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EPSTEIN-BARR VIRUS LATENT GENE EXPRESSION IN NASOPHARYNGEAL AND GASTRIC CARCINOMA

Dissertação de Candidatura ao grau de Mestre em Oncologia submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

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PREFACE

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

A systematic review of literature is being prepared to be published: Ribeiro J, Oliveira C, Sousa H. **EBV LATENCY PATTERN IN GASTRIC CARCINOMAS: A SYSTEMATIC REVIEW**.

The results obtained in this study are being prepared to be included in different publications to be submitted in the near future.

AGRADECIMENTOS

Esta tese não teria certamente sido possível, sem o apoio, motivação e encorajamento, que fui tendo ao longo da sua realização. Deixo aqui os meus agradecimentos a todos, que de uma forma ou de outra, contribuíram para a sua execução.

Em primeiro lugar gostaria de agradecer ao meu orientador, Doutor Hugo Sousa por me ter aceite no Grupo de Oncologia Molecular e Patologia Viral do Instituto Português de Oncologia do Porto (IPO Porto FG EPE), pelos novos conhecimentos adquiridos, por toda a ajuda e disponibilidade fornecida e por ter deste modo ter enriquecido o meu percurso académico. Não menos importante, á minha co-orientadora, Mestre Joana Ribeiro, por ter de igual forma ter contribuído para que este projeto fosse possível.

Queria também agradecer ao serviço de Anatomia Patológica do IPO-PORTO, em especial ao Dr. Luis Pedro, á Dra. Ana Gallaghar, ao Dr. Manuel Jácome, á Técnica Fernanda Silva e a Técnica Ana tavares, por toda a ajuda e disponibilidade que forneceram, que tornaram este trabalho possível.

Às minhas companheiras de laboratório, Nádia Neto e Ana Bela Campos, pelos momentos de desespero passados em conjunto, pelas horas infindáveis a moldar os sofás da entrada, mas principalmente pelas gargalhadas e por todas as situações de descontração que tornaram este ano muito mais fácil de aguentar. Um especial agradecimento também á Mariana Malta, que na altura do aperto, foi uma ajuda preciosa no laboratório.

Aos meus amigos, pelos convívios, pelas saídas, por todos os bons momentos, mas mais importante do que isso, por todo o apoio que ao longo destes anos todos nunca falhou.

Aos meus pais e família, por terem permitido que eu realizasse todo este percurso sem nunca terem duvidado de mim.

Por último, quero agradecer àqueles que ao longo deste ano aturaram todo o meu mau humor e, que á sua maneira, sempre souberam sempre como o mudar.

A todos, um sincero obrigada!

RESUMO

O EBV é um vírus associado a cancro que infecta cerca de 90% da população mundial, sem causar sintomas na maioria dos portadores ao longo da vida. A infeção por EBV pode adotar quatro diferentes padrões de latência, a latência 0, I, II, e III, que parecem estar correlacionados com os diferentes tipos de doenças associadas ao EBV. . No entanto, há algumas dúvidas sobre a expressão de genes latentes do EBV em diferentes doenças tais como o carcinoma nasofaríngeo e o carcinoma gástrico.

Foi realizado um estudo retrospetivo com 23 casos de carcinoma da nasofaringe e 9 casos de carcinoma gástrico associados ao EBV, a fim de avaliar a expressão de proteínas do EBV nos diferentes tumores e tentar estabelecer uma correlação clínica entre a latência viral e a malignidade. A deteção do EBV foi feita por EBER-IHS e a deteção de proteínas do EBV (LMP1 e LMP2a) foi realizada por imuno-histoquímica. Todos os casos utilizados neste estudo foram EBV positivos.

LMP1 esteve presente em 95,5% dos casos de carcinoma da nasofaringe, mas nenhum dos casos de carcinoma gástrico mostrou ter expressão LMP1. Em relação á LMP2a, ela foi expressa em 100% dos casos de carcinoma da nasofaringe, enquanto no carcinoma gástrico estava presente em 44,4%. A expressão proteica de cada um dos tumores levou a diferentes tipos de latência. No carcinoma da nasofaringe, 21 casos (95,5%), apresentaram expressão de LMP1 e LMP2a, o que corresponde a um padrão de latência II, e apenas 1 caso (4,5%) expressou um padrão de latência distinto semelhante à latência II, mas sem a expressão de LMP1. No carcinoma gástrico, 5 casos (55,5%) não apresentavam expressão de LMP1 e LMP2a, que corresponde a um padrão de latência I. Os restantes 4 casos, tiveram o mesmo padrão de latência que um caso de carcinoma da nasofaringe onde há expressão de LMP2a, mas não de LMP1. Esses dados confirmam os padrões de latência associados ao carcinoma da nasofaringe (latência II) e com o carcinoma gástrico (latência I). Identificou-se também um padrão de latência novo e ainda não aceite (latency II-like) que é especialmente importante no carcinoma gástrico, e é caracterizado por a expressão de EBNA 1 e LMP2a.

Este foi o primeiro estudo realizado em Portugal para caracterizar os padrões de latência do EBV em diferentes neoplasias. Em conclusão, mesmo com a confirmação que o carcinoma da nasofaringe expressa tipicamente um padrão Latência II e o carcinoma gástrico a latência I, observou-se também que alguns casos expressam um perfil de latência diferente. Portanto, há uma necessidade de uma determinação correta da expressão de proteínas do EBV para um conhecimento correto sobre os mecanismos de

transformação. A literatura suporta a necessidade de reestruturação dos padrões de latência, considerando a expressão de todos os tipos de expressão de proteínas.

ABSTRACT

EBV is a human cancer-associated virus that infects about 90% of the global population, without causing major symptoms in the majority of lifelong carriers trough the establishment of a viral latent state. EBV infection can adopt four different patterns of latency, named type 0, I, II, and III, which seem to be correlated with the different types of EBV associated disease. Nevertheless, there is some doubt on the differential expression of EBV latent genes in different diseases such as Nasopharyngeal Carcinoma and Gastric Carcinoma.

