increase in the risk of developing GC [121, 124]. The contribution of *H. pylori* to gastric carcinogenesis is via mechanisms that induce chronic gastritis. This chronic gastritis over time may progress to severe atrophic gastritis, which in turn can develop to cancer [114]. Although *H. pylori* infection affects half of the world population, only around 0.5% of infected individuals will develop gastric adenocarcinoma [125]. Thereby, other risk factors are necessary to stomach carcinogenesis as, for example, high-salt intake that could contribute to increase the risk of persistent *H. pylori* infection [126].

Nevertheless, recently a second infectious agent has been associated with gastric carcinogenesis: the Epstein-Barr Virus [127]. *Sousa et al.* showed that the worldwide prevalence of EBV-positive gastric cancer is 8.29%, with the highest EBVaGC prevalence registered in America (11.3%) and the lowest in Europe (7.96%) [128]. Additionally, *Murphy et al.* demonstrated that this incidence is two times higher in men than in women (11.1% males vs. 5.2% females) and regardless of gender, EBV-positive tumors seem to occur more frequently in cardia or corpus than in the antrum [129]. The EBV specific mechanism of action in gastric carcinogenesis is still unknown, however it is conceivable that EBV infection occurs in atrophic gastric cells and leads to carcinoma development [125, 130].

Dietary and lifestyle risk factors include salt and salted preserved food, fruits and vegetables, tobacco, alcohol and body mass index/physical activity [131, 132].

Dietary intake of salt in excess could result in early atrophic gastritis, thereby increasing the later risk of GC. In fact, recent data suggest that high-salt consumption is responsible for a two-fold increase in the risk of GC development when compared to low-salt intake [122, 133]. Conversely, several studies have reported a protective effect of consumption of fresh fruits and vegetables, with vitamins C and E, carotenoids and selenium being highlighted as possible protective micronutrients. These reports suggest that fruits and vegetables intake contribute to a decreased risk of GC in around 20% and 30%, respectively [114, 121].

The two-lifestyle factors implicated in gastric carcinogens are tobacco smoking and alcohol. Like in other types of cancer, tobacco smoking is an unequivocal risk factor for gastric

cancer.Smoking was significantly associated with both cardia and non-cardia cancers, being responsible for a 1.5-fold increased relative risk of developing GC [121]. In contrast, no definite association exists between alcohol and gastric cancer, although some studies have showed a slightly increase in risk of gastric cancer associated with alcohol consumption [121, 125].

Other risk factors, include body mass index (BMI) above 25 reported by a meta-analysis showing that overweight and obese population have increased risk to develop non-cardia gastric cancer, with an increase in the risk of 1.4-fold for overweight and 2-fold in obese. Conversely, regular physical activity seems to be associated with lower risk of non-cardia gastric carcinoma [122, 134].

Inherited predisposition syndromes are associated to around 3% of all gastric cancers. These include, for example, hereditary diffuse gastric cancer (HDGC) and Lynch syndrome that confer 80% and 10% lifetime risk of developing gastric cancer, respectively [122]. HDGC in a rare cancer that is caused by germline mutations in the E-cadherin (*CDH1*) gene and is characterized by autosomal dominance and high penetrance [121]. On the other hand, Lynch syndrome is a hereditary predisposition that is genetically heterogeneous, caused by germline mutations in various DNA mismatch repair (MMR) genes: *MLH1*, *MSH2*, *MSH6*, or *PMS2*. Lynch syndrome, besides gastric cancer, also predispose to colorectal cancer and endometrial adenocarcinomas [135].

2.2.4EBV AND GASTRIC CANCER

EBV infection has been detected in almost 10% of all cases of GC and its incidence have regional differences [128, 136]. Moreover, the prevalence of EBV-associated gastric cancer (EBVaGC) has distinct distribution according to gender and tumor location, being more predominant in males and in proximal stomach, such as cardia and fundus [129].

Recently, due to the heterogeneity in GC and to the limited clinical utility provided by the current systems of classification of gastric tumors, two studies proposed a new classification of GC based on molecular features of tumors and categorized EBVaGC as a “new” and distinct subtype of GC [137, 138]. EBVaGC seems to exhibit an extreme hypermethylation phenotype, also known as EBV-CIMP (CpG island methylator phenotype), with the highest prevalence of DNA hypermethylation of all cancers reported by The Cancer Genome Atlas (TGCA) [138]. *PIK3CA* mutations occur in ~80% of EBVaGC, contrasting with the other subtypes wherein *PIK3CA* mutations are not so frequent. EBVaGC has also been described as having mutations in *ARID1A* (55%) and *BCOR* (23%) genes. Interesting, *TP53* mutations that occur in the majority of gastric tumors (71%) are rare in EBVaGC. Additionally, the EBV subgroup exhibits

amplification at 9p24.1 at the locus containing *JAK2* (encodes a receptor tyrosine kinase), *CD274* (encodes PD-L1) and *PDCD1LG2* (encodes PD-L2) [138].

Taking into account the characteristics of EBVaGC, a recent publication suggests that EBV coordinates with somatic gene mutations in order to induce the carcinogenesis process in gastric epithelial cells (Figure 9) [139]. In this proposed mechanism, high frequency mutations, such as in PIK3CA and ARID1A, are a requirement in the GC development and are responsible for the transformation of normal gastric cells into susceptible pre-cancerous cells, which are more likely to be infected by EBV. After viral infection and establishment of EBV-latency, other lower-frequency mutations, such as BCOR mutation or amplification of PD-L1 and PD-L2, might contribute to an increase progression and immune evasion of cancer cells [139]. Nevertheless, there is still some lack of information and further studies are necessary to clarify the coordination of virus and host cell mutations in gastric cancer carcinogenesis.

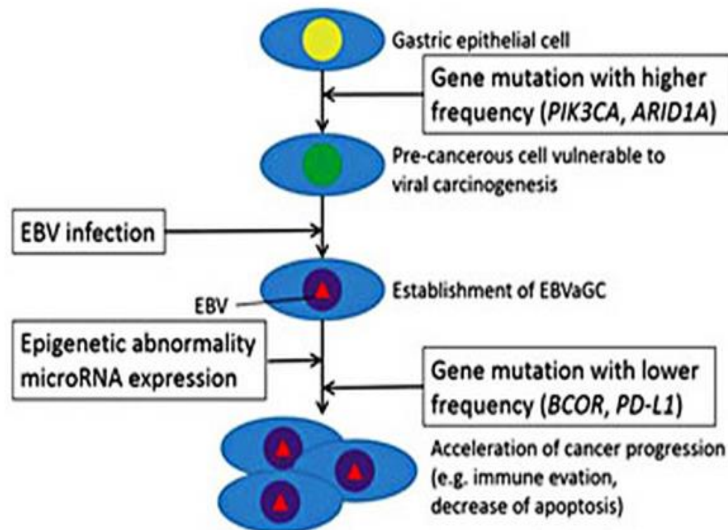

Figure 9. Coordination between EBV and somatic gene mutation in EBVaGC (*Abe et al. 2015*).

3. *TP53*

3.1 STRUCTURE AND BIOLOGICAL FUNCTIONS

The human p53 protein is 393 amino acids long and is encoded by *TP53* gene, which is located on chromosome 17p13.1. This protein has three domains: a transactivation domain, which is required for establish contacts with the transcriptional coactivators or co-repressors; a sequence-specific DNA binding domain; and a tetramerization domain that regulates the p53 oligomerization process. [140, 141].

The most important function of p53 emerged from the studies in knockout mice that showed that these mice deficient in *TP53* were susceptible to spontaneous tumorigenesis. Hence, p53 was recognized as a tumor suppressor protein extremely important in the biological activity of cells [142]. Nevertheless, in response to endogenous or exogenous stresses, p53 triggers p53-regulated responses that include cell cycle arrest, DNA repair, apoptosis and senescence (Figure 10). Together these widely studied functions of p53 converge to its main function as tumor suppressor in cancer [142].

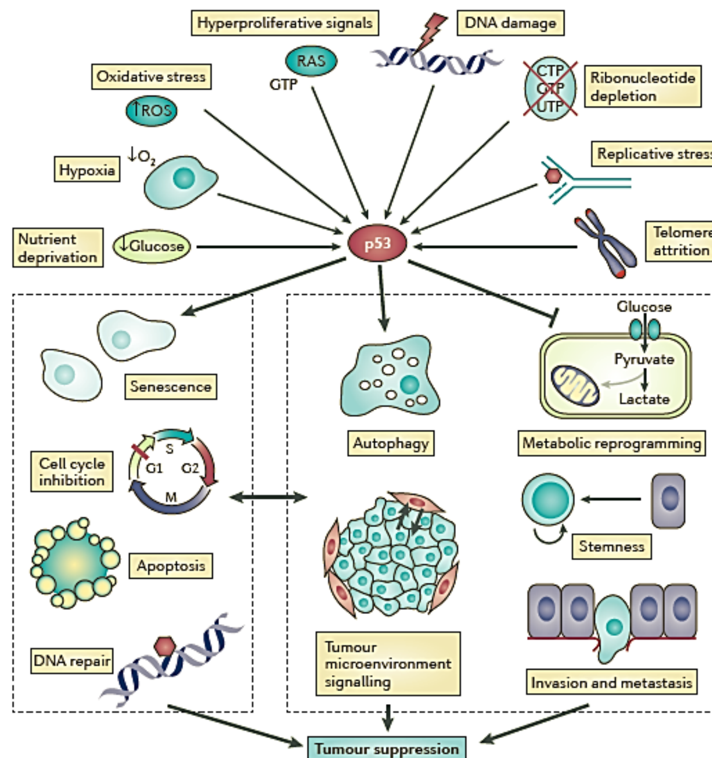

Figure 10. p53-activating signals and responses important for tumor suppression (*Bieging 2014*).

3.1.1 Cell Cycle Arrest

Cell cycle arrest is an immediate response to DNA damage that gives cells time to repair DNA, and when unsuccessful, the cell can enter apoptosis or the senescence programme permanently discontinuing the cell cycle and preventing the organism of the proliferation of these cells [143]. p53 interferes with cell cycle progression by several mechanisms that induce arrest at the G1/S border (G1 arrest) or the G2/M border (G2 arrest) [144]. Its crucial role in induction of G1 arrest occurs trough the induction of transcriptional upregulation of p21, an inhibitor of cyclin-dependent kinases (CDKs), which in turn, inhibits the CDK2 that is responsible for cell cycle progression from G1 into S-phase [144, 145]. p53 role in inhibition of G2/M progression occurs through the upregulation of several genes (p21, Gadd45A and Btg2) and despite the mechanisms are very heterogeneous, it includes interactions with CDK1 and regulation of p21 mRNA stability. However, studies have suggested that p53 is not an essential piece in the induction of G2 arrest but it appears that p53 and its target genes are required to sustain the arrest in G2 [144, 146].

3.1.2 DNA Repair

The p53 protein plays a role in DNA repair response to genotoxic stresses by activating both nucleotide excision repair (NER) and base excision repair (BER) mechanisms [144]. NER is responsible for the removal of bulky DNA adducts, such as UV-induced pyrimidine dimmers and polycyclic aromatic hydrocarbon. In NER, p53 promotes the transcriptional activation of its downstream effector genes that include Gadd45a (binds to UV-damaged chromatin and interacts with core histones and p21) and p48-XPE (the small subunit of the heterodimeric damage-specific DNA binding protein (DDB) in the NER protein complex, and as the function to bind to UV-damaged DNA) [147]. BER corrects DNA base modifications that are frequently induced by reactive oxygen species and endogenous alkylating agents [144]. p53 interacts directly with BER proteins enhancing the stability of interaction between DNA polimerase β, which performs base excision repair, and DNA abasic sites [148-150].

3.1.3 Apoptosis

Apoptosis is the most studied biological function of p53 and is induced in response to cellular stresses, such as DNA damage, hypoxia and aberrant oncogene expression [151]. The apoptotic process is a vital part of p53 tumor suppressor function and its activation can occur through the intrinsic mitochondrial pathway or through the extrinsic death receptor apoptotic program [152, 153]. In the intrinsic mitochondrial pathway, the mitochondria is target by a death

stimuli and, consequently, releases apoptogenic proteins that lead to caspase activation and apoptosis [152]. p53 is intimately involved in this process through transcription-dependent activation of bcl-2, such as PUMA, NOXA and BAX, which will disrupt the integrity of the outer mitochondrial membrane and leading to the release of apoptosis signaling factors [154]. p53 can also promote apoptosis via repression of anti-apoptotic genes, such as survivin, resulting in the caspase activation [152, 155]. In the extrinsic death receptor pathway, p53 directly activates the transcription of genes encoding death receptors, including APO1/FAS/CD95 and KILLER/DR5, which are located at the cellular membrane, recruit adaptor proteins and induce caspases activation, ultimately culminating in apoptosis [152, 156]. Although the literature pointed p53 protein as a regulator of the extrinsic apoptotic pathway, p53-mediated death through this via is not yet fully understood [151]. In fact, cells that die via p53-dependent apoptosis generally follow the intrinsic mitochondrial pathway [152].

3.1.4 Senescence

In the cellular senescence process, proliferation in damaged or potentially oncogenic cells is blocked but these cells are not eliminated from tissues [157]. p53 levels do not seem to raise during cellular senescence but the p53 DNA binding activity and its transcriptional activity were reported as being increased during senescence [158]. Moreover, p21 protein expression increases to its highest levels in senescent cells and these findings suggest that p53 may induce the senescent state through the transactivation of p21 expression [159]. Although other p53 targets and regulators have been linked to induction of senescence, the underlying molecular mechanisms are still poorly understood [157, 159].

3.2 *TP53* AND HUMAN CANCER

In cancer development, p53 inactivation occurs through different mechanisms that include genetic alterations, inactivation by binding to viral or cellular oncoproteins and sequestration of the protein in the cytoplasm. Moreover, somatic *TP53* gene mutations occur in almost every type of cancer [160]. The frequency in somatic *TP53* mutations is highly variable, ranging from around 50% in ovarian, colorectal, head and neck and lung cancers to about 5% in sarcoma, testicular cancer, malignant melanoma and cervical cancer [161]. In fact, the frequency of *TP53* mutations varies according different factor such as the stage of development of the tumor, for example in prostate cancer *TP53* mutations occurs in 10 to 20% of the primary tumors but in the metastatic stage *TP53* mutations are described in around 50% of all cases [162]. Viral and bacterial infections strongly modulate *TP53* mutation frequency due to its capability of interfere

with p53 activity [160]. Different mechanisms that interfere with p53 function have been reported in DNA tumor viruses. For example, the papillomavirus E6 protein interacts directly with p53 to promote its degradation [163]; the hepatitis B virus X protein inhibits the nuclear translocation of p53 [164]; and the adenovirus E1B protein interacts directly with p53 and inhibits its acetylation [165]. Thus, the modulation of p53 function is clearly advantageous for many viruses although *TP53* mutations are a rare event [163].

3.3 *TP53* AND EPSTEIN-BARR VIRUS

Until now, five EBV-encoded viral proteins have been shown to interact with p53: BZLF1, EBNA-LP and EBNA3C are capable of bind to p53 and directly interact with the protein; and LMP1 and EBNA1 who are implicated in indirect modulation p53 expression [166-169].

BZLF1 immediate-early protein, which is an important modulator of p53 function, binds to the C-terminus of p53 through its sequences in the C-terminus dimerization domain, inhibiting p53 transcriptional function and enhancing the ubiquitin-mediated degradation of p53 [167, 170]. However, the effect of BZLF1 on p53 function is controversial, with some studies reporting that BZLF1 increases the level of cellular p53 and enhances p53 transactivation function [171, 172]. The underlying mechanisms of BZLF1 interaction with p53 are still unclear but it is possible that BZLF1 has both activating and inhibitory effects on p53. This dual contradictory function could be the result of cell type-dependent effects of BZLF1 on p53 function or the influence of other viral proteins, whose presence might alter the effect of BZLF1 on p53 [173].

EBNA5, also referred to as EBNA-LP, deregulates cell cycle progression through binding to both Rb and p53 [166]. Recent studies have suggested that EBNA5 binds to p14ARF and MDM2, two proteins involved in p53 regulation, resulting in the downregulation of p53 levels in infected B cells. Furthermore, these studies hypothesis that inhibition of p53 transactivation function is due to formation of trimolecular complexes between EBNA5, MDM2 and p53 [174, 175].

LMP1 role in p53 expression seems to be contradictory, while some report that LMP1 can induce p53 degradation other defend that contributes to its stability and accumulation [168, 176]. *Husaini et al.* refers that LMP1 overexpression lead to increased polyubiquitination of p53, suggesting that decrease of p53 protein levels by LMP1 was due to increased degradation of the protein [168]. Contrarily, *Li et al.* describes that LMP1 promotes p53 accumulation/stability and transcriptional activity through a distinct ubiquitination process, the K63-linked ubiquitination, that results in cell cycle arrest and escape of apoptosis by tumor cells [176].

EBNA1 indirectly regulates p53 through ubiquitin-specific-processing protease 7 (USP7). USP7 is a direct MDM2 antagonist and its overexpression stabilizes p53, leading to p53-mediated growth repression and apoptosis [177]. In EBV-infected cells, EBNA1 binds to USP7 ten times more strongly than p53, interfering with p53 stabilization, and therefore indirectly destabilize p53 contributing for cell immortalization, proliferation and survival of the latently infected cells [178].

EBNA3C contributes to MDM2 stabilization and cellular accumulation by direct binding and deubiquitination of this protein. In turn, this event facilitates p53 ubiquitination and, consequently, its degradation. The repression of p53 function by EBNA3C may augment the efficiency of EBV-mediated cellular transformation [179, 180].

Together, these five EBV proteins mediate the virus interaction with p53 protein, contributing to decreased apoptosis and cell cycle arrest that ultimately promotes proliferation and survival of infected cells and contribute to EBV-mediated carcinogenesis [181].