We have performed a retrospective study with 23 NPC and 9 EBVaGC cases, in order to evaluate the expression of EBV proteins in different tumors and try to establish a clinical correlation between the viral latency and the malignances. The identification of EBV was made by EBER-ISH and the detection of EBV proteins (LMP1 and LMP2a) was performed by immunohistochemistry. All cases used in this study were EBV positive

LMP1 was present in 95.5% of NPC cases, but in none EBVaGC was shown LMP1 expression. Regarding the LMP2a, it was expressed in 100% of NPC cases, while in GC it was present in 44.4%. The protein expression that each of the tumour presented led to different latency types. In NPC, 21 cases (95.5%), had the expression of both LMP1 and LMP2a, which corresponds to a latency II pattern, and only 1 case (4.5%) expressed a distinct latency pattern similar to latency II, but without the expression of LMP1. In GC, 5 cases (55.5%) had no expression of LMP1 and LMP2a, wich corresponds to a latency I pattern. The remaining 4 cases, had the same latency pattern as one case of NPC were there is expression of LMP2a, but not LMP1. These data confirm the latency patterns associated with NPC (latency II) and with EBVaGC (latency I). We also identified a novel and yet not accepted latency (latency II-like) that is especially important in EBVaGC, characterized by the expression of EBNA1 and LMP2a.

This was the first study in Portugal to characterize the latency patterns of EBV in different diseases. In conclusion, even with the confirmation of NPC as typically expressing a Latency II pattern and EBVaGC a latency I, we observed that some cases express a different latency profile. Hence, there is an urge in the correct determination of EBV profile expression for a correct knowledge on the mechanisms of transformation. The literature supports the need for restructuring the latency patterns, considering the expression of all types of protein expression.

ABREVIATIONS LIST

BARTs - BamHI A rightward transcripts

B-CLL - B-Chronic Lymphocytic Leukemia

BL- Burkitt's Lymphoma

CSF - colony stimulating factor

EBNAs - Epstein Barr Nuclear Antigens

EBERs - Epstein-Barr Virus-encoded RNAs

eBL - Endemic BL

EBV - Epstein-Barr virus

EBVaGC - Epstein-Barr virus associated gastric carcinoma

GC - gastric cancer

gH - glycoprotein H

GIST - gastrointestinal stromal tumours

gM - glycoprotein M

gN - glycoprotein N

HHV4 - Human Herpesvirus 4

HIV - human immunodeficiency vírus

HL - Hodgkin lymphoma

HSV - herpes simplex virus

IHC - immunohistochemistry

IM - Infectious mononucleosis

IRs - internal repeat sequences

Kb - kilobase pairs

LELC - lymphoepithelioma-like gastric carcinomas

LMPs - Latent Membrane Proteins

LPHL - lymphocyte-predominant

M-CSF - macrophage colony-stimulating factor

MHC - Major histocompatibility complex

miR-BARTs – EBV microRNAs

miRNAs - microRNAs

NET - neuroendocrine tumours

NPC - nasopharyngeal carcinoma

ori-lyt - lytic origin of replication

PML - Promyelocytic Leukemia gene

PTLD - Post-transplant lymphoproliferative disease

sBL - Sporadic Burkitt's lymphoma

STAT1 - activation of transcription 1

TF - transcription factor

TLR3 - Toll-like receptor 3

TRs - terminal direct repeats

VCA - viral capsid antigen

WHO - World Health Organization

ZEBRA - Z Epstein–Barr replication activator

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I. INTRODUCTION

1. Epstein-Barr Virus

1.1. Historical Background

In 1958, Denis Burkitt identified a frequent cancer among children in Equatorial Africa [1, 2]. This tumour was dependent on climatic and geographical conditions, which led to the possibility that it could be associated with a vector-borne agent [1]. Later, in 1964, Anthony Epstein, Yvonne Barr and Bert Achong using electronic microscopy discovered typical herpesvirus particles in biopsies of the named Burkitt's Lymphoma (BL), which they have called Epstein-Barr Virus (EBV). In the late 1960s, studies revealed that BL patients had higher antibody titers to EBV antigens [3, 4] These serological assays also allowed the identification of EBV as the etiological agent of infectious mononucleosis (IM) and nasopharyngeal carcinoma (NPC) [5, 6].

1.2. EBV Structure and Genome

EBV also recognized as Human Herpesvirus 4 (HHV4), belongs to the *Herpesviridae* family, *Gammaherpesvirinae* subfamily and is the only human virus from the *Lymphocryptovirus* genus [7]. The virion of EBV is about 120-300 nm in diameter and consists of a toroid shaped protein core wrapped with linear double stranded DNA with approximately 172 kilobase pairs (kb) in an icosahedral nucleocapsid with 162 capsomeres, an outer envelope with external glycoprotein spikes and a tegument protein between the nucleocapsid and envelope (Figure 1) [8-11].

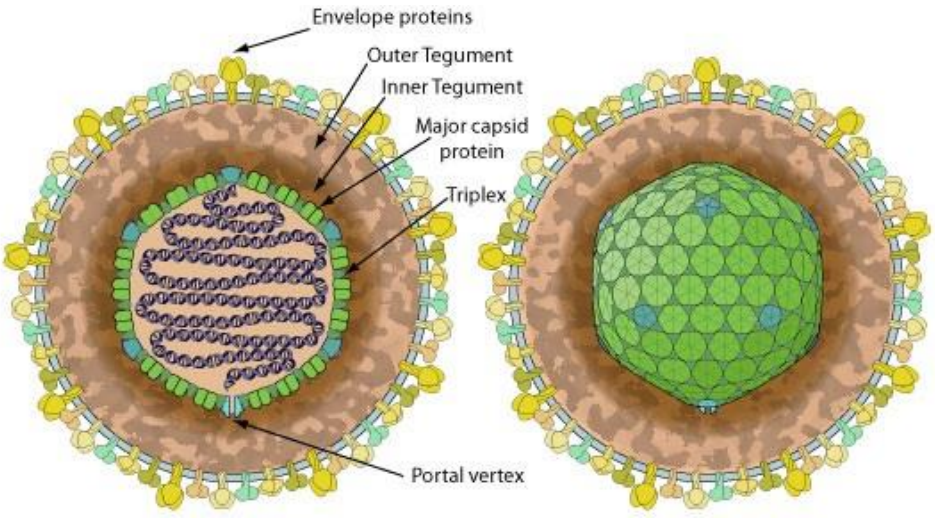

Figure 1: EBV virion structure (adapted from *http://viralzone.expasy.org/viralzone/all_by_species/185.html*)

EBV genome has several terminal repeats (TRs) and internal repeat sequences (IRs) that divide the genome into short and long unique sequence domains (Figure 2) [12]. The EBV genome is linear, but once it reaches the nucleus of the infected cell, it adopts an

episomal structure through the binding of the TRs, which is required for replication of the viral genome [13].

Only a very small fraction of the EBV genome that enters the cell is able to reach the nucleus and an even smaller fraction is able to circularize. During cellular proliferation, linear viral genomes are gradually lost while circular DNA is maintained [14, 15] Indeed, during latency, EBV DNA acts like the celular DNA, being associated with histones and replicating once during the S phase, depending only on the cellular machinery and being equally transmitted to daughter cells [13].

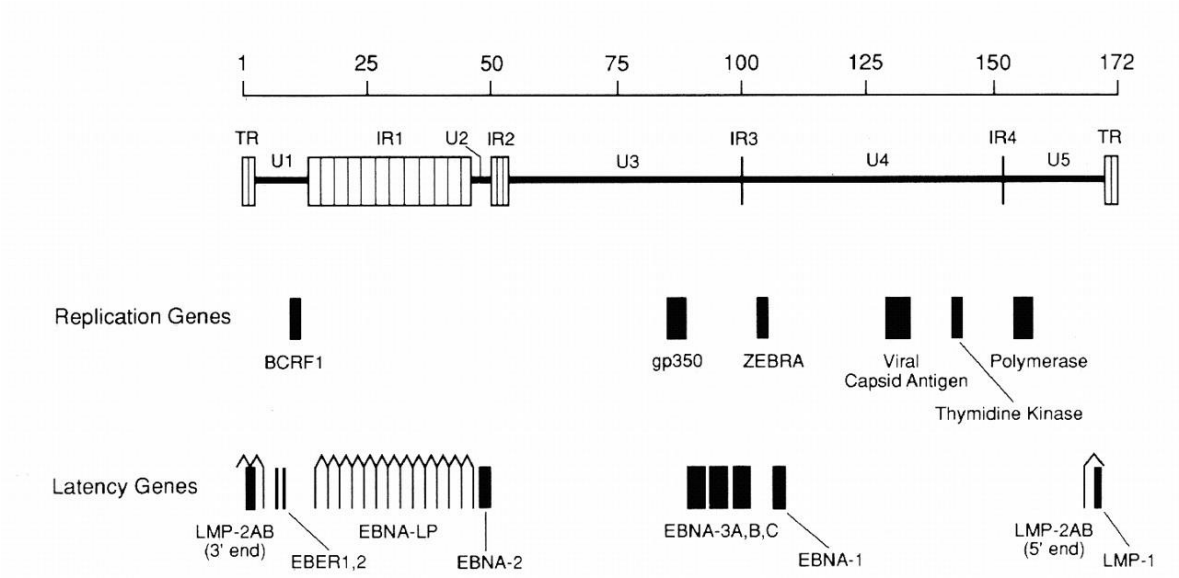

Figure 2: Map of EBV genome (Straus, 1993)

By gene sequencing of different EBV isolates it was possible to identify two different EBV subtypes, which were classified as type 1 and type 2 (or type A and type B, respectively) [16]. The difference between these two subtypes are mainly on genetic polymorphism in the Epstein Barr Nuclear Antigens (EBNAs), but other single base changes may be observed in other regions of the genome [9, 17-19]. Type 1 is observed in almost all populations, being predominant in Europe, America, South America and Asia; while type 2 is mainly found in Central Africa, New Guinea and in Alaskan Eskimos [20]. The influence of these EBV subtypes in disease development is not yet understood, but type 1 virus seems to prevail in most EBV positive associated diseases, whereas type 2 is mainly associated with immunocompromised patients [21].

1.3. EBV Infection

EBV infection is restricted to humans, and is transmitted almost exclusively by saliva [5]. The primary infection usually occurs early in life and the family is frequently responsible for the transmission, although in developed countries this infection can be delayed until adolescence or adulthood and here it can lead to the development of a strong immune response medically known as IM [5, 22, 23]. After controlled, the infection is asymptomatic due to the establishment of a viral latency within the memory B lymphocytes [24]. In fact, EBV establishes a persistent lifelong infection, in most cases without consequences, in more than 90% of the world population [25, 26].

It is assumed that the primary infection occurs in the oropharynx, due to the interaction of the viral membrane glycoprotein gp85/42, with a Major histocompatibility complex (MHC) class II receptor on the surface of lymphoepithelial cells of the Waldeyer's ring [27]. Subsequently, EBV starts a short period of lytic replication, after which it is released into the saliva and infects adjacent epithelial cells (Figure 3) [27].