In contrast to the majority of epithelial malignancies, *TP53* mutations are an infrequent event in EBV-associated neoplasias [34]. In nasopharyngeal carcinomas mutations of *TP53* are a rare event, occurring in less than 10% of all cases [182-184]. However, p53 overexpression has been reported in more than 85% of NPC cases [185, 186]. Although the reason for high p53 levels in NPC is unclear, these findings suggest that other mechanisms different from mutations, such as epigenetic modulation induced by EBV proteins, are responsible for p53 overexpression [187, 188]. In gastric cancer, p53 pathway dysregulation is due to mutations of p53 in approximately 70% of all cases [137, 189]. In contrast, in EBV-associated gastric cancer mutations in p53 are infrequent but CpG islands methylation is a common event suggesting that aberrant methylation might be an important mechanism of EBV-related gastric carcinogenesis [137, 138]. Additionally, EBVaGC had lower rate of p53 overexpression than gastric cancer non-associated with EBV indicating that abnormal p53 expression could be associated with EBV infection [190].

AIMS

Although there are a few studies regarding p53 accumulation in EBV-associated neoplasias, there are no data on p53 mRNA expression in these tumors and moreover there is a lack of clarification concerning the influence of EBV on p53 modulation in neoplasias.

The aim of this study is to characterize p53 accumulation and mRNA expression in EBV-associated epithelial tumors: gastric and nasopharyngeal carcinomas.

MATERIALS AND METHODS

1.Study Population

A retrospective study was performed using 53 patients attended at Portuguese Oncology Institute of Porto (IPO-Porto): 10 with EBV-associated NPC and 43 with GC, being 12 EBV-positive and 31 EBV-negative. All cases were histologically confirmed by a pathologist from our institution and categorized according to the WHO classification systems for each type of cancer. NPC cases were randomly selected from a cohort of patients of our institution [23, 24]. GC cases were selected from a cohort of patients diagnosed with GC in 2011 in our institution (unpublished data), including 12 EBV-positive cases and 31 matched (histological type, age and stage of disease) EBV-negative cases. Positive cases were detected using in situ hybridization for the detection of EBV-encoded small RNA (EBER-ISH). Tumor tissues samples were collected from the institution archives and histological sections from formalin-fixed paraffin-embedded (FFPE) tissue blocks were used for immunohistochemistry and for RNA extraction. This study did not interfere with clinical decisions. Clinicopathological data was collected from individual clinical records and inserted on a database with unique codification. All 111 procedures were approved by the ethical committee of IPO Porto (CES IPO 74/2015).

1.1. Characterization of Population

NPC group of patients (n=10) included 7 males and 3 females with mean age of 51 years old. All NPC cases were undifferentiated nonkeratinizing carcinomas (Table 3).

EBVaGC group of patients (n=12) included 9 males and 3 females with mean age of 69 years old. In this group, half of the cases were tubular adenocarcinomas and the other half were distributed by the other histological subtypes. Regarding tumor localization the EBVaGC were evenly distributed (Table 4).

EBVnGC group of patients (n=31) included 18 males and 13 females with mean age of 63 years old. EBVnGC were equally distributed by histological subtypes. This group was also characterized by a predominance of antral tumor localization and infiltrative invasion pattern (Table 4).

Table 3. Characterization of nasopharyngeal carcinoma cases

	NPC
Gender	*n (%)*
Male	7 (70.0%)
Female	3 (30.0%)
Age	
Mean± sd	51±16.1
Maximum	74
Minimum	20
Global Stage	*n (%)*
II	1 (10.0%)
III	2 (20.0%)
IVa	3 (30.0%)
IVb	1 (10.0%)
IVc	2 (20.0%)
Missing	1 (10.0%)

NPC, nasopharyngeal carcinoma.

Table 4. Characterization of gastric carcinoma cases.

	EBVaGC	*EBVnGC*
Gender	*n (%)*	*n (%)*
Male	9 (75.0%)	18 (58.1%)
Female	3 (25.0%)	13(41.9%)
Age		
Mean ± sd	69±9.62	63±9.86
Range	52- 82	40- 81
Histology WHO	*n (%)*	*n (%)*
Mixed adenocarcinoma	2 (16.7%)	10 (32.3%)
Tubular adenocarcinoma	6 (50.0%)	10 (32.3%)
Poorly cohesive carcinoma	1 (8.3%)	11 (35.4%)
Carcinoma with lymphoid stroma	2 (16.7%)	-
Adenosquamous carcinoma	1 (8.3%)	-
Tumor Localization	*n (%)*	*n (%)*
Antrum	3 (25.0%)	20 (64.5%)
Cardia	2 (16.7%)	3 (9.7%)
Body	4 (33.3%)	8 (25.8%)
Pylorus	1 (8.3%)	-
Missing	2 (16.7%)	-
Invasion Pattern	*n (%)*	*n (%)*
Expansive	6 (50.0%)	8 (25.8%)
Infiltrative	3 (25.0%)	22 (71.0%)
Missing	3 (25.0%)	1 (3.2%)
Global Stage	*n (%)*	*n (%)*
Ia	1 (8.3%)	7 (22.6%)
Ib	2 (16.7%)	1 (3.2)
IIa	2 (16.7%)	3 (9.6%)
IIb	1 (8.3%)	6 (19.7%)
IIIa	5 (41.7%)	4 (12.8%)
IIIb	1 (8.3%)	5 (16.1%)
IIIc	-	3 (9.6%)
IV	-	2 (6.4%)

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

2.p53 accumulation

IHC was used to investigate the accumulation of p53 protein, using 3 µm sections from FFPE tissue blocks with the monoclonal antibody DO-7 (DAKO, Glostrup, Denmark). Tissue samples were submitted to deparaffinization/rehydration using the following sequence: xylene for 2 x 4 minutes; 100% v/v ethanol for 2 x 4 minutes; 96% v/v ethanol for 2x4 minutes; 70% v/v ethanol for 4 minutes and water for 5 minutes. After that, antigen retrieval was performed using a heat induced epitope retrieval method, where the slides were submersed in a citrate-based antigen unmasking solution (VECTOR, Burlingame, CA 121 USA) and heated in the microwave for 15 minutes at medium power. Slides were allowed to cold down to room temperature, rinsed in the unmasking solution for almost 30 minutes. Then, samples were washed in phosphate-buffered saline (PBS) containing 0.02% Tween 20 (PBS-T) and the endogenous peroxidase was blocked with 3% hydrogen peroxide (H_2O_2) for 10 minutes. Subsequently, the slides were washed 2x in PBS-T for 5 minutes, 126 treated with UV-block solution from UltraVision Large Volume Detection System Anti- 127 Polyvalent, HRP (THERMO SCIENTIFIC, Fremont, USA) for 10 minutes to block nonspecific protein binding and incubated overnight at 4ºC with DO-7 mouse anti-human p53 monoclonal antibody diluted 1:200 (DAKO, Glostrup, Denmark). Slides were then rinsed in PBS-T, incubated with Biotinylated Goat Anti-Polyvalent Antibody (THERMO SCIENTIFIC, Fremont, USA) in a humid chamber at room temperature for 10 minutes, washed 2x with PBS-T for 5 minutes and incubated with Streptavidin Peroxidase (THERMO SCIENTIFIC, Fremont, USA) for 10 minutes at room temperature. Detection of hybrids was achieved by an enzymatic reaction using 3,3'-diaminobenzidine (DAB) ImmPACTTM DAB (VECTOR, Burlingame, CA USA) diluted at 3:100 and incubated during 4 minutes at room temperature. The final wash was performed with distilled water for 5 minutes. Mayer's hemalum solution (Millipore, Darmstadt, Germany) was used as counterstain. After coloration, slides were washed in running water for 5 minutes and the following step was sequential dehydration in 70% v/v ethanol for 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). Nuclear p53 accumulation was defined as negative (>5% cells). Tumors with positive p53 staining were semi-quantitatively categorized into four categories: 5-25%, 25-50%, 50-75% and >75% of nuclei staining positive.

3. *TP53* mRNA expression

RNA was extracted from 10 µm sections using the Absolutely RNA FFPE Kit (Agilent Technologies, San Diego CA, USA) and quantified using the NanoDrop 1000 Spectrophotometer v3.7 (Thermo Scientific, Wilmington DE, USA). *TP53* and GAPDH were analyzed by two-step real-time PCR using hs01034249_m1 and hs02758991_g1 TaqMan Gene Expression Assays (Applied Biosystems, Foster CA, USA), respectively. Reverse transcriptase reactions, with 20 µL final volume, were performed using High-Capacity cDNA Reverse Transcription Kit (PN 4368814; Applied Biosystems, Foster CA, USA) according to the manufacturer's instructions. The amplification conditions were as follows: annealing at 25ºC for 10 min, extension at 37ºC for 120 min and RT inactivation at 85ºC for 5 min. All reverse transcriptase reactions included no-template controls. qPCRs were performed in duplicates in independent reactions 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 10-100 ng of cDNA (RT product). Amplification was run in Applied Biosystems Step-One Real Time PCR System (Applied Biosystems, Foster CA, USA) with the following thermal cycling conditions: 10 min at 95°C followed by 45 cycles of 15 sec at 95°C and 1 min at 60°C. The relative quantification of p53 expression was analyzed using the $2^{-\Delta\Delta Ct}$ method, also known as Livak method. In this method, Ct from the target RNA (p53) in both test and control cases were adjusted in relation to the Ct of a normalizer RNA (GAPDH) resulting in ΔCt. For the comparison between EBVaGC and EBVnGC we have calculated ΔΔCt value, which allows us to determine the differences in p53 expression.

4. Statistical analysis

Results were analysed using the computer software IBM SPSS Statistics for Windows, Version 22.0 (IBM Corp, Armonk NY, USA). Data from all cases were compared by Student's t-test and ANOVA considering a statistical significance of 5% ($p<0.05$).

RESULTS

1. p53 accumulation

The results from IHC analysis are shown in Table 5 and Figure 11 where is described, for each group, the percentage of cells with p53 accumulation/reactivity. Figure 12 illustrates examples of p53 accumulation in the different groups. All cases included in this study, except one GC case, were positive for the presence of p53 accumulation by immunohistochemistry. NPC cases are a homogeneous group, with all samples having more than half of cells with p53 accumulation: 3 cases (30%) showed 50-75% of cells with p53 accumulation and 7 (70%) presented more than 75% of cells with p53 accumulation. In EBVnGC cases, p53 was frequently found with 16.7%, 33.3%, 26.7% and 23.3% of cases found with 5-25%, 25-50%, 50-75%, and >75% of accumulation, respectively. Similarly to NPC, EBVaGC showed a strong p53 accumulation, with 58.3% of cases having more than 75% of cells with p53 accumulation, 16.7% with 50-75% and only 25% having less than 50% of cell with p53 accumulation. Results showed that p53 accumulation in NPC and EBVaGC is not significantly different (p=0.501) while there is a statistically significant difference between EBVaGC and EBVnGC (p=0.027). Regardless of EBV status, the analysis of all gastric cancer cases revealed that there is no statistical differences between the histological subtypes in the p53 accumulation in tissue (p=0.856) (data not shown). Similarly, the comparison of all gastric cancer cases according to tumor localization and invasion pattern indicated no statistical differences in the expression of p53 (p=0.723 and p=0.171, respectively) (data not shown).

Table 5. Distribution of percentage of cells with p53 accumulation in nasopharyngeal and gastric carcinomas

	Percentage of cells			
	5-25% *n(%)*	*25-50%* *n(%)*	*50-75%* *n(%)*	*>75%* *n(%)*
EBVnGC (n=30)	5 (16.7)	10 (33.3)	8 (26.7)	7 (23.3)
EBVaGC (n=12)	2 (16.7)	1 (8.3)	2 (16.7)	7 (58.3)
NPC (n=10)	0 (0.0)	0 (0.0)	3 (30.0)	7 (70.0)
Total	7 (13.4)	11 (21.2)	13 (25.0)	21 (40.4)

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

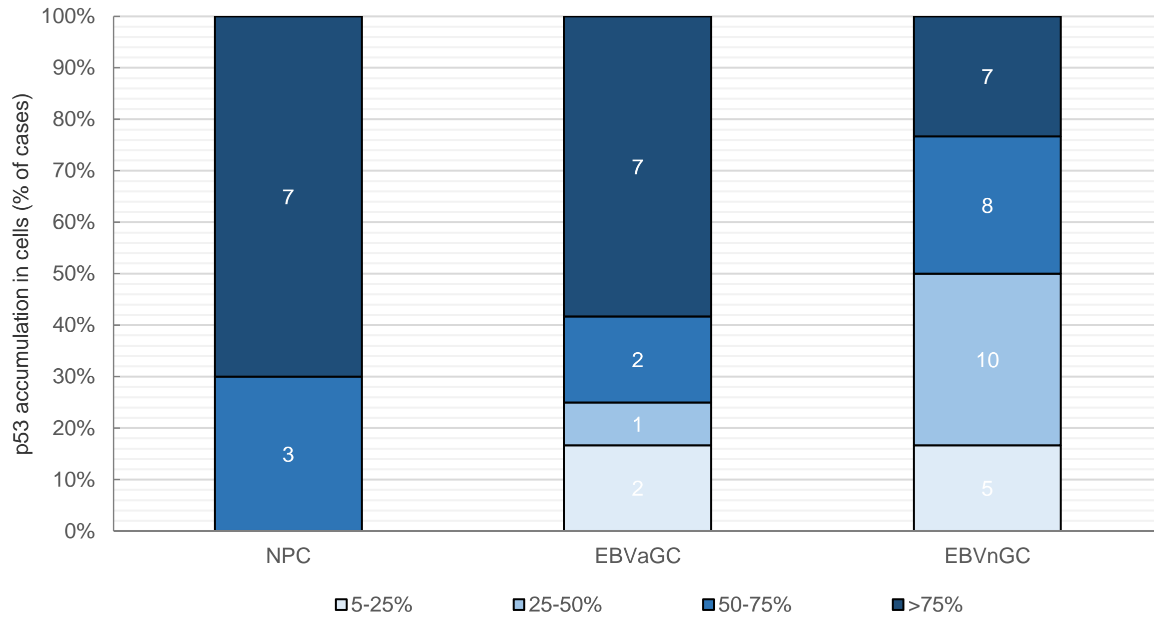

Figure 11. Percentage of cells with p53 accumulation in nasopharyngeal and gastric carcinomas.

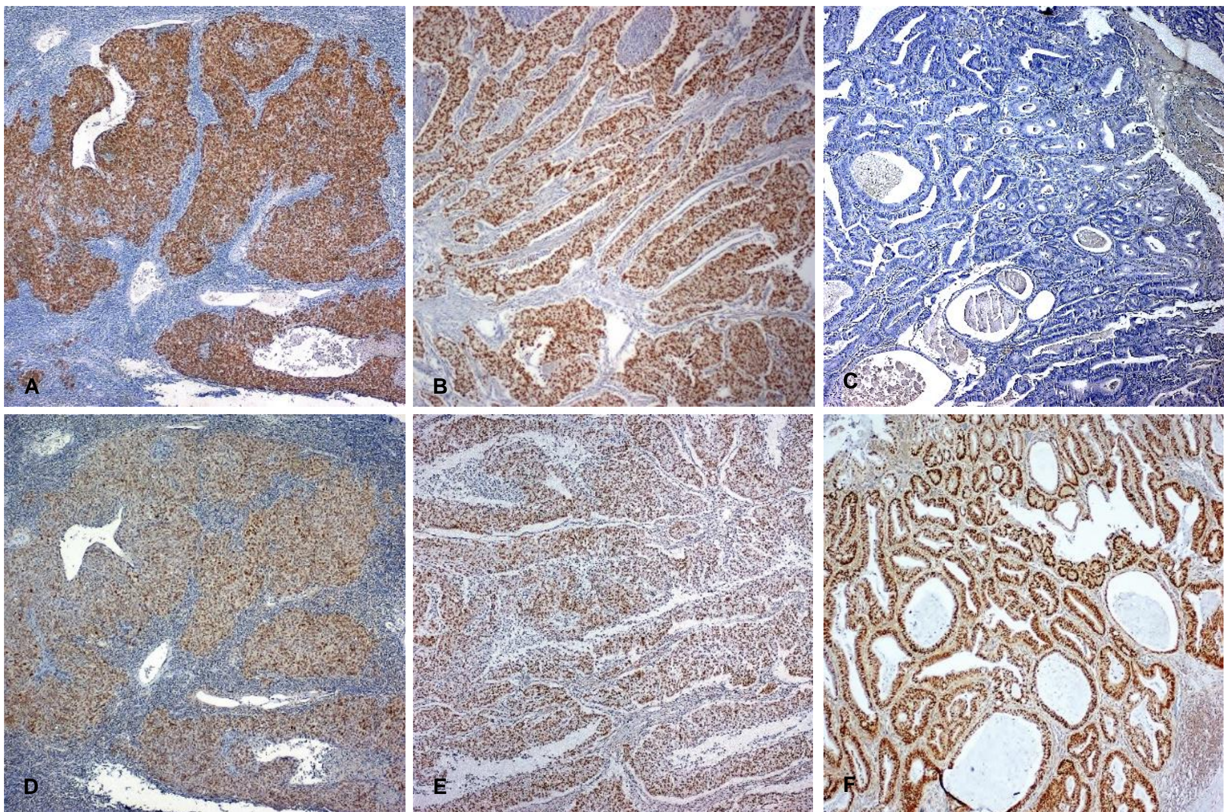

Figure 12. Expression of EBERs and p53 in NPC, EBV-associated and EBV-negative gastric cancers.
A-B) EBER-ISH positive staining in NPC and EBVaGC;
C) Negative result of EBER-ISH in EBVnGC;
D-F) Representative tumors with strong p53 accumulation.