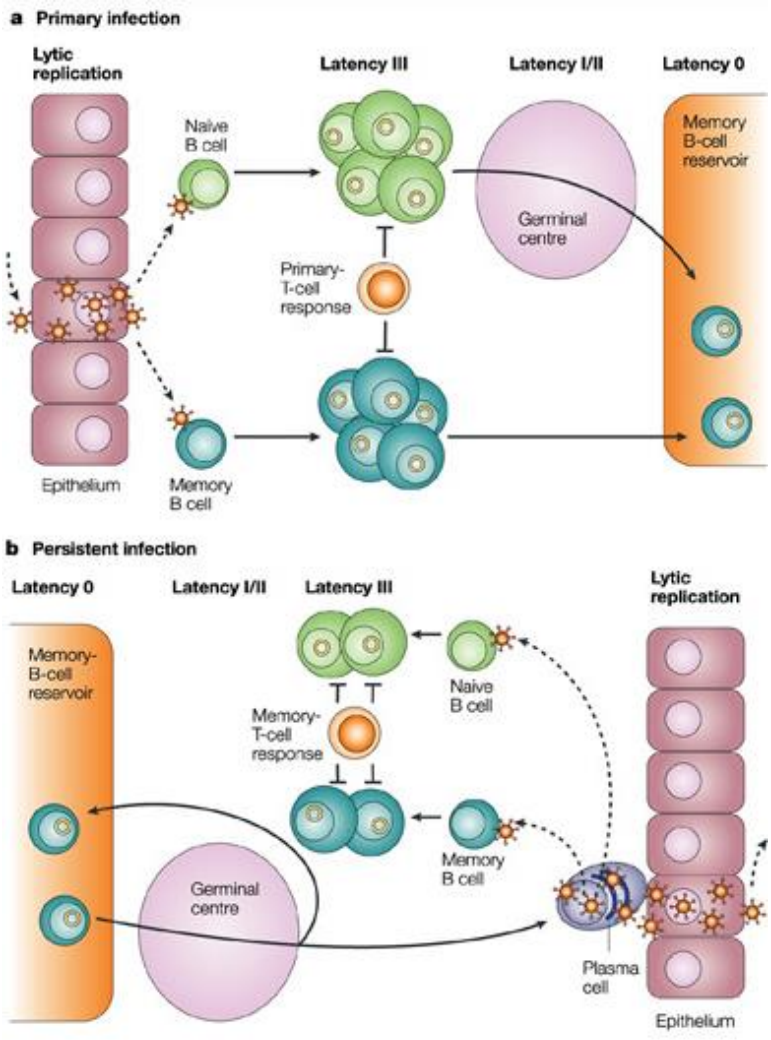

Figure 3: Interactions between Epstein–Barr virus and host cells (Young, 2004)

Then, after the infection of epithelial cells in the oropharynx, EBV infects immature B lymphocytes that are nearby, process only possible due to the binding of EVB's membrane glycoprotein gp350 / 220 with the CD21 molecule of B lymphocytes [28]. The infected B-lymphocytes will be transformed into lymphoblastoid cells with latent EBV, proliferating without control. Many of these proliferating cells are killed by cytotoxic T-lymphocyte response, nevertheless some escape through downregulation of antigen expression and the establishment of a stable reservoir of memory B lymphocytes where viral antigen expression is almost nonexistent [29].

1.4. Viral gene expression

As all other herpesviruses, EBV has a life cycle with different viral gene expression programs: a latent one, on which no viral particles are formed; and a lytic one, on which new infectious viruses are produced [16]. There are more than 90 viral proteins coded by the EBV genome and the great majority has no known function [30].

1.4.1. Lytic genes

EBV establishes a latent persistent lifelong infection in most cases, nevertheless, for reasons not yet understood, the virus can reactivate and restart its lytic cycle in specific conditions [24]. By analogy with other herpesviruses, the proteins expressed in this phase are classified as immediate-early, early, and late lytic proteins: Immediate-early genes are transcribed after infection in the presence of protein synthesis inhibitors; Early genes are expressed in the presence of viral DNA synthesis inhibitors; and late genes are not transcribed when these inhibitors are present.

EBV lytic genes are named by BamHI fragment within which they are located, whether they are expressed in a leftward (L) or rightward direction (R), and the number of their position in the BamHI fragment. For example, BZLF1 is the first transcript expressed in the leftward direction in the BamHI Z fragment of EBV (Figure 4) [31].

1.4.1.1. Immediate-Early Lytic Proteins

Immediate-early genes are important for regulating gene expression in the virus. The major immediate-early proteins of EBV are encoded by BZLF1 and BRLF1, also named by Z Epstein–Barr replication activator (ZEBRA) or Zta and Rta, respectively. BZLF1 and BRLF1 proteins activate transcription of viral early genes [32, 33]. BZLF1 protein inhibits transcription from the EBNA Cp promoter and may facilitate the switch from latent to lytic

infection [34]. BZLF1 protein also downregulates the interferon gamma receptor and inhibits the ability of interferon gamma to activate its target genes including IRF-1, CIITA, and MHC class II [31].

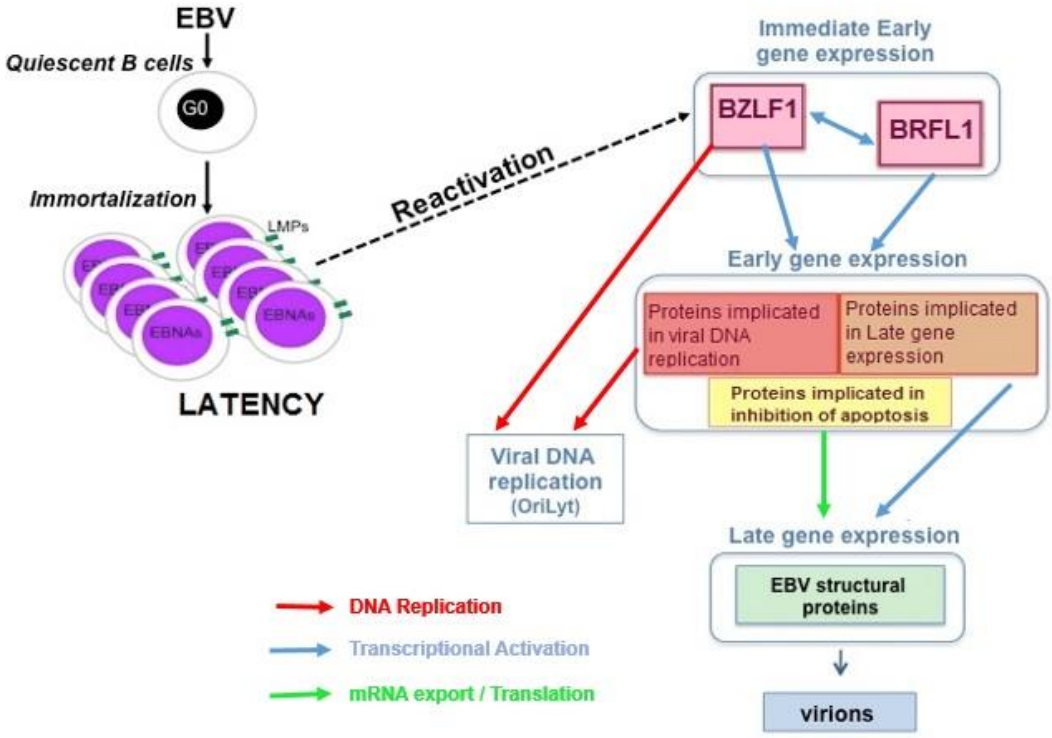

Figure 4: EBV lytic cycle activation

1.4.1.2. Early Lytic Proteins

EBV early lytic proteins include enzymes that are important for viral DNA replication, inhibition of apoptosis, and activation of gene expression. Six viral proteins have been identified as replication proteins originated by the lytic origin of replication (ori-lyt) [35]. These replication proteins are the viral DNA polymerase, encoded by BALF5, the DNA polymerase processivity factor, encoded by BMRF1, the single-stranded DNA-binding protein homolog, encoded by BALF2, the primase homolog, encoded by BSLF1, the helicase homolog, encoded by BBLF4, and the helicase-primase homolog, encoded by BBLF2/3. The viral thymidine kinase, encoded by BXLF1, phosphorylates acyclovir and results in activation of the drug with inhibition of the viral DNA polymerase and viral DNA replication0 [35]. Other early viral proteins important for viral DNA replication are the ribonucleotide reductase proteins encoded by BORF2 and BARF1 and the uracil DNA glycosylase encoded by BKRF3 [35].

1.4.1.3. Late Lytic Proteins

Late genes encode structural proteins of the virion: glycoproteins, nucleocapsid proteins, and a viral cytokine [36]. Most of the viral capsid antigen (VCA) is comprised of the major nucleocapsid protein, which is encoded by BcLF1 [36].

EBV encodes several glycoproteins including gp350, gp110, gp85, gp42, and gp25 [37]. The gp350, encoded by BLLF1, is the major viral envelope protein and it is able to bind CD21 contributing to the virus entry into the host B-cells. Deletion of gp350 from the virus markedly reduces the infectivity of the virus, and therefore purified recombinant gp350 is being studied as a vaccine candidate [37]. EBV gp110, encoded by BALF4, is the homolog of *herpes simplex virus* (HSV) glycoprotein B, which is required for HSV entry into cells. The three remaining EBV glycoproteins, gp85, gp42, and gp25, form a trimolecular complex, which is responsible for virion penetration of the B-cell membrane [38]. EBV gp85, encoded by BXLF2, is the homolog of HSV glycoprotein H (gH), which is essential for the fusion of the virus to B-cells and absorption to epithelial cells [39, 40]. EBV gp25, the product of BKRF2, acts as a viral chaperone to transport gp85 to the cell membrane [41], and gp42, encoded by BZLF2, binds to MHC class II molecules [42] and functions as a co-receptor for virus entry in B-cells [43]. EBV also encodes homologs of HSV glycoprotein N (gN), encoded by BLRF1, and glycoprotein M (gM), encoded by BBRF3, that are important for egress of virus from the cell [44].

BCRF1 protein, also termed viral IL-10, shares over 80% amino acid identity with human IL-10 [45]. This viral IL-10 inhibits interferon gamma secretion by peripheral blood mononuclear cells and release of IL-12 from macrophages protecting virus-infected cells from cytotoxic T-cells. [46]. Viral IL-10 also stimulates growth of B-cells and inhibits the activity of dendritic cells [47, 48].

1.4.2. Latent genes

From all viral proteins coded by EBV genome, only a restricted group may be expressed in different combinations during latency. [49]. Therefore, latency results from a tight interplay between viral and host transcription factors, leading to the distinct use of three viral promoters (Cp, Wp and Qp) which regulate the transcription of different viral genes [50]. The viral genes expressed during latency include: six EBV Nuclear Antigens (EBNA1, 2, 3A, 3B, 3C and LP); three Latent Membrane Proteins (LMP1, LMP2A. and LMP2B); two small nonpolyadenylated Epstein-Barr Virus-encoded RNAs (EBER1 and 2) and highly spliced BamHI A rightward transcripts (BARTs) (Figure 5) [13, 51, 52].

1.4.2.1 EBV-nuclear antigens

EBNA1

EBNA1 is the only nuclear EBV antigen expressed in both latent and lytic modes and it is essential for efficient EBV genome replication, persistence and transcription in dividing cells. [53, 54]. This protein is expressed in all virus-associated tumours [55, 56]. Literature have referred several functions to EBNA1, such as binding to viral DNA elements and cellular promoters, activation EBV viral Cp and Wp promoters, inhibition of Qp promoters, upregulation signal transducers and activation of transcription 1 (STAT1), downregulation of tumour growth factor-β signaling pathways, reduction of SMAD2, upregulation of CCL20 [57], inhibition the canonical NF-KB pathway, and enhancing activity of the AP-1 transcription factor (TF) [56-63].

EBNA1 contributes to the latent infection as it suppresses spontaneous lytic reactivation and induces a family of microRNAs (miRNAs), which in turn decrease the level of cellular protein Dicer, inhibiting reactivation of latent EBV [64, 65]. EBNA1 has also been recognized as inducing the loss of Promyelocytic Leukemia gene (PML) nuclear bodies, and decreased p53 activation and apoptosis in response to DNA damage in NPC and EBV-associated gastric cancer (EBVaGC) [66].

EBNA2

EBNA2 exists in two allelic forms, which differ in the amino acid sequence of the central region of the molecule [67]. Interestingly, each EBV subtype has a different EBNA2 allelic form. This may probably account for the differences observed in the growth transformation abilities of each EBV subtype. [68].

EBNA2 expression results from the same pre-mRNA as EBNA-LP, after an alternative splicing. These two viral proteins are the first EBV proteins expressed during EBV infection and B cells transformation, nevertheless, only EBNA2 and LMP1 essential for.EBV-induced immortalization [69, 70]. EBNA2 by itself is responsible for the expression of several proteins such as LMP1, LMP2A, LMP2B and also the ones arising from the EBNA's Cp promoter [71-74]. EBNA2 activation of gene expression does not occur though direct binding of EBNA2 to DNA, but rather through the interaction with the cellular CBF1 (RBP-J11) protein complex. One important target of EBNA2 is the cellular oncogene c-Myc, this link may be relevant for EBV induced proliferation and immortalization of B lymphocytes [75].