2. *TP53* mRNA expression

The results from qPCR analysis are shown in Table 6. *TP53* mRNA and GAPDH mRNA (reference gene) were evaluated for all cases and 6 (1 NPC and 5 EBVnGC) were excluded of the analysis because *TP53* mRNA expression was not detected. The analysis of NPC cases revealed the presence of *TP53* mRNA – Figure 13. When analysing the expression of *TP53* in EBVaGC, we observed a significant decrease ($2^{-\Delta\Delta Ct}$=0.21; p=0.010) in *TP53* mRNA expression in comparison with EBVnGC – Figure 13. Further analysis subdividing EBVnGC according to histological subtypes revealed that EBVaGC *TP53* mRNA expression was significantly decreased when compared with EBVnGC poorly cohesive and EBVnGC tubular histological subtypes ($2^{-\Delta\Delta Ct}$=0.11; p<0.001 and $2^{-\Delta\Delta Ct}$=0.20; p=0.008, respectively); despite not statistically significant, the reduction of expression also occurs when comparing with EBVnGC mixed types ($2^{-\Delta\Delta Ct}$=0.43; p=0.162) - Figure 13. Moreover, the comparison of *TP53* mRNA expression between GC histological subtypes, regardless of EBV status, revealed a difference with statistical significance between mixed adenocarcinomas and poorly cohesive carcinomas ($2^{-\Delta\Delta Ct}$=0.27; p=0.014). In addition, the analysis of GC cases according to tumor localization and invasion pattern indicated no statistical significant differences in *TP53* mRNA expression.

Table 6. qPCR data analysis and expression profile data for *TP53* mRNA in nasopharyngeal and gastric cancers

	Ct GAPDH	*VC*	*Ct TP53*	*VC*	*ΔCt ± sd (range)*
EBVaGC (n=12)	26.31 ± 1.19 (24.27 – 27.97)	0.05	34.42 ± 1.59 (31.78 – 36.68)	0.05	8.10 ± 1.83 (3.84 – 10.34)
EBVnGC (n=26)	28.06 ± 2.51 (23.41 – 34.57)	0.09	33.90 ± 1.69 (30.49 – 37.27)	0.05	5.84 ± 1.73 (2.71 – 10.61)
EBVnGC poorly cohesive (n=9)	29.71 ± 1.69 (27.29 – 32.60)	0.06	34.68 ± 1.24 (32.53 – 36.25)	0.04	4.97 ± 1.20 (3.60 – 7.35)
EBVnGC tubular (n=9)	27.07 ± 3.16 (23.41 – 34.57)	0.12	32.86 ± 1.95 (30.49 – 37.27)	0.06	5.80 ± 1.72 (2.71 – 8.93)
EBVnGC mixed (n=8)	27.31 ± 1.58 (25.29 – 30.10)	0.06	34.19 ± 1.35 (32.36 – 35.90)	0.04	6.88 ± 1.86 (4.41 – 10.61)
NPC (n=9)	27.83 ± 1.55 (25.14 – 29.76)	0.05	33.39 ± 1.74 (30.51 – 36.08)	0.05	5.56 ± 1.06 (4.07 – 7.19)

Ct, cycle threshold; sd, standard deviation; VC, variation coefficient; EBVaGC, EBV-associated gastric carcinoma; EBVnGC, EBV non-associated gastric carcinoma; NPC, nasopharyngeal carcinoma.

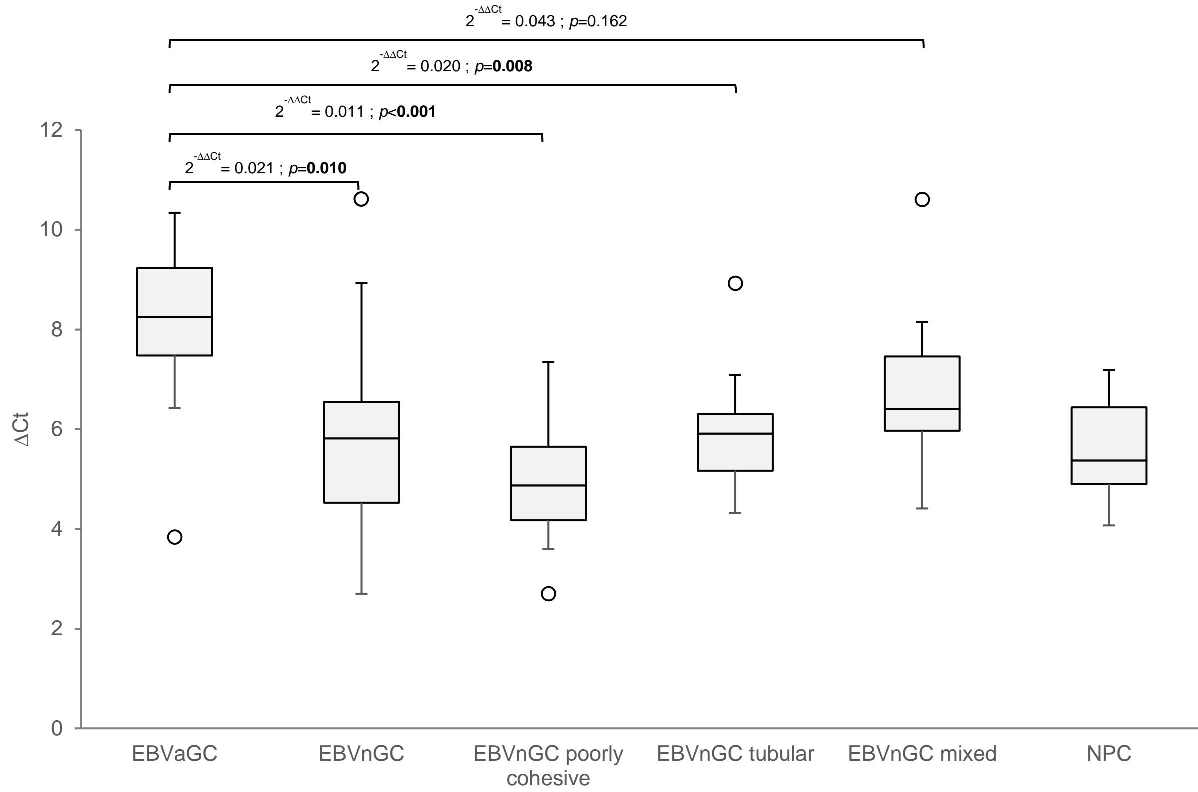

Figure 13. Expression profile of *TP53* mRNA in nasopharyngeal and gastric carcinomas.

DISCUSSION

Despite the great number of genes involved in human carcinogenesis, *TP53* gene has been considered as one of the most important genes, being crucial in the regulation of signalling processes of tumor development [191]. The p53 protein is activated in response to endogenous or exogenous stresses, inducing cell cycle arrest, DNA repair, apoptosis and senescence of cells [142]. Therefore, the modulation of *TP53* is considered a key hallmark for cancer development and there are several mechanisms that contribute for its deregulation [191]. *TP53* gene mutations are one of the most frequent alterations in human cancers, occurring in almost every type of cancer at rate of 10% to 100% [142, 161]. Furthermore, viruses have been considered as able to modulate p53 pathway, either by direct inactivation of the protein or by gene-gene interaction [160].

EBV, a ubiquitous herpesvirus, infects approximately 90% of the human adult population worldwide, being the infection asymptomatic in the majority of lifelong carriers [10]. Based on its role in the development of malignant disorders, EBV has been classified as a group 1 carcinogen by the International Agency for Research on Cancer (IARC) [34, 192].

EBV has been described in the pathogenesis of lymphoproliferative disorders as well as epithelial malignancies such as NPC and GC [19, 58]. One of the potential targets of EBV in carcinogenesis processes is the p53 pathway and previous studies have shown that the frequency of *TP53* gene mutations in EBV-associated neoplasias is low [137, 138, 182-184] . In fact, *TP53* is rarely mutated suggesting that other mechanisms different from mutations could be responsible for p53 deregulation [187, 188]. Understanding the mechanisms of EBV-associated p53 deregulation in these cancers would allow a better knowledge of the carcinogenesis model in epithelial tumors.

In our study, the detection of p53 accumulation was performed by IHC for p53 using the DO-7 antibody, that recognizes both wild-type and mutant forms of p53 [193]. This is a cheap and rapid method widely used in routine procedures, being the commonest method to infer *TP53* mutational status in ovarian carcinoma [194] since it is more likely to detect mutated p53 due to its longer half-life usually considering that is related to *TP53* gene mutation [195]. However, in other models studies that have used IHC as a surrogate marker for *TP53* mutation have failed to demonstrate consistent results [161] by showing a large number of misclassified cases (false-positive and false-negative) [161, 196]. In our study p53 staining was nuclear in tumor cells and absent in normal cells. Nuclear p53 staining was classified as p53 accumulation and this accumulation could not be directly correlated with *TP53* mutations. Alterations in p53 signalling pathway might lead to functional p53 stabilization or inhibition of p53 degradation, ultimately resulting in p53 accumulation not related to *TP53* mutations [197].

Concerning NPC, we have found p53 accumulation in 100% of cases and all of them showed more than 50% of cells with p53 accumulation. Previous studies also showed a high p53 accumulation, ranging between 64.7% and 95.9% [185, 186, 188, 198-202]. The relationship between accumulation of p53 in NPC and EBV infection is still controversial [185, 188, 199-201]. Although the mechanism by which EBV induced p53 expression has not been elucidated, some authors suggested that LMP1, a highly expressed EBV-latent protein in NPC, seem to be responsible for p53 accumulation in NPC without *TP53* gene mutation [108]. Indeed, LMP1 seems to induce wild-type p53 stabilization through the promotion of K63-linked ubiquitination, which is a cellular pathway regulator and thus enhancing the half-life of the protein [176, 203]. Furthermore, LMP1 also blocks MDM2-mediated p53 degradation through the suppression of K48-linked ubiquitination [176]. Together this dual function of LMP1 could explain the wild-type p53 accumulation in NPC tissue [176].

Regarding p53 accumulation in GC, our study revealed an overall high rate of p53 accumulation (100% in EBVaGC and 96.7% in EBVnGC). Similar results were found by other authors: *Wang et al.* reported a high p53 accumulation in both EBVaGC (84.6%) and EBVnGC (86.7%) [190] ; and *Kim et al.* showed more p53 accumulation in EBVaGC (100%) compared to EBVnGC (85.0%) and with a predominance of >50% of cells with p53 accumulation in both EBVaGC (83.3%) and EBVnGC (75.0%) [204]. Other studies showed contradictory results reporting less p53 accumulation in EBVaGC compared to EBVnGC [205-207] and one meta-analysis study demonstrated a lower rate of p53 accumulation, although not statistical significant, in EBVaGC (36.2%) when compared with in EBVnGC (47.9%) [208]. Our results also showed a significant difference of p53 accumulation in EBVaGC comparing with EBVnGC (p=0.027), with p53 accumulation in more than 50% of cells in 9/12 EBVaGC comparing with an equally distribution between p53 accumulation groups in EBVnGC. Contrarily to other studies, our data seems to indicate that the percentage of cells with p53 accumulation in gastric cancer is dependent of EBV infection [209-211].

In the present study, comparison of gastric cancers, regardless of EBV status, revealed no differences in terms of p53 accumulation between tumor histology subtypes. With the exception of two studies that reported no significant association between the histology of tumor and p53 accumulation [212, 213], the majority of publications found a significant correlation of histological subtype with p53 accumulation in gastric cancer tissue [214-216]. All these reports utilized Lauren's histological classification, comparing intestinal and diffuse type. Accumulation of p53 occurred significantly more in the intestinal type, with p53 accumulation in 55.9% of intestinal type and 27.3% of diffuse type [214, 216]. Although our results were not statistically

different, we found that the majority (58.3%) of tubular adenocarcinomas (Lauren's intestinal type) had more than 50% of cells with p53 accumulation while 66.7% of poorly cohesive carcinomas (Lauren's diffuse type) had less than 50% of cells with p53 accumulation.

The results regarding p53 accumulation according to tumor localization and invasion pattern also showed no statistical differences, which is in agreement with the literature, suggesting that neither tumor location or invasion pattern influence p53 accumulation in gastric cancer tissue [212, 217].

In order to better understand if the accumulation of p53 was a consequence of increased *TP53* transcription, we have also investigated the *TP53* mRNA by qRT-PCR. As far as we know, this is the first study using qRT-PCR methodology to evaluate the levels of *TP53* gene expression in EBVaGC.

Among GC, we found a significant decrease in *TP53* mRNA expression in EBVaGC, which had less 80% of *TP53* mRNA expression when compared with EBVnGC. A further subdivision of EBVnGC in different histological subgroups showed that EBVaGC had a significant downregulation of *TP53* mRNA expression independent of histological subtypes. This result could be explained by the potential of some EBV latent proteins to destabilize p53: EBNA1 has been described to compete with p53 for the same binding site of USP7, a cellular ubiquitin-specific protease that has been reported to bind and regulate p53, and its higher affinity interferes with the stabilization of p53 contributing for lower p53 levels [104, 218, 219]; EBNA3C reported as capable to directly bind p53 and repress its apoptotic and transcriptional activities [179]; and EBNA5, which is capable of binding to p14ARF and MDM2, two proteins involved in p53 regulation, resulting in the downregulation of p53 levels by the formation of trimolecular complexes between EBNA-5, MDM2 and p53 [174, 175]. Therefore, EBNA1, EBNA3C, EBNA5 or the combined effect of these three EBV proteins could be the cause of decrease in *TP53* mRNA expression that we found in EBVaGC. Although there are no previous studies, taking into account that deregulations induced by these EBV latent proteins do not always lead to p53 degradation, they could also be responsible for p53 accumulation in cells. Due to this interaction of EBV proteins with p53, it will be important to evaluate the *TP53* mutational status to understand if p53 accumulation is a result or not of *TP53* mutations in EBV-positive cells.Our results are mostly important to corroborate the idea that with the current knowledge on molecular features of GC, histological classification has limited value to distinguish these tumors. In fact, due to the heterogeneity of GC, a new classification based on molecular features of tumors have been proposed, with EBVaGC categorized as a "new" and distinct subtype of gastric cancer [137, 138]. In addition, *Cristescu et al.* proposed a new classification

wherein p53 status has a pivotal role: *TP53*+ (without *TP53* gene mutation) and *TP53*- (with *TP53* gene mutations). Interestingly the group of *TP53*+ was closely linked to EBV infection and had a better overall survival [220]. These data is in accordance with previous reports of no *TP53* mutations in EBV positive gastric carcinomas [137, 138]. Our study gives new insight on modulation of p53 by EBV but further studies are needed in order to understand the mechanisms by which EBV modulates p53 expression and accumulation in cells.

CONCLUSION

In EBV-associated epithelial malignancies, p53 is differently modulated comparing to non-viral neoplasias, whereas *TP53* mutations are common. With this study, it was possible to confirm that in our population all NPC and EBVaGC had p53 accumulation in tissue and EBVaGC and EBVnGC had a significant difference of p53 accumulation. We were also able to identify that EBVaGC had a significant decrease of *TP53* mRNA comparing with EBVnGC.

Our results gave new insight in the molecular features of NPC and EBVaGC and demonstrated that p53 is not differently accumulated or expressed according to the histological groups. This data corroborate previous studies defending that classification of gastric carcinomas based on histology is a method with limited utility and that propose a new gastric cancer classification based on its molecular features [138].

Furthermore, our results demonstrated that although EBV-associated neoplasias showed p53 accumulation, in EBVaGC *TP53* mRNA seems to be significantly diminished when compared to EBV non-associated neoplasias. These findings support the hypothesis that the carcinogenesis mechanism is different depending if the tumor is associated or not to EBV.

This is the first study regarding *TP53* mRNA expression in EBVaGC and further studies are required to confirm these evidences, especially with different populations. It is also important to study the mutational status of *TP53* gene to know if the p53 accumulation found in this work is related or not with mutations in this gene. Additionally, it would be interesting to study other genes in order to understand the mechanisms by which EBV modulates both oncogenes and tumor suppressor genes to promote carcinogenesis.

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APPENDIX I

Poster EBV 2016 International Symposium

p53 EXPRESSION IN EPSTEIN-BARR VIRUS ASSOCIATED GASTRIC CANCER AND NASOPHARYNGEAL CARCINOMA

THU-PS10-30

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Introduction

Epstein-Barr virus (EBV) is a human cancer-associated virus that infects approximately 90% of the human adult population worldwide. Nevertheless, EBV has a great carcinogenic potential and has been associated with epithelial tumours such as Nasopharyngeal Carcinoma (NPC) and more recently to Gastric Carcinoma.

The development of EBV-associated malignancies is dependent on the expression of viral proteins, such as LMP1 and LMP2a, that modulate multiple signalling pathways.

TP53 is a tumour suppressor gene frequently mutated in human cancers. However, in EBV-associated epithelial malignancies TP53 mutations are uncommon, suggesting that other mechanisms different from mutations could be responsible for p53 deregulation .

This study aimed to evaluate p53 accumulation and mRNA expression in NPC and EBV-associated gastric carcinoma (EBVaGC) tissues and compare with EBV LMP1 and LMP2a expression in tumours.

Methods

p53 expression and accumulation was evaluated in 3 groups of patients: 10 with EBV-associated NPC (mean age: 51±16); 12 EBVaGC (mean age: 64±10) and 31 EBV non-associated gastric carcinomas (EBVnGC) (mean age: 63±10).

The expression of p53 mRNA was evaluated by qRT-PCR and its relative quantification was determined using the Livak method, with GAPDH mRNA as normalizer.

Accumulation of p53 and LMP1 and LMP2a expression were assessed by immunohistochemistry (IHC) using monoclonal antibodies (DO-7, NCLEBV-CS1-4 and 15F9, respectively). LMP1 and LMP2a expression was classified as positive or negative. Nuclear p53 accumulation was defined as negative (<5% cell) or positive (>5% cells). Tumours with positive p53 staining were semi-quantitatively categorized into four groups as follows: 5-25%, 25-50%, 50-75% and >75% of nuclei staining positive.

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Results

IHC for p53 showed its accumulation in all cases with the exception of one EBV non-associated gastric cancer (EBVnGC) that was negative. The EBVnGC cases were uniformly distributed between the four groups of percentages while EBVaGC and NPC showed a strong p53 accumulation (Figure 1). The majority of EBVaGC cases had more than 75% of cells with p53 accumulation. The NPC cases form a homogeneous group, with all samples having more than 50% of cells with p53 accumulation.

In contrast, qRT-PCR results revealed a significant decreased expression of TP53 mRNA in EBVaGC ($2^{-\Delta\Delta Ct}$=0.21; *p*=0.010) when compared with EBVnGC (Table 1). In all NPC cases the presence of TP53 mRNA was also observed.

The expression analysis of LMP1 and LMP2a in EBVaGC and NPC tumors, demonstrated different profiles. LMP1 is expressed in all NPC and in none of EBVaGC cases, while LMP2a is present in 100% and 58.3%, respectively (Figure 2; Figure 3).

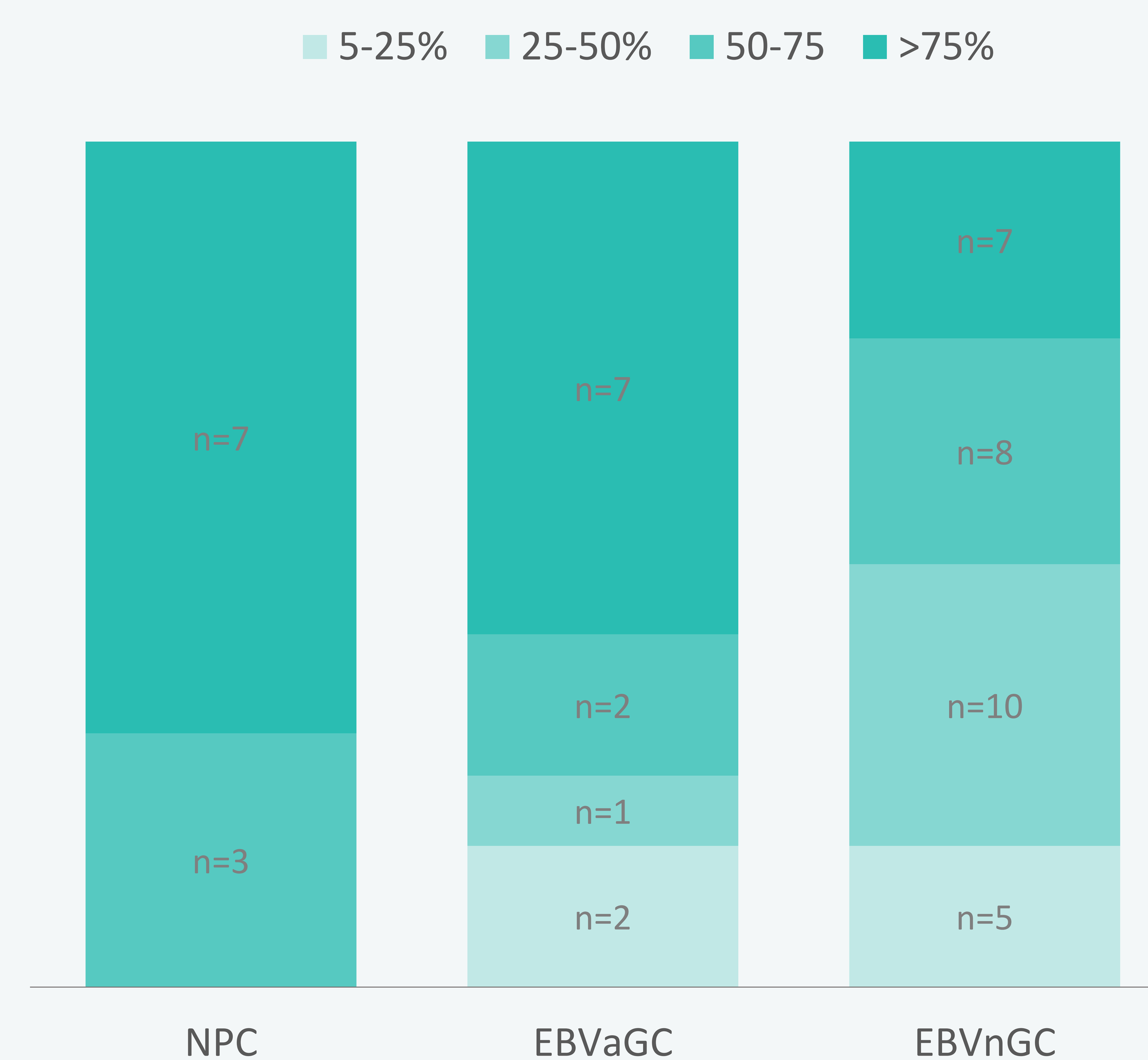

Figure 1: Percentage of cells with p53 accumulation in different neoplasias.

Table 1: Expression profile data for p53 mRNA in gastric cancer.

	ΔCt ± sd	$2^{-\Delta\Delta Ct}$	*p value*
EBVnGC (n=26)	5.84 ± 1.73	Reference (1)	-----
EBVaGC (n=12)	8.10 ± 1.83	0.21	0.010

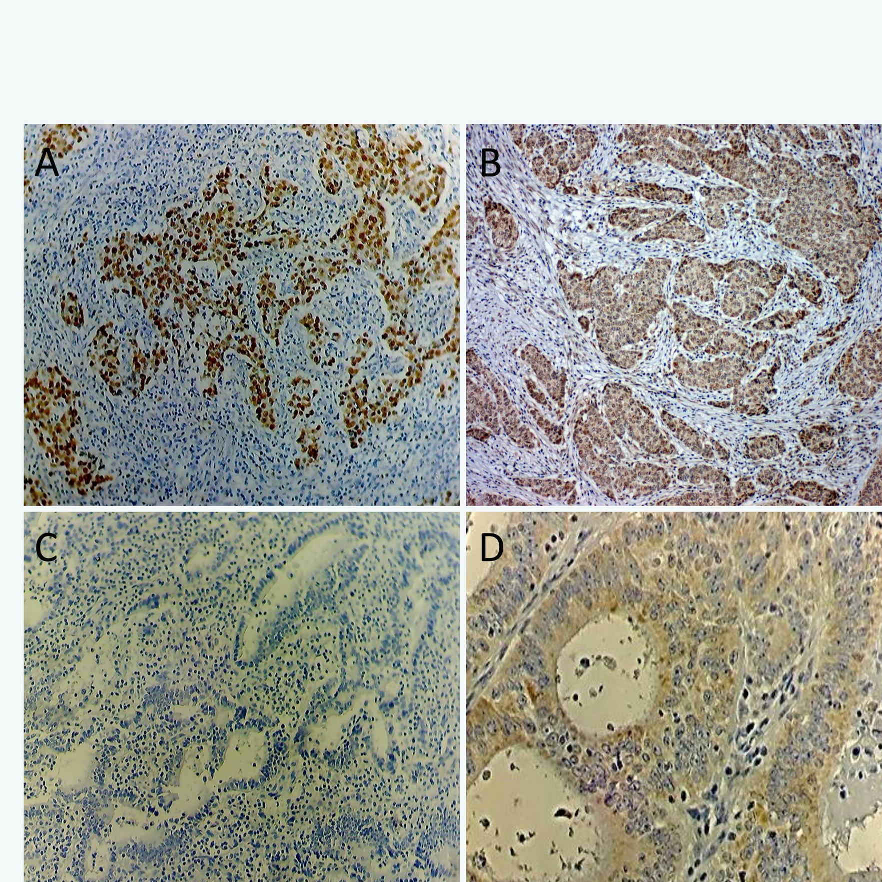

Figure 2: Examples of immunohistochemistry on EBVaGC: A. EBERs; B. p53; C. LMP1; D. LMP2a.

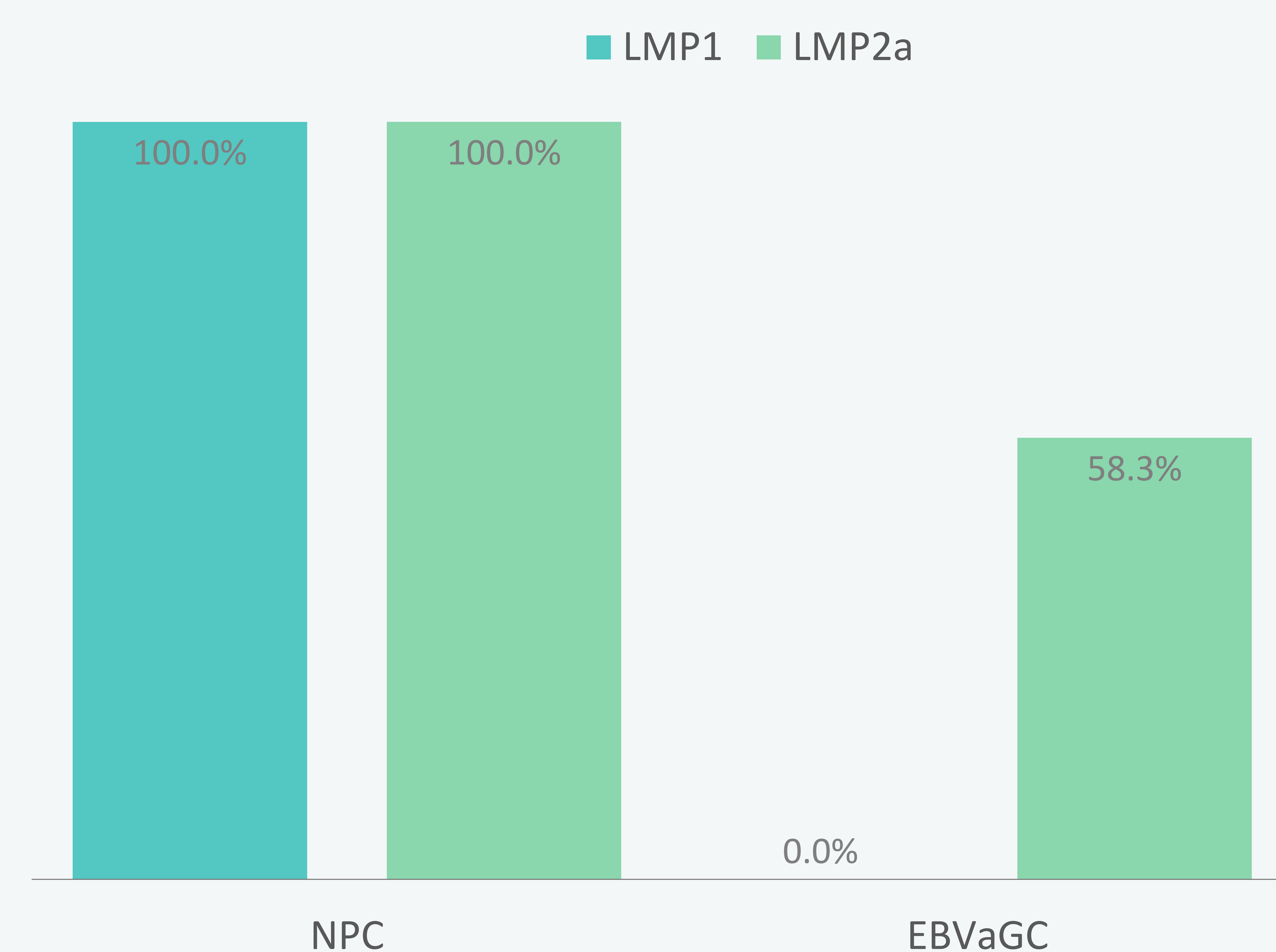

Figure 3: Percentage of cases with LMP1 and LMP2a expression in NPC and EBVaGC.

Conclusion

p53 accumulation is observed in all EBV-associated epithelial malignancies and in 96.8% EBV-negative gastric cancers. However, our study revealed that p53 mRNA expression decreases significantly when comparing EBV-positive and EBV-negative gastric carcinomas. These results suggest that **EBV-mediated carcinogenesis interferes with p53 pathway**.

In addition, **NPC and EBVaGC were characterized by different profiles of LMP1 and LMP2a expression suggesting a distinct EBV-mediated carcinogenesis**.

APPENDIX II

Article I

TITLE: *p53 ACCUMULATION AND EXPRESSION IN EPSTEIN-BARR VIRUS ASSOCIATED EPITHELIAL TUMORS: GASTRIC AND NASOPHARYNGEAL CARCINOMA*

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29 **ABSTRACT (250 words)**

30 **Background:** Epstein-Barr virus (EBV) has been associated with the development of
31 epithelial tumors such as Nasopharyngeal Carcinoma (NPC) and more recently to Gastric
32 Carcinoma (GC). *TP53* is a tumor suppressor gene frequently mutated in human cancers;
33 nevertheless, in EBV-associated epithelial malignancies mutations are uncommon even
34 with frequent deregulation of the p53 pathway. In this study, we aimed to characterize p53
35 accumulation and mRNA expression in NPC and EBV-associated gastric carcinoma
36 (EBVaGC) tissues.

37 **Methods:** p53 expression and accumulation was evaluated by qRT-PCR and
38 immunohistochemistry (IHC), in a retrospective study with tissues from 10 NPC, 12
39 EBVaGC and 31 EBV-negative GC (EBVnGC) cases.

40 **Results:** IHC showed that p53 is accumulated in 42/43 GC and all 10 NPC cases, with
41 more than 50% of cases showing 50-100% of cells with p53 accumulation. This high rate of
42 p53 accumulation was more common in NPC and EBVaGC rather than EBVnGC.
43 Regarding the expression of TP53, in NPC it was observed the presence of TP53 mRNA.
44 Furthermore, in GC the *TP53* expression analysis revealed that the levels of TP53 mRNA
45 in EBVaGC are almost 80% lower ($2^{-\Delta\Delta Ct}$=0.21; *p*=0.010) when compared with EBVnGC,
46 and these results were independent of the histological subtypes.

47 **Conclusion:** Our results showed that p53 accumulation was observed in 100% of EBV-
48 associated epithelial malignancies (NPC and EBVaGC) and in 96.8% of EBVnGC. The
49 significant decrease of *TP53* mRNA in EBVaGC comparing with EBVnGC, suggests that
50 viral carcinogenesis interferes with the p53 pathway and that this seems to occur
51 independently of the presence of mutations.

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INTRODUCTION

Approximately 16% of all cancer cases are attributable to infectious agents, with Epstein-Barr virus (EBV) being one of the most important [1]. EBV is a ubiquitous pathogen that is harbored by approximately 90% of all adults throughout the world, although the infection is asymptomatic in the majority of lifelong carriers [2]. Based on its role in the development of malignant disorders, EBV has been classified as a group 1 carcinogen by the *International Agency for Research on Cancer* (IARC) and has been related with malignancies of both lymphoid and epithelial cell origin [3, 4]. Despite more frequently associated with lymphoid tumors, EBV has been consistently found in tumor cells of Nasopharyngeal Carcinoma (NPC) cases from endemic regions [5], and more recently it was associated with Gastric Cancer (GC), where EBV infection has been detected in around 10% of all cases [6].

TP53, the gene encoding for the tumor suppressor protein p53, is the most frequently mutated gene in human cancer [7]. Dysregulation of the TP53 pathway involve different mechanisms that include genetic alterations, inactivation by binding to viral or cellular oncoproteins and sequestration of the protein in the cytoplasm [8]. The frequency in somatic *TP53* mutations is highly variable, ranging from around 80% in squamous cell lung carcinoma to only 5% in testicular and cervical cancer [8, 9]. Viral and bacterial infections strongly modulate *TP53* mutation frequency due to its capability of interfere with p53 activity [10]. For example, the papillomavirus E6 protein interacts directly with p53 to promote its degradation [11]; the hepatitis B virus X protein inhibits the nuclear translocation of p53 [12]; and the adenovirus E1B protein interacts directly with p53 and inhibits its acetylation [13]. Thus, the modulation of p53 function is clearly advantageous for many viruses [11].

EBV-associated epithelial malignancies are characterized by rare *TP53* mutations [14, 15]. In NPC, mutations of *TP53* are a rare event, occurring in less than 10% of all cases [16-18]. In GC, TP53 pathway dysregulation is due to *TP53* mutations in approximately 70% of all cases [19, 20], nevertheless in the EBV-associated gastric cancers, mutations in *TP53* are infrequent [14, 19]. These findings suggest that other mechanisms different from mutations,

88 such as epigenetic modulation induced by EBV proteins, could be responsible for p53
89 deregulation [21, 22].

90 The aim of this study was to characterize p53 accumulation and *TP53* mRNA expression in
91 EBV-associated nasopharyngeal and gastric carcinomas contributing for the knowledge of
92 the molecular mechanisms of *TP53* silencing in EBV-associated malignancies.
93

94 **SUBJECTS, MATERIALS, AND METHODS**

95 **Study population**

96 A retrospective study was performed using 53 patients attended at Portuguese Oncology
97 Institute of Porto (IPO-Porto): 10 with EBV-associated NPC (**Table 1**) and 43 with GC,
98 being 12 EBV-positive and 31 EBV-negative (**Table 2**). All cases were histologically
99 confirmed by a pathologist from our institution and categorized according to the WHO
100 classification systems for each type of cancer.