EBNA3 FAMILY

There are three EBNA3 proteins, EBNA3A, EBNA3B, and EBNA3C. Studies have demonstrated that, in contrast with EBNA3B, EBNA3A and EBNA3C are required for B-cell transformation [76, 77]. The EBNA3 proteins are responsible for increased expression of both cellular and viral genes. EBNA3C increases the expression of CD21 and co-activates the LMP1 promoter in conjunction with EBNA2, it interacts with the human metastatic suppressor protein Nm23-HI and inhibits the ability of the latter to suppress the migration of BL cells [78]. EBNA3C has also been shown to mediate the degradation of the retinoblastoma protein (pRb), with the assistance of the SCF^{SKP2} complex, in transiently or stably transfected cells [79, 80]. On the other hand, EBNA3B seems to upregulate CD40 and bcl-2 expression [81-83]. These funtions reforce the ability of EBNA3C family in the maintenance of EBV latency and transformation [81-83].

EBNA-LP

Even though the role of EBNA-LP in EBV-induced B-cell transformation is still uncertain, it is thought that it plays an important role in the establishment of B-cell immortalization, once mutant viruses lacking the unique carboxy-terminal domain, are much less efficient at immortalizing B cells than wild-type viruses [84, 85]. EBNALP coactivates EBNA2 enhancing the expression of the major viral oncoprotein LMP1 [85, 86]. While EBNA-LP has been shown to bind to p53 and the retinoblastoma protein, it is unclear what significance these interactions have for the role of EBNALP in B-cell transformation [87, 88]. In addition, EBNA-LP binds to a number of other cellular proteins including heat shock protein 70, DNA protein kinase catalytic subunit, HA95 a nuclear protein that may be involved in mitosis, and in a and b tubulin [87, 89].

In newly infected B cells, a range of EBNA-LP isoforms are expressed, but over time, the number of expressed isoforms decreases [85]. In addition, EBNA-LP localizes diffusely throughout the nucleus within the hours post-infection, but with time, it associates with PML nuclear bodies [90].

1.4.2.1. Latent membrane proteins

LMP1

LMP1 develops a key role in the immortalization of B cells and is considered a major EBV oncoprotein [91, 92]. It is a membrane protein composed of six hydrophobic clusters forming three membrane spanning domains connected to a short N-terminal cytoplasmic domain and also to a long C-terminal cytoplasmic tail of 200 amino acids [93, 94]. It is

relatively highly expressed in most EBV associated tumours, but its expression is rare in EBV infected healthy individuals [18, 95].

Several studies have demonstrated the LMP1 is involved in proliferation, apoptosis, angiogenesis, invasion and modulation of immune response, leading to dysregulation of various cellular pathways in tumour cells, and also affecting the tumour microenvironment [95-97]. Thus, LMP1 is highly regulated and presents various functions in different cellular processes [94, 95, 97, 98]. Although it is a latent protein, its transcription is also observed during lytic infection [99, 100].

L M P 2

LMP2A and LMP2B are LMP2 isoforms transcribed from 2 different promoters and differing only in the first exon [101, 102]. Both LMP2A and LMP2B are membrane proteins responsible for the maintenance of EBV latency and pathogenicity [101, 103].

LMP2A is known to regulate several signaling pathways, like proliferation and survival of B cells even in the absence of normal BCR [104, 105]. LMP2A has been shown to interfere with switch from latency into lytic EBV infection, either by blocking the activation of protein tyrosine kinases usually associated with BCR or by providing surrogate BCR receptor signaling [103]. Moreover, it has been shown that LMP2A expression increases the signaling capacity of LMP1 in epithelial cells. [106, 107].

Although the LMP2A isoform has been well studied, the LMP2B function in EBV infection has not been yet uncovered, mainly due to technical limitations associated with inability to produce antibodies against this protein [101, 103]. Nevertheless, some *in vitro* studies indicate a co-localization of this protein with LMP2A at the cellular membrane. It seems that LMP2B negatively negatively regulates the function of LMP2A in preventing the switch from latent to lytic EBV replication, , resulting in increased susceptibility to induction of lytic EBV infection through modulation of BCR and downstream signaling [103].

1.4.3. Other transcripts

BARTs

BARTs, are a family of multispliced rightward transcripts from the BamH1 A region of the EBV genome [108]. It has been suggested that they may play a special role in epithelial malignances, once they are expressed at high levels in EBV-infected epithelial cancers, but not in EBV-transformed lymphocytes [109, 110].

EBV encodes a number of microRNAs (miR-BARTs), that are transcribed from the same BART transcript [111]. These miR-BARTs are believed to play a key role in tumourigenesis by targeting multiple viral and cellular genes, and prevention of apoptosis is its major function in epithelial cancers. In addition miR-BARTs are able to protect EBV-infected malignant epithelial cells by weakening the host immune response. [108, 111].

Three BART cluster-1 miRNAs, miR-BART1-5p, -16 and -17-5p, can affect the growth-promoting and pro-apoptotic actions of LMP1 by downregulation of its expression [112]. miR-BART3, on the other hand, targets a nuclear importer receptor, importin 7 (IPO7), for immune evasion [113]. Recently studies showed that miR-BART22 suppressed expression of LMP2A in order to protect NPC cells from immunological attack [114]. miR-BART3-5p promotes cellular growth by targeting the DICE1 tumour-suppressor gene and miR-BART9 targets E-cadherin to enhance invasiveness and metastatic capacity in NPC cells [115, 116]. Moreover, the miR-BARTs facilitate EBV latency by limiting the expression of multiple lytic genes like BZLF1, BRLF1 and BALF5 in infected epithelial cells [117, 118].

BARF1

BARF1, encoded in the BamH1 A region, is a homologue of CSF1R, and blocks CSF-1 mediated signaling, a pathway of innate immunity [119]. It is considered a major viral oncogene in epithelial cells, and is highly expressed in NPC and EBV associated gastric carcinoma (EBVaGC) [120, 121].

BARF1 may drive carcinogenesis by transforming and mortalizingin epithelial cells, and enable cell survival, by upregulating anti-apoptotic Bcl-2 [122]. Also, secreted hexameric BARF1 inhibits macrophage colony-stimulating factor (M-CSF), manipulating this way myeloid cell growth and functions [123]. In B cells and lymphomas, BARF1 expression is restrained to the viral lytic replication cycle [123].