101 NPC cases were randomly selected from a cohort of patients of our institution [23, 24]. GC
102 cases were selected from a cohort of patients diagnosed with GC in 2011 in our institution
103 (unpublished data), including 12 EBV-positive cases and 31 matched (histological type, age
104 and stage of disease) EBV-negative cases. Positive cases were detected using *in situ*
105 *hybridization* for the detection of EBV-encoded small RNA (EBER-ISH). Tumor tissues
106 samples were collected from the institution archives and histological sections from
107 formalin-fixed paraffin-embedded (FFPE) tissue blocks were used for
108 immunohistochemistry and for RNA extraction.

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

112

113 **p53 accumulation**

114 IHC was used to investigate the accumulation of p53 protein, using 3µm sections from
115 FFPE tissue blocks with the monoclonal antibody DO-7 (DAKO, Glostrup, Denmark).

116 Tissue samples were submitted to deparaffinization/rehydration using the following
117 sequence: xylene for 2 x 4 minutes; 100% v/v ethanol for 2 x 4 minutes; 96% v/v ethanol
118 for 2x4 minutes; 70% v/v ethanol for 4 minutes and water for 5 minutes. After that, antigen
119 retrieval was performed using a heat induced epitope retrieval method, where the slides
120 were submersed in a citrate-based antigen unmasking solution (VECTOR, Burlingame, CA
121 USA) and heated in the microwave for 15 minutes at medium power. Slides were allowed
122 to cold down to room temperature, rinsed in the unmasking solution for almost 30 minutes.

Then, samples were washed in phosphate-buffered saline (PBS) containing 0.02% Tween 20 (PBS-T) and the endogenous peroxidase was blocked with 3% hydrogen peroxide (H2O2) for 10 minutes. Subsequently, the slides were washed 2x in PBS-T for 5 minutes, treated with UV-block solution from *UltraVision Large Volume Detection System Anti-Polyvalent, HRP* (THERMO SCIENTIFIC, Fremont, USA) for 10 minutes to block nonspecific protein binding and incubated overnight at 4ºC with DO-7 mouse anti-human p53 monoclonal antibody diluted 1:200 (DAKO, Glostrup, Denmark). Slides were then rinsed in PBS-T, incubated with *Biotinylated Goat Anti-Polyvalent Antibody* (THERMO SCIENTIFIC, Fremont, USA) in a humid chamber at room temperature for 10 minutes, washed 2x with PBS-T for 5 minutes and incubated with *Streptavidin Peroxidase* (THERMO SCIENTIFIC, Fremont, USA) for 10 minutes at room temperature. Detection of hybrids was achieved by an enzymatic reaction using 3,3'-diaminobenzidine (DAB) *ImmPACTTM DAB* (VECTOR, Burlingame, CA USA) diluted at 3:100 and incubated during 4 minutes at room temperature. The final wash was performed with distilled water for 5 minutes. *Mayer's hemalum solution* (Millipore, Darmstadt, Germany) was used as counterstain. After coloration, slides were washed in running water for 5 minutes and the following step was sequential dehydration in 70% v/v ethanol for 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).

Nuclear p53 accumulation was defined as negative (<5% cell) or positive (>5% cells). Tumors with positive p53 staining were semi-quantitatively categorized into four categories: 5-25%, 25-50%, 50-75% and >75% of nuclei staining positive.

TP53 mRNA expression

RNA was extracted from 10µm sections using the *Absolutely RNA FFPE Kit* (Agilent Technologies, San Diego CA, USA) and quantified using the *NanoDrop 1000 Spectrophotometer v3.7* (Thermo Scientific, Wilmington DE, USA).

TP53 and GAPDH were analyzed by two-step real-time PCR using hs01034249_m1 and hs02758991_g1 *TaqMan Gene Expression Assays* (Applied Biosystems, Foster CA, USA), respectively. Reverse transcriptase reactions, with 20 µL final volume, were performed

153 using *High-Capacity cDNA Reverse Transcription Kit* (PN 4368814; Applied Biosystems,
154 Foster CA, USA) according to the manufacturer's instructions. The amplification conditions
155 were as follows: annealing at 25ºC for 10 min, extension at 37ºC for 120 min and RT
156 inactivation at 85ºC for 5 min. All reverse transcriptase reactions included no-template
157 controls.

158 qPCRs were performed in duplicates in independent reactions with a 10µl final volume
159 mixture containing 1X of *TaqMan® Universal PCR Master Mix* (Applied Biosystems,
160 Foster City, California USA), 1X RNA Assay (Applied Biosystems, Foster City, California
161 USA), and 10-100 ng of cDNA (RT product). Amplification was run in *Applied Biosystems*
162 *Step-One Real Time PCR System* (Applied Biosystems, Foster CA, USA) with the
163 following thermal cycling conditions: 10 min at 95°C followed by 45 cycles of 15 sec at
164 95°C and 1 min at 60°C.

165 The relative quantification of p53 expression was analyzed using the $2^{-\Delta\Delta Ct}$ method, also
166 known as Livak method. In this method, Ct from the target RNA (p53) in both test and
167 control cases were adjusted in relation to the Ct of a normalizer RNA (GAPDH) resulting
168 in ΔCt. For the comparison between EBVaGC and EBVnGC we have calculated ΔΔCt
169 value, which allows us to determine the differences in p53 expression.

170

171 **Statistical Analysis**

172 Results were analyzed using the computer software *IBM SPSS Statistics for Windows,*
173 *Version 22.0* (IBM Corp, Armonk NY, USA). Data from all cases were compared by
174 Student's t-test and ANOVA considering a statistical significance of 5% (p<0.05).

175

176

RESULTS

p53 accumulation

The results from IHC analysis are shown in **Figure 1** where is describe, for each group, the percentage of cells with p53 accumulation. **Figure 2** illustrates examples of p53 accumulation in the different groups.

All cases included in this study, except one GC case, were positive for the presence of p53 accumulation by immunohistochemistry. NPC cases are a homogeneous group, with all samples having more than half of cells with p53 accumulation: 3 cases (30%) showed 50-75% of cells with p53 accumulation and 7 (70%) presented more than 75% of cells with p53 accumulation. In EBVnGC cases, p53 was frequently found with 16.7%, 33.3%, 26.7% and 23.3% of cases found with 5-25%, 25-50%, 50-75%, and >75% of accumulation, respectively. Similarly to NPC, EBVaGC showed a strong p53 accumulation, with 58.3% of cases having more than 75% of cells with p53 accumulation, 16.7% with 50-75% and only 25% having less than 50% of cell with p53 accumulation.

Results showed that p53 accumulation in NPC and EBVaGC is not significantly different (p=0.501) while there is a statistically significant difference between EBVaGC and EBVnGC (p=0.027). Regardless of EBV status, the analysis of all gastric cancer cases revealed that there is no statistical differences between the histological subtypes in the p53 accumulation in tissue (p=0.856) (data not shown). Similarly, the comparison of all gastric cancer cases according to tumor localization and invasion pattern indicated no statistical differences in the expression of p53 (p=0.723 and p=0.171, respectively) (data not shown).

TP53 mRNA expression

The results from qPCR analysis are shown in **Table 3**. *TP53* mRNA and GAPDH mRNA (reference gene) were evaluated for all cases and 6 (1 NPC and 5 EBVnGC) were excluded of the analysis because *TP53* mRNA expression was not detected.

The analysis of NPC cases revealed the presence of *TP53* mRNA – **Figure 3**. When analysing the expression of *TP53* in EBVaGC, we observed a significant decrease ($2^{-\Delta\Delta Ct}$=0.21; p=0.010) in *TP53* mRNA expression in comparison with EBVnGC – **Figure 3**.

206 Further analysis subdividing EBVnGC according to histological subtypes revealed that
207 EBVaGC *TP53* mRNA expression was significantly decreased when compared with
208 EBVnGC poorly cohesive and EBVnGC tubular histological subtypes ($2^{-\Delta\Delta Ct}$=0.11;
209 p<0.001 and $2^{-\Delta\Delta Ct}$=0.20; p=0.008, respectively); despite not statistically significant, the
210 reduction of expression also occurs when comparing with EBVnGC mixed types ($2^{-\Delta\Delta Ct}$=0.43; p=0.162) - **Figure 3**.
211

212 Moreover, the comparison of *TP53* mRNA expression between GC histological subtypes,
213 regardless of EBV status, revealed a difference with statistical significance between mixed
214 adenocarcinomas and poorly cohesive carcinomas ($2^{-\Delta\Delta Ct}$=0.27; p=0.014). In addition, the
215 analysis of GC cases according to tumor localization and invasion pattern indicated no
216 statistical significant differences in *TP53* mRNA expression.

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222 **DISCUSSION**

223 Despite the great number of genes involved in human carcinogenesis, *TP53* gene has been
224 considered as one of the most important genes, being crucial in the regulation of signalling
225 processes of tumor development [25]. The p53 protein is activated in response to
226 endogenous or exogenous stresses, inducing cell cycle arrest, DNA repair, apoptosis and
227 senescence of cells [26]. Therefore, the modulation of *TP53* is considered a key hallmark
228 for cancer development and there are several mechanisms that contribute for its
229 deregulation [25]. *TP53* gene mutations are one of the most frequent alterations in human
230 cancers, occurring in almost every type of cancer at rate of 10% to 100% [8, 26].
231 Furthermore, viruses have been considered as able to modulate p53 pathway, either by
232 direct inactivation of the protein or by gene-gene interaction [10].

233 EBV has been described in the pathogenesis of lymphoproliferative disorders as well as
234 epithelial malignancies such as NPC and GC [27, 28]. One of the potential targets of EBV
235 in carcinogenesis processes is the p53 pathway and previous studies have shown that the
236 frequency of *TP53* gene mutations in EBV-associated malignancies is low [14, 15]. In fact,
237 some studies have shown that in NPC and EBVaGC, *TP53* is rarely mutated suggesting that
238 other mechanisms different from mutations could be responsible for p53 deregulation [14,
239 16-19]. Understanding the mechanisms of EBV-associated p53 deregulation in these
240 cancers would allow a better knowledge of the carcinogenesis model in epithelial tumors.

241 Concerning NPC, we have found p53 accumulation in 100% of cases and all showed more
242 than 50% of cells with p53 accumulation. Previous studies also showed a high p53
243 accumulation, ranging between 64.7% and 95.9% [22, 29-35]. The relationship between
244 accumulation of p53 in NPC and EBV infection is still controversial [22, 30-33]. Although
245 the mechanism by which EBV induced p53 expression has not been elucidated, some
246 authors suggested that LMP1, a highly expressed EBV-latent protein in NPC, seem to be
247 responsible for p53 accumulation in NPC without TP53 gene mutation [36]. Indeed, LMP1
248 seems to induce wild-type p53 stabilization through the promotion of K63-linked
249 ubiquitination, which is a cellular pathway regulator and thus enhancing the half-life of the
250 protein [37, 38]. Furthermore, LMP1 also blocks MDM2-mediated p53 degradation

251 through the suppression of K48-linked ubiquitination [37]. Together this dual function of
252 LMP1 explains the wild-type p53 accumulation in NPC tissue [37].

253 Regarding p53 accumulation in GC, our study revealed an overall high rate of p53
254 accumulation (100% in EBVaGC and 96.7% in EBVnGC). Similar results were found by
255 other authors: *Wang et al.* reported a high p53 accumulation in both EBVaGC (84.6%) and
256 EBVnGC (86.7%) [39]; and *Kim et al.* showed more p53 accumulation in EBVaGC (100%)
257 compared to EBVnGC (85.0%) and with a predominance of > 50% of cells with p53
258 accumulation in both EBVaGC (83.3%) and EBVnGC (75.0%) [40]. Other studies showed
259 contradictory results reporting less p53 accumulation in EBVaGC compared to EBVnGC
260 [41-43] and one meta-analysis study demonstrated a lower rate of p53 accumulation,
261 although not statistical significant, in EBVaGC (36.2%) when compared with in EBVnGC
262 (47.9%) [44]. Our results seem to suggest that accumulation of p53 in gastric cancer is
263 independent of EBV infection [45-47].

264 In our study, the detection of p53 accumulation was performed by IHC for p53 using the
265 DO-7 antibody, that recognizes both wild-type and mutant forms of p53 [48]. This is a
266 cheap and rapid method widely used in routine procedures, being the commonest method to
267 infer *TP53* mutational status in ovarian carcinoma [49] since it is more likely to detect
268 mutated p53 due to its longer half-life usually p53 considering that accumulation is related
269 to *TP53* gene mutation [50]. However, in the majority of studies that have used IHC as a
270 surrogate marker for TP53 mutation have failed to demonstrate consistent results [8, 51] by
271 showing a large number of misclassified cases (false-positive and false-negative) [8]. In our
272 study p53 staining was nuclear in tumor cells and was absent in normal cells and
273 considering that in EBVaGC and NPC *TP53* mutations are rare[14, 16, 18-20, 52, 53] it is
274 difficult to reconcile with the observation of p53 accumulation

275 In order to better understand if the accumulation of p53 was a consequence of increased
276 *TP53* transcription, we have also investigated the *TP53* mRNA by qRT-PCR. As far as we
277 know, this is the first study using qRT-PCR methodology to evaluate the levels of *TP53*
278 gene expression in EBVaGC.

279 Among GC, we found a significant decrease in *TP53* mRNA expression in EBVaGC,
280 which had less 80% of *TP53* mRNA expression when compared with EBVnGC. A further

281 subdivision of EBVnGC in different histological subgroups showed that EBVaGC had a
282 significant downregulation of *TP53* mRNA expression independent of histological
283 subtypes. This result could be explained by the potential of some EBV latent proteins to
284 destabilize p53: EBNA1 has been described to compete with p53 for the same binding site
285 of USP7, a cellular ubiquitin-specific protease that has been reported to bind and regulate
286 p53, and its higher affinity interferes with the stabilization of p53 contributing for lower
287 p53 levels [54-56]; EBNA3C reported as capable to directly bind p53 and repress its
288 apoptotic and transcriptional activities [57]; and EBNA5, which is capable of binding to
289 p14ARF and Mdm2, two proteins involved in p53 regulation, resulting in the
290 downregulation of p53 levels by the formation of trimolecular complexes between EBNA-
291 5, Mdm2 and p53 [58, 59]. Therefore, EBNA1, EBNA3C, EBNA5 or the combined effect
292 of these three EBV proteins could be the cause of decrease in *TP53* mRNA expression that
293 we found in EBVaGC. Although there are no previous studies, taking into account that
294 deregulations induced by these EBV latent proteins do not always lead to p53 degradation,
295 they could also be responsible for p53 accumulation in cells.

296 Our results are mostly important to corroborate the idea that with the current knowledge on
297 molecular features of GC, histological classification has limited value to distinguish these
298 tumors. In fact, due to the heterogeneity of GC, a new classification based on molecular
299 features of tumors have been proposed, with EBVaGC categorized as a "new" and distinct
300 subtype of gastric cancer [14, 19]. In addition, *Cristescu et al.* proposed a new classification
301 wherein p53 status has a pivotal role: TP53+ (without *TP53* gene mutation) and TP53-
302 (with TP53 gene mutations). Interestingly the group of TP53+ was closely linked to EBV
303 infection and had a better overall survival [60]. These data is in accordance with previous
304 reports of no TP53 mutations in EBV positive gastric carcinomas [14, 19]. Our study gives
305 new insight on modulation of p53 by EBV but further studies are needed in order to
306 understand the mechanisms by which EBV modulates p53 expression and accumulation in
307 cells.

308

309

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314

315 **AUTHORSHIP CONTRIBUTIONS**

316 MM and JR participated in the sample selection, IHC, RT-PCR and manuscript writing;
317 CO, participated in IHC; AG, LPA participated in the sample selection and data collection;
318 MM, JR and HS contributed in the data analysis and manuscript writing; RM and HS were
319 responsible for the scientific supervision of the study; All authors were able to provide
320 critical review and revision of the manuscript.

321

322 **FINANTIAL DISCLOSURE**

323 All authors declare no competing financial interests.

324

325

FIGURES

Figure 1. Percentage of cells with p53 accumulation in nasopharyngeal and gastric carcinomas.

NPC, Nasopharyngeal carcinoma; EBVaGC, Epstein-Barr Virus-associated Gastric Carcinomas; EBVnGC, Epstein-Barr Virus-negative Gastric Carcinomas.

Figure 2. Examples of immunohistochemistry staining on nasopharyngeal and gastric carcinomas.
EBER-ISH (40x): A) NPC; B) EBVaGC; C)EBVnGC.
p53 (40x): D) NPC; E) EBVaGC; F) EBVnGC.

NPC, Nasopharyngeal carcinoma; EBVaGC, Epstein-Barr Virus-associated Gastric Carcinomas; EBVnGC, Epstein-Barr Virus-negative Gastric Carcinomas.

Figure 3. Expression profile of TP53 mRNA in nasopharyngeal and gastric carcinomas.

NPC, Nasopharyngeal carcinoma; EBVaGC, Epstein-Barr Virus-associated Gastric Carcinomas; EBVnGC, Epstein-Barr Virus-negative Gastric Carcinomas. Bold values are statistically significant different.

TABLES

Table 1. Characterization of nasopharyngeal carcinoma cases.

Table 2. Characterization of gastric carcinoma cases.

Table 3. qPCR data analysis and expression profile data for TP53 mRNA in nasopharyngeal and gastric cancers.

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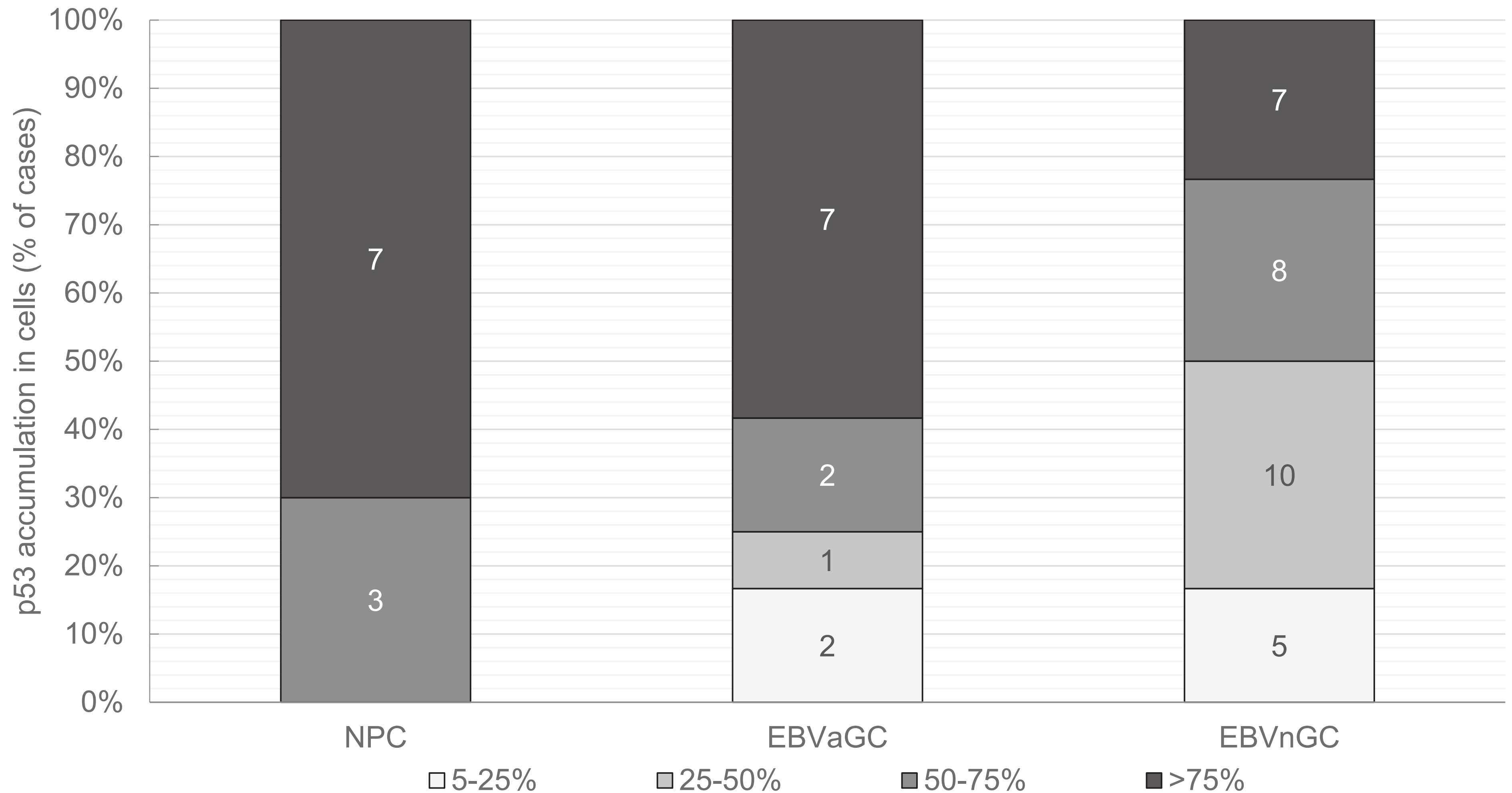

Figure 1. Percentage of cells with p53 accumulation in nasopharyngeal and gastric carcinomas.

NPC, Nasopharyngeal carcinoma; EBVaGC, Epstein-Barr Virus-associated Gastric Carcinomas; EBVnGC, Epstein-Barr Virus-negative Gastric Carcinomas.

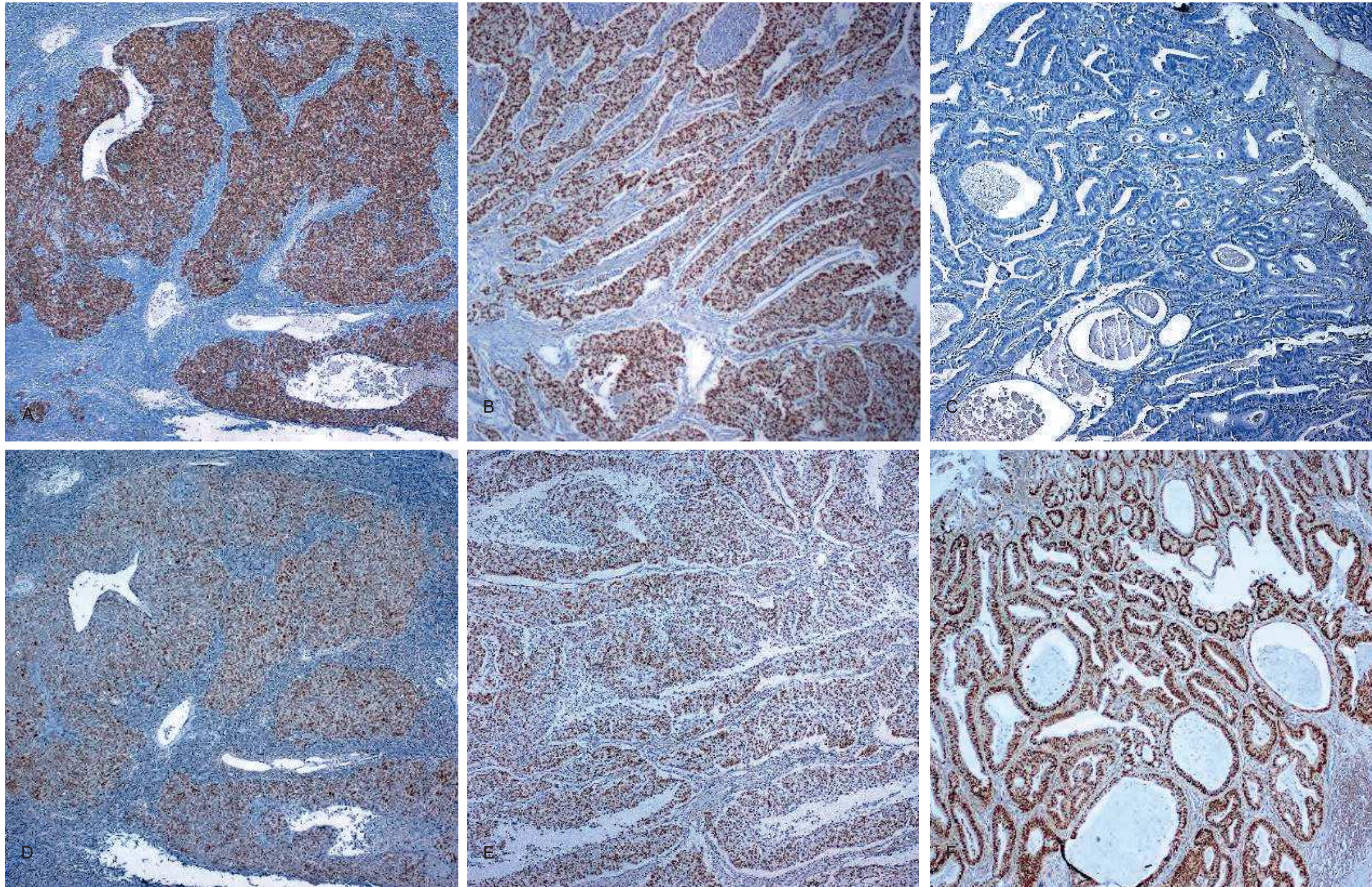

Figure 2. Examples of immunohistochemistry staining on nasopharyngeal and gastric carcinomas.
EBER-ISH (40x): A) NPC; B) EBVaGC; C)EBVnGC.
p53 (40x): D) NPC; E) EBVaGC; F) EBVnGC.

NPC, Nasopharyngeal carcinoma; EBVaGC, Epstein-Barr Virus-associated Gastric Carcinomas; EBVnGC, Epstein-Barr Virus-negative Gastric Carcinomas.

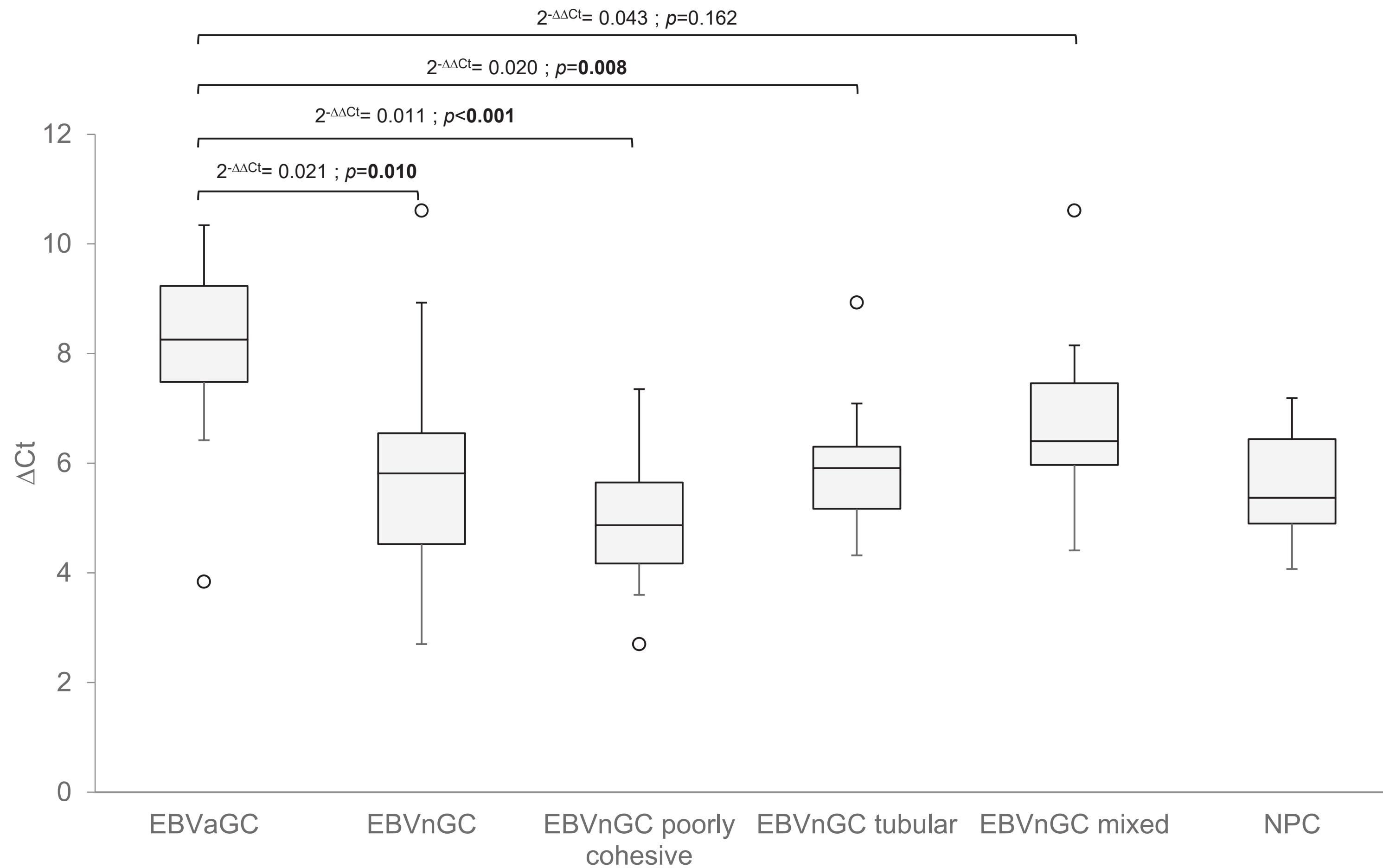

Figure 3. Expression profile of TP53 mRNA in nasopharyngeal and gastric carcinomas.

NPC, Nasopharyngeal carcinoma; EBVaGC, Epstein-Barr Virus-associated Gastric Carcinomas; EBVnGC, Epstein-Barr Virus-negative Gastric Carcinomas. Bold values are statistically significant different.

Table 1. Characterization of nasopharyngeal carcinoma cases.

	NPC
Gender	*n (%)*
Male	7 (70.0%)
Female	3 (30.0%)
Age	*years*
Mean ± sd	51±16.1
Median (min-max)	51.0 (20 – 74)
Global Stage	*n (%)*
II	1 (10.0%)
III	2 (20.0%)
IVa	3 (30.0%)
IVb	1 (20.0%)
IVc	2 (20.0%)
Missing	1 (10.0%)

Table 2. Characterization of gastric carcinoma cases.

	EBVaGC	*EBVnGC*
Gender	*n (%)*	*n (%)*
Male	9 (75.0%)	18 (58.1%)
Female	3 (25.0%)	13(41.9%)
Age	*years*	*years*
Mean ± sd	69±9.62	63±9.86
Median (min-max)	68.50 (52 – 82)	64.0 (40 – 81)
Histology WHO	*n (%)*	*n (%)*
Mixed adenocarcinoma	2 (16.7%)	10 (32.3%)
Tubular adenocarcinoma	6 (50.0%)	10 (32.3%)
Poorly cohesive carcinoma	1 (8.3%)	11 (35.5%)
Carcinoma with lymphoid stroma	2 (16.7%)	-
Adenosquamous carcinoma	1 (8.3%)	-
Tumor Localization	*n (%)*	*n (%)*
Antrum	3 (25.0%)	20 (64.5%)
Cardia	2 (16.7%)	3 (9.7%)
Body	4 (33.3%)	8 (25.8%)
Pylorus	1 (8.3%)	-
Missing	2 (16.7%)	-
Invasion Pattern	*n (%)*	*n (%)*
Expansive	6 (50.0%)	8 (25.8%)
Infiltrative	3 (25.0%)	22 (71.0%)
Missing	3 (25.0%)	1 (3.2%)
Global Stage	*n (%)*	*n (%)*
Ia	1 (8.3%)	7 (22.6%)
Ib	2 (16.7%)	1 (3.2)
IIa	2 (16.7%)	3 (9.7%)
IIb	1 (8.3%)	6 (19.7%)
IIIa	5 (41.7%)	4 (12.9%)
IIIb	1 (8.3%)	5 (16.1%)
IIIc	-	3 (9.7%)
IV	-	2 (6.5%)

Table 3. *qPCR* data analysis and expression profile data for TP53 mRNA in nasopharyngeal and gastric cancers.

	Ct GAPDH	*VC*	*Ct TP53*	*VC*	*ΔCt ± sd (range)*
EBVaGC (n=12)	26.31 ± 1.19 (24.27 – 27.97)	0.05	34.42 ± 1.59 (31.78 – 36.68)	0.05	5.84 ± 1.73 (2.71 – 10.61)
EBVnGC (n=26)	28.06 ± 2.51 (23.41 – 34.57)	0.09	33.90 ± 1.69 (30.49 – 37.27)	0.05	8.10 ± 1.83 (3.84 – 10.34)
EBVnGC poorly cohesive (n=9)	29.71 ± 1.69 (27.29 – 32.60)	0.06	34.68 ± 1.24 (32.53 – 36.25)	0.04	4.97 ± 1.20 (3.60 – 7.35)
EBVnGC tubular (n=9)	27.07 ± 3.16 (23.41 – 34.57)	0.12	32.86 ± 1.95 (30.49 – 37.27)	0.06	5.80 ± 1.72 (2.71 – 8.93)
EBVnGC mixed (n=8)	27.31 ± 1.58 (25.29 – 30.10)	0.06	34.19 ± 1.35 (32.36 – 35.90)	0.04	6.88 ± 1.86 (4.41 – 10.61)
NPC (n=9)	27.83 ± 1.55 (25.14 – 29.76)	0.05	33.39 ± 1.74 (30.51 – 36.08)	0.05	5.56 ± 1.06 (4.07 – 7.19)

APPENDIX III

Article II

Cellular and Molecular Life Sciences

EPSTEIN-BARR VIRUS GENE EXPRESSION AND LATENCY PATTERN IN GASTRIC CARCINOMAS: A SYSTEMATIC REVIEW

--Manuscript Draft--

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Abstract:	Our study aimed to summarize, with a systematic review of literature, Epstein-Barr Virus (EBV) gene expression patterns in gastric carcinomas (GC). A systematic search of literature regarding the expression of EBV proteins and EBV latency pattern in gastric carcinomas was performed. The search retrieved 247 papers, of which 25 papers matched the inclusion criteria. Data regarding background characteristics of population, histologic information, type of sample, viral genes expression and methodologies were extracted from included papers. The analysis reveals that not all studies evaluate the necessary proteins for the characterization of latency profiles in GC. The most frequently expressed EBV latent proteins are EBNA1 (98.1%) and LMP2A (53.8%), while LMP1 and LMP2B are only present in 10% of cases. The combination of protein expression showed that the most frequent pattern found in GC (44.4%) does not fit to the "standard" viral latency patterns. Moreover, lytic proteins, such as BARF0 and BARF1 (100% and 63.9%, respectively), and other lytic transcripts are present in almost half of cases. Our review showed that EBV-associated GC (EBVaGC) seems to display a unique transcription pattern. More studies combining information regarding latent and lytic proteins may provide significant information to better understand EBVaGC carcinogenesis.
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TITLE

EPSTEIN-BARR VIRUS GENE EXPRESSION AND LATENCY PATTERN IN GASTRIC CARCINOMAS: A SYSTEMATIC REVIEW

RUNNING TITLE

SYSTEMATIC REVIEW OF EBV GENES EXPRESSION IN GASTRIC CANCER

AUTHORS

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ABSTRACT (max 150-250words)

Our study aimed to summarize, with a systematic review of literature, Epstein-Barr Virus (EBV) gene expression patterns in gastric carcinomas (GC). A systematic search of literature regarding the expression of EBV proteins and EBV latency pattern in gastric carcinomas was performed. The search retrieved 247 papers, of which 25 papers matched the inclusion criteria. Data regarding background characteristics of population, histologic information, type of sample, viral genes expression and methodologies were extracted from included papers. The analysis reveals that not all studies evaluate the necessary proteins for the characterization of latency profiles in GC. The most frequently expressed EBV latent proteins are EBNA1 (98.1%) and LMP2A (53.8%), while LMP1 and LMP2B are only present in 10% of cases. The combination of protein expression showed that the most frequent pattern found in GC (44.4%) does not fit to the "standard" viral latency patterns. Moreover, lytic proteins, such as BARF0 and BARF1 (100% and 63.9%, respectively), and other lytic transcripts are present in almost half of cases. Our review showed that EBV-associated GC (EBVaGC) seems to display a unique transcription pattern. More studies combining information regarding latent and lytic proteins may provide significant information to better understand EBVaGC carcinogenesis.