EBERs

EBV encodes two small RNAs, EBER1 and EBER2, which are non-polyadenylated, noncoding and expressed abundantly in all forms of cells latently infected with EBV [124]. Despite its abundance and well characterized structure, the function and mechanism of action is poorly understood. EBERs are reported to be involved in several cellular activities such as inhibition of apoptosis, increase cell proliferation and induction of tumour formation [125-127]. Literature suggests also, that EBER-1, which is the most abundant and stable of the two [128, 129], is excreted from cells as an RNA-protein complex and is able to induce pro-inflammatory cytokines such as IL-12 via Toll-like receptor 3 (TLR3) [128-130].

EBERs can be used as target molecules for the detection of EBV-infected cells and are considered a reliable marker of the presence of EBV. [131].

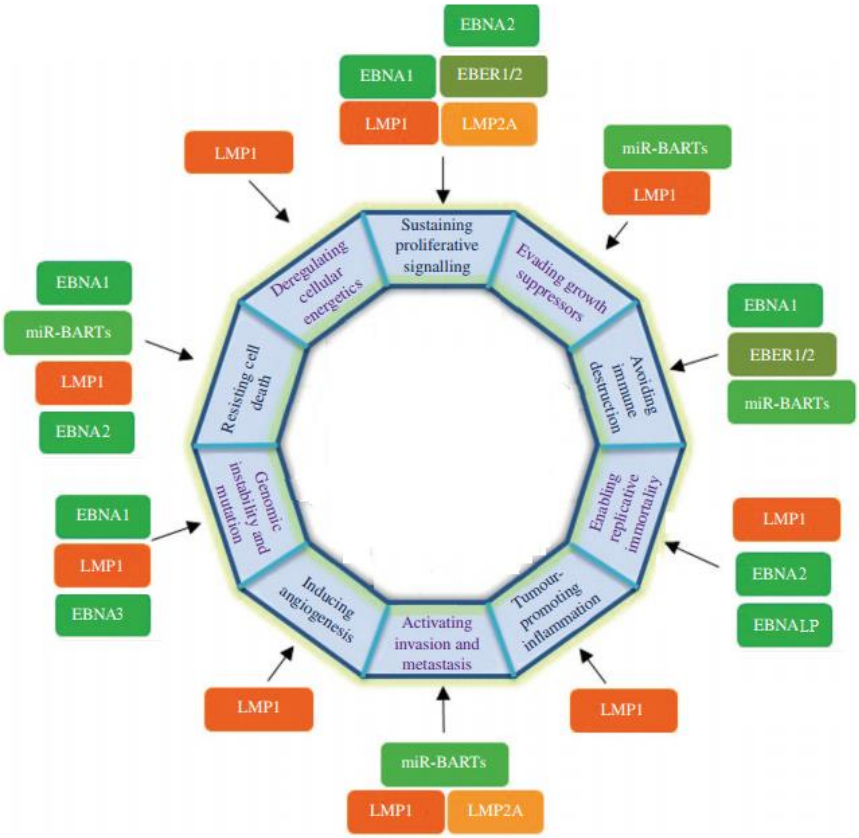

Figure 5: EBV latent genes target cancer hallmarks, *adapted from* (Tsao, 2015)

1. EBV-Associated Malignancies

EBV is currently associated with lymphoid and solid tissue malignancies in both immunocompetent and immunocompromised individuals. The presence of EBV infection in tumour cells and viral genes capable of usurping normal cellular growth control, suggest that EBV infection plays a causal role in the development of these associated malignancies [52].

2.1. Burkitt's Lymphoma

BL was first described as sarcoma of the jaw, but soon it became evident that this was a distinct form of Non Hodgkin's lymphoma [132]. The highest incidence and mortality rates of BL are seen in Eastern Africa, and it affects mainly children of male gender [132].

There are several forms of BL according to its geographic distribution, incidence magnitude and risk factors (Table 1). BL is a B-cell lymphoma genetically characterized by a chromosomal translocation that results in deregulation of the c-MYC oncogene [133].

Table 1: Overview of Burkitt's lymphoma clinical variants.

Characteristics	Endemic BL	Sporadic BL	HIV associated BL
Epidemiology	Equatorial Median age 7 yrs Associated with malaria/Climate	Median age 30yrs Children (30%) Older adults (1%) Low Socio Economical Status	HIV risk groups Median age 10-19 yrs
Clinical Presentation	Facial skeleton (50%), Central Nervous System (33%), other organs	Abdominal, ileo-coecal (80%) Bone marrow (20%) Other organs also affected	Organ and nodal presentation
Pathology/Morphology	Germinal centre B-cell		
Chromosomal translocations	Monomorphic medium sized B cells with basophilic cytoplasm and multiple mitotic figures		
EBV association	95-100%	30%	30-50%

Endemic BL (eBL) is the disease originally described by Burkitt and 95% of the cases are found in equatorial Africa (Figure 6), characteristically affecting the facial skeleton in children between ages two to nine [134]. Sporadic Burkitt's lymphoma (sBL) is the form usually described outside the endemic region and accounts for 1–2% of adult lymphoma in Western Europe and America. It is morphologically similar to eBL but affects mainly abdominal viscera. sBL can be detected at any age and no specific co-factor has been described [134]. A third subtype of BL has been proposed based on its association with the human immunodeficiency virus (HIV) infection. Though well described in the developed world and known among HIV positive adults in Africa, the childhood form of the disease among HIV positive children has not been well characterized [132].

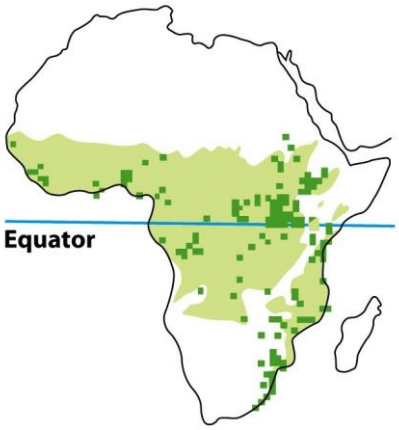

Figure 6: Map of Africa showing the 'lymphoma belt' in which eBL occurs at high incidence.