Keywords: EBVaGC, EBV Latency, Lytic genes, Latent Genes

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INTRODUCTION

Epstein-Barr Virus (EBV) is a human cancer-associated virus that infects about 90% of the global population, and despite its wide distribution, does not cause major symptoms in the majority of lifelong carriers [1-3]. Nevertheless, EBV has a great carcinogenic potential and has been described as the aetiological agent of several malignancies, especially B-cell neoplasias, but has also been associated with epithelial tumours such as Nasopharyngeal Carcinoma (NPC) and more recently to Gastric Carcinoma [4,1].

Gastric cancer (GC) is the sixth most common malignancy and the second leading cause of cancer death worldwide [5,6]. GC has a distinct geographic distribution suggesting that genetic and environmental factors play important roles on its development [7,6]. Gastric carcinogenesis is a multistep process, where different factors are involved and where EBV seems to be involved in the mechanism of some cases [5,8,9]. Literature evidences have shown that almost 10% of GC cases are associated to EBV infection, which lead the scientific community to debate the role of EBV infection on GC [10-12]. Indeed, EBV infection has been detected in both gastric adenocarcinomas and lymphoepithelioma-like carcinomas (LELC) [13,9]. The evidence for involvement of EBV in gastric carcinoma is based on the presence of viral gene products such as EBV-encoded small RNAs (EBER) in tumour cells but not in the surrounding non-neoplastic epithelium [14], the presence of clonal EBV in tumour cells [15] and elevated EBV antibodies in prediagnostic sera of patients with EBV-associated gastric carcinoma [16]. Moreover, recent studies have suggested that the EBV-associated gastric cancer (EBVaGC) is a distinct subgroup of gastric cancers with specific molecular features [17,9,18].

The development of EBV-associated malignancy is dependent on the expression of viral proteins that modulate cell proliferation, immune response and apoptosis [19,20]. Indeed, EBV has several latent proteins involved in this processes that are expressed in different conditions in EBV-associated malignancies [21,22]. There are different patterns of viral latent gene expression: Latency I, present in Burkitt's lymphoma; Latency II present in Nasopharyngeal carcinoma, Hodgkin's disease and natural killer (NK)/T-cell lymphomas; and Latency III which is found in Post-transplant lymphoma and AIDS-associated non-Hodgkin's lymphoma [2].

Regarding EBVaGC, some studies suggested that it is usually associated with latency I pattern, although, controversial reports suggest different latency patterns of viral gene expression [23-25]. The clarification of which both lytic and latent EBV genes are expressed in GC assumes a great importance for the development of future studies regarding EBV-associated carcinogenesis in gastric cells. The aim of this study was to summarize EBV

gene expression patterns in gastric carcinomas by performing a systematic review of literature.

MATERIAL AND METHODS

Articles search

In this systematic review we revised all published studies regarding the expression of EBV proteins in gastric carcinomas. A systematic PubMed/Medline and EMBASE search was performed to identify all published reports from January 1980 to December 2015 using the following query: ebv[All Fields] OR ("herpesvirus 4, human"[MeSH Terms] OR “human herpesvirus 4"[All Fields] OR ("epstein"[All Fields] AND "barr"[All Fields] AND "virus"[All Fields]) OR "epstein barr virus"[All Fields]) AND ("stomach"[MeSH Terms] OR "stomach"[All Fields] OR "gastric"[All Fields]) AND ("latent"[All Fields] OR "Lytic"[All Fields] OR ("micrornas"[MeSH Terms] OR "micrornas"[All Fields] OR "mirna"[All Fields]) OR (ebv[All Fields] AND latency[All Fields])). Furthermore, a hand search of abstract books from scientific meetings and the reference list of review manuscripts was also performed.

Inclusion and exclusion criteria

All studies performed to evaluate the expression of EBV proteins and EBV latency patterns in gastric carcinomas were evaluated. Articles were excluded if they met one or more of the following criteria: 1) reviews, meta-analysis, or systematic reviews; 2) not related with gastric cancer; 3) *in vitro* studies; and 4) studies with methods and results not available in English, Portuguese, Spanish or French - Figure 1.

Studies selection and data extraction

The study selection was performed in two steps (screening and data extraction) independently by two authors and disagreements were solved consulting a third author. Firstly, the title and abstract were screened following an evaluation of relevant full-text studies based on the inclusion and exclusion criteria. A request via email was sent by authors when full-texts were not available or when results were written in other language than English, Portuguese, Spanish or French. All included manuscripts were revised by two authors, in order to extract the necessary data to perform the analysis: first author, journal name, year of publication, data from characterization of population (ethnicity, median age, country), source of sample (formalin-fixed and paraffin-embedded tissues; frozen tissues), number of cases, histological types of GC (when reported), expression of EBV proteins (each) and methodology used for the detection of protein expression (Immunochemistry, real-time polymerase chain reaction, *in situ* hybridization).

RESULTS

A total of 247 articles were evaluated from which only 25 were included in the final analysis. The motifs for exclusion were: 56 reviews articles, 58 *in vitro* studies, 54 did not evaluate the expression of EBV proteins, 41 were not related to gastric cancer, 5 were not available in either full-text or at least the results section, 4 were written in Chinese, 3 had replicated data from previous papers and 1 was found to be a case report – Figure 1.

Table I describes the principal baseline characteristics of included studies, namely the population of study, type of samples, number of cases, proteins analysed and EBV latency pattern, when described by authors [26,15,27-31,23,32-39,24,40-42,25,43-47]. The majority of studies were performed in Asiatic populations (Japan, China, Hong Kong, Korea and India); three studies were from European populations (United Kingdom, France and Netherlands); and one study from USA and Central America. Regarding the histological classification of gastric carcinomas, only 16 studies provided this information. The detection of EBV was performed by *in situ* hybridization for Epstein-Barr virus-encoded RNA (EBER-ISH) in the majority of studies, while only two studies evaluate EBV presence by Polymerase Chain Reaction (PCR) and one used a RNA compass technology. Studies analysed different viral proteins and used different approaches for the detection of the different proteins (Immunochemistry, reverse transcriptase PCR, within others).

Expression of EBV latent infection genes

The expression of EBV latent proteins in the cases included in studies are described in Table II. As expected, the majority of included studies (n=23) have analysed at least one of EBV latent proteins: EBNA1 was reported in 16 studies and detected in 98.1% of all cases (254/259), revealing strong homogeneity amongst results; EBNA2 was analysed in 15 studies and despite it was detected in 3.1% of cases (6/193), the positivity was observed only in one study with 42.9% of cases being positive (6/14); LMP1 was analysed in 18 studies with 10.6% of cases positive (21/199), nevertheless, results showed that only four studies have positive cases, and in those there was a wide range of positivity (between 19-100%); LMP2A was analysed by 12 studies and was positive in 53.8% cases (113/210) revealing a great heterogeneity; and LMP2B was only analysed by 3 studies with an overall positivity of 13.6% (3/22) explained only by one study with positive results.

Expression of EBV lytic infection genes

The literature shows that in addition to the several EBV latency proteins, there are several authors which have identified different transcripts from EBV lytic genes: EBV immediate-early genes BZLF1 and BRLF1; early genes BARF1 and BHRF1; late genes BcLF1 and BLLF1; and some also analyse BALF5 and BXLF1. The description of results regarding the detection of EBV lytic gene products is described in Table III.

The revised data showed that the expression of EBV lytic proteins is very more heterogeneous than latent proteins: BZLF1 was studied in 14 studies and was expressed in 34.2% (63/184) of cases, however there is a great heterogeneity among results varying between 0%-92.9%; two studies investigated the presence of BARF0 and both detected 100% positivity (14/14); BARF1 transcripts were analysed in 7 studies and were positive in 71.0% (76/107) of cases; six studies analysed BHRF1 transcripts and only 8.02% (9/56) (8.02%) were positive; BRLF1 was detected by 4 studies in a total of 56.2% (18/32), but with despair results among studies; 61.1% (33/54) exhibited BcLF1 as reported by two studies; 45 of 76 cases (59.2%), described in 6 studies, expressed BLLF1; 12 of 25 (48.0%) cases were positive for BMRF1; and 29 of 48 (60.4%) cases exhibited BARTs transcripts. BALF5, BXLF1 and BCRF1 were also detected by one study, which found BALF5 and BXLF1 transcripts in 14 of 14 cases and BCRF1 in 11 of 14).

Shinozaki-Ushiku and colleagues in addiction to detect the overall expression of BARTs, they have used TaqMan MiRNA assays to evaluate a relative quantification of each ebv-mir, including microRNAs from BHRF1 region [46].

EBV Latency Profiles

As previously described, latency patterns are characterized by the expression of specific EBV latent proteins [2]: Latency I, characterized by expression of EBERs1/2, EBNA1 and BARTs; Latency II is defined by EBERs1/2, EBNA1, LMP1 and LMP2 expression; and Latency III is characterized by EBERs1/2, BARTs, all six EBNA proteins and LMP1 and LMP2 proteins.

Overall, of the 25 included studies, 17 did not provide enough information for the determination of EBV latency pattern. Nevertheless, 6 studies evaluated the latent proteins expression despite did not describe the latency pattern, and only 2 were able to describe the EBV latency pattern for the cases. We have revised all data and managed to reclassify the latency profiles of the 8 studies with sufficient information – Table IV.

EBV latency I was found in 7/8 studies and representing almost 42.9% of cases; and latency II and III were only identified in one study. Additionally, studies identify a distinct latency pattern, characterized by the expression of EBERs, EBNA1, LMP2 and the absence of LMP1 and EBN2A, which some authors have named latency II-like [23,2]. This pattern was found in 44.4% of all cases being the most frequent latency pattern in EBVaGC.

DISCUSSION

EBV establishes a latent infection expressing a restrict set of viral proteins that confer to the infected cells a survival advantage and proprieties to escape immune surveillance [48]. Literature suggest that EBV can adopt mainly four distinct latent infections according the type of cells (epithelial or lymphoid): latency 0, I, II, and III [21]. The different latency programs are characterized by specific viral gene expression pattern and are dependent on several cell-specific factors such as epigenetic events, which include DNA methylation, histone modifications and chromatin organization [49,50]. The profile of expression of latent viral proteins is crucial for the transformation of cells being the major responsible for the carcinogenesis [48]. The different EBV latency programs have been correlated with different EBV-associated diseases [48,2]. Studies have shown that in NPC, EBV is characterized by latency II; nevertheless in other EBV-associated epithelial malignancies it may express different latency patterns.

EBV has been suggested to be associated with the development of at least part of gastric cancer (EBVaGC); however the impact of EBV latent and lytic genes on gastric carcinogenesis remains controversial and unclear [11,12]. The selective expression of EBV genes contributes to the malignant transformation of epithelial cells by disrupting various cellular processes and signalling pathways [51-53]. Indeed, the distinct mutation signature and methylation pattern identified in EBVaGC illustrate that EBV infection facilitates a unique and alternate tumorigenic process in epithelial malignancies [54,9]. Hence, this systematic review intends to resume the data published regarding EBV gene expression in gastric tumors and clarify the latency pattern that characterizes best EBVaGC in order to improve the knowledge on the carcinogenesis mechanism.

By searching literature, we have found 25 papers matching the inclusion criteria and exploring the expression of EBV proteins in gastric cancer. Two independent authors performed the screening and data extraction from included papers in order to minimize errors. The first problem found in this systematic review was the difficulty to summarize the baseline characteristics of the studies due to the great heterogeneity. Indeed, the lack of standardization of EBV detection methodologies and the methodological limitations, including smaller sized samples, constitute a major limitation for this review.

The incidence of gastric cancer is substantially higher in Asia fostering the research in this field among Asiatic populations, and as expected, the majority included studies were performed in Asiatic populations [55,15,31,32,34-36,39,24,40,44-47]. Nevertheless, there are other areas of the world with significant impact of GC that should promote the development of more studies to characterize better the impact of EBVaGC worldwide.

The selection of the methodology for EBV detection relies on several factors, such as the type of sample available, and therefore different tests may be used. The detection of EBV has been performed by identifying the presence of the virus in tissues samples, especially in paraffin embedded formalin fixed tissue (FFPE) sections. The in situ hybridization with EBV EBERs (EBER-ISH) has been considered the gold standard method [56] and it was chosen by the majority of studies included in this systematic review. Nevertheless, other methods with different specificities and sensitivities were also used (western-blot, PCR, RT-qPCR for EBNA1 and RNA CoMPASS) [15,33,35,36,42,43]. The methodologies used to EBV detection among gastric tissues are an important factor, which must be taken into account during the comparative analysis of EBV prevalence between different populations or studies.

Other important limitation found was the impossibility to compare the expression profile among different histological types, which has assuming great importance. The association between EBV and GC was firstly reported among gastric medullary carcinomas with lymphoid infiltration (lymphoepitheliomas/LELC), described by World Health Organization (WHO) as an uncommon subtype and is not represented in the classification system most widely used in GC (Lauren 's classification) [57]. Several authors have identified morphological features similar to the undifferentiated nasopharyngeal carcinoma (UNPC), and some refer it as gastric medullary carcinoma with lymphoid infiltration (GMCL) [58,26,27,39]. This tumour, which has been characterized by uniform proliferation of cancer cells throughout the lymphoid stroma, represents about 4% of all gastric carcinomas and more than 80% of all cases are associated with EBV [13]. Nevertheless, EBV has been detected in other histological subtypes of GC with distinct associations dependent on the histological type [23,9]. In this systematic review we observed that only 16 studies provide information regarding the histologic types, with a strong heterogeneity regarding histological classification applied in each study. The different classifications and definitions contribute to the great heterogeneity among study populations making the analysis of association between EBV proteins expression and gastric cancer histological types extremely difficult. According to the literature, there are no evidences that EBV latency pattern may vary among different histological types. The summary of this information is important to overcome differences, specially comparing LELCs and others histological types. This limitation reflects the lack of information regarding gastric cancer histology in studies.

EBV protein expression in Gastric Cancers

EBV protein expression and latency patterns can be directly influenced by the method selected for the analysis since it may lead to different interpretations concerning if they are

analysing proteins and/or transcripts. The studies included in this systematic review detected EBV proteins including different methods including, immunochemistry (IHC), western-blot (WB), reverse transcriptase PCR (RT-PCR), RNA CoMPASS and arrays – Table I. While some methods directly identify the presence of the protein in the tissue (IHC, WB or IF), others detect the presence of transcripts from the genes (RT-PCR, RNA CoMPASS and arrays). This may lead to a misconception, since the presence of a transcript in a tissue does not imply the protein expression, because post-transcriptional modifications and RNA degradation or inhibition may occur. Nevertheless, it is not possible to point which of the strategies is correct. Actually, they have both advantages and disadvantages: whereas RT-qPCR and RNA CoMPASS are considered the methods with highest sensitivity detecting low levels of RNA; IHC allows to detect proteins and to analyse their location in the tissues [43]. Considering that there are differences on the expression of EBV proteins/transcripts, the correct interpretation of data from studies considering the methods may help to explain different results between studies.

As expected in all forms of EBV latency, EBNA1 is expressed in almost all cases (98.1%) of EBVaGC. In fact, this protein is a DNA binding nuclear phosphoprotein, which plays a crucial role in the replication and maintenance of the episomal EBV genome [59]. Some have been suggesting that EBNA1 also contributes to the transcription of other viral latency genes contributing to the carcinogenesis [60,61]. In contrast to EBNA1, the expression of EBNA2 was not expected in gastric malignancies because EBNA2 seems to be B cell specific [62]. EBNA2 acts as a transcriptional coactivator factor that coordinates viral gene expression in latency III and also can transactivates cell genes playing a critical role in cell immortalization [1]. In fact, only one study have detected EBNA 2 in some cases of EBVaGC and authors explained that they have used a sensitive array, which found "low albeit detectable levels" [25]. Our review also shows that LMP1 is generally absent in EBVaGC except for the data reported in three studies, which have detected LMP1 expression in some cases of EBVaGC [38,25,43]. Although LMP1 protein was previously reported to be absent in EBVaGC, recent approaches detecting LMP1 mRNA have pointed for its presence in almost 100% of all specimens [25,43]. The explanation for LMP1 detection in some cases could be due to differences between methods sensitivities and the amount of LMP1 in the tissues. Indeed, two of the three studies that detected LMP1 used arrays and RNA CoMPASS methodologies, which detect RNA transcripts and not the protein [38,25]. Moreover, recent have data suggested that LMP1 may itself get silenced by methylation of its own promoter, explaining the lack of LMP1 expression [63].

EBV LMP2A was found in half of EBVaGC cases and this fact has boosted the research on this field, since it has been suggested that LMP2A contributes to gastric carcinogenesis by

inducing the genome hypermethylation through phosphorylation of STAT3 and up-regulation of DNMT1 [64,17].

Our systematic review also analysed the information regarding the expression of EBV lytic genes in EBVaGC. Surprisingly, several lytic proteins/transcripts were found (BZLF1, BcLF1, BLLF1, BHRF1, BRLF1, BMRF1), including glycoproteins and proteins from the replication core. Nevertheless, as for latent genes, the methods used may influence the conclusions. The majority of studies detect mRNA and not the expressed protein (RT-PCR, arrays and RNA CoMPASS) requiring future studies to clarify if the activation of EBV lytic genes is important or not during carcinogenesis. We found curious that using RNA-based methods, BZFL1 transcripts were not detected by seven studies [31,23,35,38,39,24,45] while other seven studies revealed that it is present in up to 93% of the cases [26,32,34,36,42,25,43]. In addition, studies that performed IHC and IFA failed to detect BZLF1. BARF1 was also detected by several studies suggesting that in EBVaGC malignancies, as well as it happens in NPC carcinomas without LMP1 expression, BARF1 may be acting as a viral oncogene [23,34-36,42,25,44]. Actually, literature has revealed that in epithelial tumours BARF1 acts as a latent, rather than lytic gene and have shown to be oncogenic and capable of inducing malignant transformation [65,66]. Overall, EBV lytic transcripts have been detected in gastric cancer tissues suggesting that EBV lytic cycle is activated in a small portion of EBV-infected carcinoma cells [32,25,43]. However, there is a possibility that the presence of EBV lytic proteins/transcripts may be explained by the presence of a small percentage of other cells such as infiltrating lymphocytes in the tumor tissues and not in the malignant cells. The role of EBV lytic gene expression in EBVaGC remains unclear and more functional studies are required to understand their role on epithelial malignancies.

Although it has not possible to make a quantitative analysis of these results, we have summarize the EBV proteins profile in individually cases from different studies, when it was described by authors in the manuscript (Supplementary Table I). The Supplementary Table I allows to compare the expression of different EBV proteins in cases individually.

EBV latency profiles in Gastric Cancers

Some review articles have associated EBVaGCs with latency type I and II [67-69], nevertheless our systematic review reveals another latency pattern that has been found among EBVaGC cases. To clarify the latency pattern of EBVaGC cases, we have described the data regarding the expression of EBV proteins individually (Supplementary Table I) and summarized the data considering the latency profiles suggested by Young *et al* [21] and described the latency profiles in Table IV.

These data showed that Latency I is one the most frequent latency found in EBVaGC. In addition studies also revealed that, in a large number of EBVaGCs, EBV assumes a unique and distinct latency pattern [31,23,32,34,40]. This distinct latency pattern, also designated by latency II-like, is characterized by the expression of EBERs, EBNA 1 and LMP2A [23] differing from latency II since it does not expresses LMP1 [21]. The presence of LMP2A in these tumors could assume a crucial role on gastric carcinogenesis. LMP2A is a viral oncogene, capable to promote the transformation of epithelial cells by the induction and maintenance cell proliferation and decreasing apoptosis [51]. Furthermore, recent studies have shown that LMP2A have a strong influence on aberrant methylation of tumor-related genes in gastric cancer development [64,17].

Considering only latent proteins, the results are consistent attributing EBV latency II-like and I to gastric carcinomas. Only Tang *et al* have identified some EBVaGC cases expressing EBNA2 that allows to characterize these cases as latency III [25]. Authors have referred that the methodology used in their experiments has high sensitivity capable to detect “low albeit detectable levels” of LMP1 and EBNA2 [25]. As mentioned above, the expression of both LMP1 and LMP2A is heterogeneous in terms of prevalence and distribution within the tumors. These results point for different latency patterns among EBVaGC cases and reinforce the needed of more studies to define these speculative roles of LMP proteins in the development of EBV-associated epithelial cancers.

Conclusion

This systematic review demonstrates that EBVaGC are often associated with a distinct latency pattern, characterized by the expression of EBERs, EBNA1 and LMP2A. This profile does not really fit to the standard latency patterns and it has been named by latency II-like. Following latency II-like, latency I is the most frequently EBV latency pattern found among EBVaGCs. The clarification of the different latency patterns is important to allow directed strategies in future treatment options. Furthermore, some studies have shown the expression of EBV lytic genes in EBVaGC and therefore its role is still unclear. These data evidences that further studies are required to mechanisms of viral carcinogenesis in EBVaGC.

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Figure 1

PRISMA 2009 Flow Diagram

Identification

Records identified through database searching (n =247)

Additional records identified through other sources (n =0)

Screening

Records after duplicates removed (n =0)

Records screened (n =247)

Records excluded (n =190)

- Do not evaluate the expression of EBV proteins (n=39)
- *In vitro* studies (n=55)
- Not related with gastric cancer (n=40)
- Review (n=56)

Eligibility

Full-text articles assessed for eligibility (n =57)

Full-text articles excluded (n =32)

- Do not evaluate the expression of EBV proteins (n=15)
- *In vitro* studies (n=3)
- Articles in Chinese (n=4)
- Case report (n=1)
- No access to full-text (n=5)
- Replicated data (n=3)
- MALT lymphoma (n=1)

Included

Studies included in qualitative synthesis (n = 25)

Table I

Table I: Baseline characteristics of included studies.

STUDY	Population	Type of Sample	Histological information	EBV+ (n)	Methodology of EBV detection	Latent proteins analysed (method of analysis)	Others genes analysed (method of analysis)
Shinozaki-Ushiku A. *et al.* 2015 [29]	Japan	FFPE	ND	10	EBER-ISH		BHRF1, BARTs (RT-PCR)
Cheng N. *et al.* 2015 [30]	China	FFPE	LELCs	8	EBER-ISH	EBNA1, EBNA2, LMP1, LMP2A (IHC)	BZLF1 (IHC)
Zhang YW. *et al.* 2015 [31]	China	FFPE	Adenocarcinomas: Diffuse type (n=60) Intestinal type (n=18)	78	EBER-ISH	LMP2A (IHC)	
Zhu S. *et al.*2013 [32]	China	Tissue	ND	13	RT-PCR*	EBNA1, EBNA2, LMP1 (RT-PCR*)	BARF1, BHRF1 (RT-PCR*)
Strong M. *et al.* 2013 [33]33]	ND	Tissue	ND	4	RNA compass	EBNA1, EBNA2, LMP1, LMP2A, LMP2B (RNA compass)	BRLF, BARF0, BZLF1 (RNA compass)
Shukla SK. *et al.* 2012 [34]	India	Frozen tissue	Adenocarcinomas: Diffuse type (n=22), Intestinal type (n=18)	40	EBNA1-RT-PCR	EBNA1 (RT-PCR)	BARF1, BZLF1, BCLF1 (RT-PCR)
Tang W. *et al.* 2012 [25]	USA, Central America and Japan	FFPE	Adenocarcinomas not classified	14	EBER-ISH	EBNA1, EBNA2, EBNA3C, EBNA-LP, LMP1, LMP2A (array)	BARF1, BZLF1, BMRF1, BHRF1, BCRF1, BRLF1, BLLF1, BALF5, BXLF1 (array)
Han J. *et al.* 2012 [35]	China	FFPE	Adenocarcinomas: Diffuse type (n=45), Intestinal type (n=8)	53	EBER-ISH	LMP2A (IHC)	
Lee J M. *et al.* 2011 [36]	Korea	FFPE	Undifferentiated Adenocarcinomas (n=2) LELCs (n=2)	4	EBER-ISH	EBNA1, EBNA2, LMP1, LMP2A (RT-PCR)	BARTs (RT-PCR)
Chen J. *et al.* 2011 [23]	China	FFPE	Adenocarcinomas: Diffuse type (n=8)	8	EBER-ISH	EBNA1, EBNA2, LMP1, LMP2A (IHC)	BZLF1 (IHC)
Chen J. *et al.* 2010 [37]	China	FFPE	Adenocarcinomas: Diffuse type (n=37) Intestinal type (n=18)	45	EBER-ISH	EBNA1, EBNA2, LMP1 (IHC)	BZLF1 (IHC)
Ryan J. *et al.* 2009 [38]	USA and Central America	FFPE	Adenocarcinomas: Diffuse type (n=6), Intestinal type (n=5),	11	EBER-ISH	LMP1, LMP2A (IHC)	BMRF1, BZLF1 (IHC)

			Mixed type (n=1)				
Kim D. *et al.* 2007 [39]	Japan	FFPE	ND	4	EBER-ISH		BARTs, BHRF1 (Northern blot)
Luo B. *et al.* 2005 [40]	China	FFPE	ND	11	EBER-ISH	EBNA1, EBNA2, LMP1, LMP2A, LMP2B (RT-PCR*)	BZLF1, BRLF1, BARF1, BHRF1, BcLF1, BLLF1 (RT-PCR*)
Wang Y. *et al.* 2005 [41]	China	FFPE	Adenocarcinomas (n=12) Signet ring carcinoma (n=1)	13	EBER-ISH	EBNA 1, EBNA2, LMP1 (RT-PCR*)	BARF1, BHRF1, BZLF1 (RT-PCR*)
Seto E. *et al.* 2005 [42]	Japan	Tumor biopsies	ND	6	EBER-ISH	EBNA1 (RT-qPCR)	BARF1, BZLF1 (RT-qPCR)
Lee M. *et al.* 2004 [43]	Korea	FFPE	Adenocarcinomas: Diffuse type (n=1), Intestinal type (n=2) Mixed type (n=1)	4	EBNA1 (PCR)	LMP1 (IHC)	
Hoshikawa Y. *et al.* 2002 [44]	Japan	FFPE	Adenocarcinomas not classified	3	EBER-ISH	EBNA1, EBNA2, LMP1, LMP2A (RT-PCR)	BZLF1, BRLF1, BcLF1, BLLF1 (RT-PCR)
zur Hausen A. *et al.* 2000 [24]	Netherlands	FFPE	Adenocarcinomas not classified	10	EBER-ISH	EBNA1, LMP1 (RT-PCR and IHC) EBNA2, LMP2A (RT-PCR)	BARF1 (NASBA) BARF0, BHFR1 (RT-PCR) BZLF1 (RT-PCR and IHC)
Sugiura M *et al.* 1996 [27]	Japan	Frozen tissue	LELC (n=1) Well differentiated (n=1) Poorly differentiated (n=2) Moderated differentiated (n=3)	7	EBER-ISH	EBNA1, EBNA2, LMP1, LMP2A, LMP2B (RT-PCR*)	BZLF1 (IFA)
Selves J. *et al.* 1996 [46]	France	FFPE	LELCs (n=4), Well differentiated (n=1)	5	EBER-ISH	LMP1 (IHC)	BHRF1 (ISH)
Gulley M *et al.* 1996 [47]	USA	FFPE	Adenocarcinomas: Diffuse type (n=7) Intestinal type (n=4),	11	EBER-ISH	LMP1 (IHC)	
Imai S. *et al.* 1994 [15]	Japan	FFPE and Frozen	ULCs (n=8), Adenocarcinomas: poorly differentiated (n=27) mod. to well-differentiated (n=35)	20	EBER-ISH	EBNA1(western-blot and IF) EBNA2, LMP1 (western-blot)	
Takano Y *et al.* 1994 [26]	Japan	FFPE	Medullary carcinomas with lymphoid infiltration (n=27)	27	EBER-ISH	LMP1 (IHC)	
Rowlands D. *et al.* 1993 [28]	UK and Japan	FFPE	UCNT (n=6) Intestinal type (n=1) Mixed type (n=2)	9	EBER-ISH	EBNA2, LMP1 (IHC)	BZLF1 (IHC)

Table II

Table II: Expression of EBV latent proteins

Study	**n**	**EBNA1 n (%)**	**EBNA2 n (%)**	**LMP1 n (%)**	**LMP2A n (%)**	**LMP2B n (%)**
Cheng N, *et al*. 2015 [30]	7	7 (100)	0 (0)	0 (0)	5 (71.4)	----
Zang YW, *et al*. 2015 [31]	78	----	----	----	37 (47.4)	----
Zhu S, *et al*. 2013 [32]	13	13 (100)	0 (0)	0 (0)	----	----
Strong M, *et al*. 2013 [33]	4	3 (75.0)	0 (0)	3 (75.0) *	3 (75.0)	3 (75.0)
Han J, *et al*. 2012 [35]	53	----	----	----	29 (54.7)	----
Tang W, *et al*. 2012 [25]	14	14 100)	6 (42.9) *	14 (100)	14 (100)	----
Shukla SK, *et al*. 2012 [34]	40	40 (100)	----	----	----	----
Lee JM. *et al*. 2011 [36]	4	4 (100)	0 (0)	0 (0)	1 (25.0)	----
Chen J. *et al*. 2011 [23]	8	7 (87.5)	0 (0)	0 (0)	5 (62.5)	----
Chen J *et al*. 2010 [37]	45	42 (93.3)	0 (0)	0 (0)	----	----
Ryan J. *et al*. 2007 [39]	11	----	----	1 (9.0)	4 (36.4)	----
Seto E, *et al*. 2005 [42]	6	6 (100)	----	----	----	----
Wang Y, *et al*. 2005 [41]	13	13 (100)	0 (0)	0 (0)	----	----
Luo B, *et al*. 2005 [40]	11	11 (100)	0 (0)	0 (0)	4 (36.4)	0 (0)
Lee M, *te al*. 2004 [43]	4	4 (100)	----	0 (0)	----	----
Hoshikawa Y, *et al*. 2002 [44]	3	3 (100)	0 (0)	0 (0)	2 (66.7)	----
zur Hausen A, *et al*. 2000 [24]	10	10 (100)	0 (0)	0 (0)	6 (60.0)	----
Gulley M, *et al*. 1996 [47]	11	----	----	3 (27.3)	----	----
Selves J, *et al*. 1996	5	----	----	0 (0)	----	----
Sugiura M, *et al*. 1996 [27]	7	7 (100)	0 (0)	0 (0)	3 (42.9)	0 (0)
Imai S, *et al*. 1994 [15]		70/70 (100)	0/20 (0)	0/20 (0)	----	----
Takano Y, *et al*. 1994 [26]	27	----	0 (0)	----	----	----
Rowlands D, *et al*. 1993 [28]		----	0/7 (0)	0/9 (0)	----	----
TOTAL		254/259 (98.1)	6/193 (3.1)	21/199 (10.6)	113/210 (53.8)	3/22 (13.6)

* Low albeit detectable level

Table III

Table III: Expression of EBV lytic genes

Study	N	BARF0 n (%)	BARF1 n (%)	BHRF1 n (%)	BARTs n (%)	BLLF1 n (%)	BMRF1 n (%)	BcLF1 n (%)	BRLF1 n (%)	BZLF1 n (%)
Cheng N, *et al*. 2015 [30]	8	----	----	----	----	----	----	----	----	0 (0)
Zhu S, *et al*. 2013 [32]	13	---	8 (46.2)	2 (15.4)	---	---	---	---	---	----
Strong M, *et al*. 2013 [33]	4	4 (100)	----	----	----	----	----	----	3 (75.0)	3 (75.0)
Tang W, *et al*. 2012* [25]	14	----	14 (100)	----	----	13 (92.9)	12 (85.7)	----	14 (100)	13 (92.9)
Shukla SK, *et al*. 2012 [34]	40	----	30 (75.0)	----	25 (62.5)	25 (62.5)	----	25 (62.5)	----	31 (77.5)
Lee JM. *et al*. 2011 [36]	4	----	----	----	0 (0)	0 (0)	----	----	----	----
Chen J. *et al*. 2011 [23]	4	----	----	----	----	----	----	----	----	0 (0)
Chen J, *et al*. 2010 [37]	45	----	----	----	----	----	----	----	----	0 (0)
Kim D. *et al*. 2007 [39]	4	----	----	0 (0)	4 (100)	4 (100)	----	----	----	----
Ryan J. *et al*. 2009 [38]	11	----	----	----	----	----	0 (0)	----	----	0 (0)
Seto E, *et al*. 2005 [42]	6	----	5 (83.3)	----	----	----	----	----	----	0 (0)
Wang Y, *et al*. 2005 [41]	13	----	6 (46.2)	2 (15.4)	----	----	----	----	----	6 (46.2)
Luo B, *et al*. 2005 [40]	11	----	5 (45.5)	2 (18.2)	----	0 (0)	----	7 (63.6)	0 (0)	5 (45.5)
Hoshikawa Y, *et al*. 2002 [44]	3	----	----	----	----	3 (100)		1 (33.3)	1 (33.3)	2 (66.7)
zur Hausen A, *et al*. 2000 [24]	10	10 (100)	10 (100)	2 (20.0)	----	----	----	----	----	0 (0)
Selves J, *et al*. 1996 [46]	5	----	----	1 (20.0)	----	----	----	----	----	----
Sugiura M, *et al*. 1996 [27]	7	----	----	----	----	----	----	----	----	0 (0)
Rowlands D, *et al*. 1993 [28]	8	----	----	----	----	----	----	----	----	3 (37.5)
TOTAL		14/14 (100)	78/122 (63.9)	9/56 (8.02)	29/48 (60.4)	45/76 (59.2)	12/25 (48.0)	33/54 (61.1)	18/32 (56.2)	63/184 (34.2)

* Tang W, *et al*. also observed the expression of BALF5 and BXLF1in all cases and BCRF1 in 11 of 14 cases.

Table IV

Table IV – Description of latency patterns

Author, Year	Latency I n	Latency II n	Latency III n	Latency II-like n
Cheng N, *et al*. 2015 [30]	2/7	----	----	5/7
Tang W, et al. 2012 [25]	----	8/14	6/14	----
Chen J, et al.2011 [23]	2/7	----	----	5/7
Lee J M, 2011 [36]	3/4	----	----	1/4
Luo B, et al, 2005 [40]	7/11	----	----	4/11
Hoshikawa Y, et al, 2002 [44]	1/4	----	----	3/4
zur Hausen A, 2000 [24]	2/9 [1]	----	----	7/9
Sugiura M, 1996 [27]	4/7	----	----	3/7

[1]The classification of these cases into latency I is based on the presence of both EBNA1 and LMP2A and absence of EBNA2 and LMP1. Authors also showed that BARF1 is present in all tested cases so this is not a typical latency I pattern.