BL has a very close association with EBV, with approximately 95% of eBL showing the presence of the EBV genome in their tumour cells, while the other form are associated with EBV in 30-50% of all cases [135].

EBV plays a role in the pathogenesis of BL by deregulation of c-MYC activity and clonal expansion, direct mutagenesis and immune inactivation. Indeed, EBV is known to transform resting B cells into latently infected lymphoblastoid cells [132]. The majority of BL's, show EBV latency I pattern. This cells carry a wild-type EBV genome and express only EBNA1 from the latent promoter Qp [136]. However, around 15% of endemic tumours, carry an EBNA2 gene-deleted genome and express EBNA1, -3A, -3B, and -3C from the Wp latent promoter [137].

2.2. Hodgkin's Lymphoma

Hodgkin lymphoma (HL) was first discribed it in 1832 by Doctor Thomas Hodgkin. HL is rare type of cancer, being only diagnosed 66,000 new cases of Hodgkin lymphoma worldwide in 2012, accounting to 0.5% of total cancer cases. HL has a global spread, being more common in Asia and Europe (Figure 7) [138] HL is characterized by the presence of malignant multinucleated Reed Sternberg cells in a background of reactive inflammatory cells [13]. HL exists in five forms: mixed-cellularity, nodular-sclerosis, lymphocyterich, lymphocyte-depleted and lymphocyte-predominant (LPHL) subtypes. LPHL is the only one not associated with EBV [13].

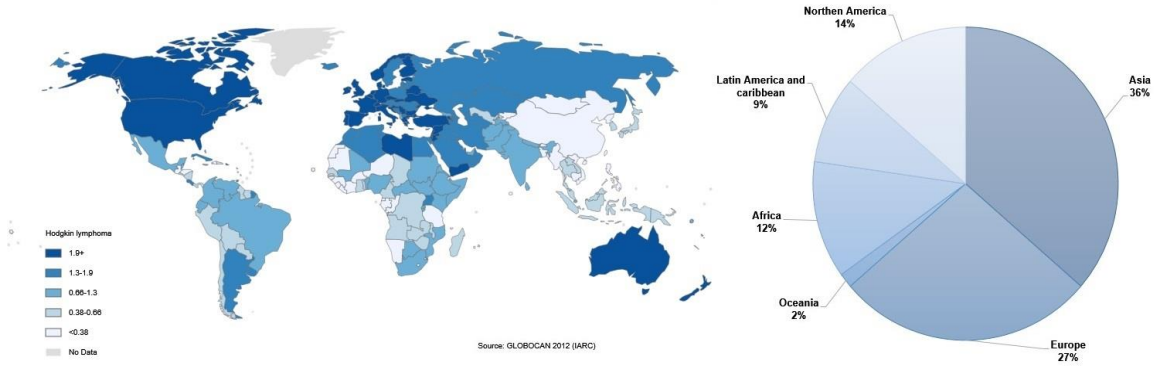

Figure 7: Hodgkin Lymphoma incidence worldwide, both sexes, all ages, Globocan 2012

HL presentation can be very different, since, it can appear in all lymphoid nodes in the body (Figure 8). The most common place for Hodgkin lymphoma is in the lymph nodes in the neck, and >50% of diagnosis are at stage I or II (Appendix II) [139].

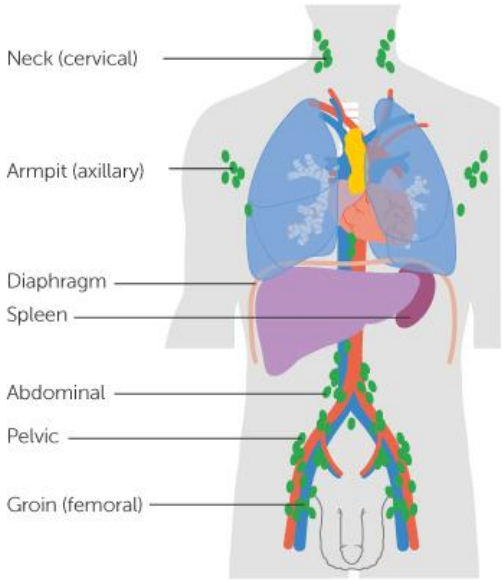

Figure 8: Hodgkin Lymphoma body locations, from cancer research UK

Studies suggest that 40% of HL cases, are associated with EBV. In these EBV infection exhibits a type II form of latency, being limited to the EBERs, EBNA1, LMP1, LMP2, and BARTs [140].

2.3. Post-transplant lymphoproliferative disease

Post-transplant lymphoproliferative disease (PTLD) is a well-recognized complication of both solid organ transplantation and allogeneic hematopoietic stem cell transplantation [13]. This terms is used to describe lymphoproliferations, that are not always monoclonal and whose morphologic features often differ from those of lymphomas [141].

PTLD are the second most frequent neoplasia in transplant patients, and its incidence varies depending on the organ transplanted, and ranges from 0.5% in adult kidney or liver recipients to more than 10% in lung, intestinal recipients or pediatric transplant patients (table 2) [142].

Table 2: Reported incidence of PTLD by organ system and recipient age

Organ	Recipient Age	1 year (%)	3 years (%)	5 years (%)	>5 years (%)
Kidney	Adult[a)]	0.46	0.87	1.18	ND
	Pediatric [a)]	1.73	2.45	ND	ND
Liver	Adult [a)]	1.1	3	4	4.7
	Pediatric [a)]	ND	ND	6	ND
Heart	Adult [b)]	0.67	ND	1.3	2.0
	Pediatric [b)]	1.7	ND	4.6	7.9
Lung	Adult [b)]	1.6	ND	2.1	5.6
	Pediatric [b)]	4.8	ND	11.1	10.3

*ND- Not Described; a) ISHLT 2008; b) French PTLD registry

Literature suggest that EBV infection has a major pathogenic role in PTLDs, infecting 60%-80% of PTLD patients, including 100% of early-onset PTLD patients [28]. Up to 2/3 of PTLD cases are associated with EBV infection of B cells, either because of reactivation of the virus or from primary EBV infection [13, 102]. Recipients who are EBV seronegative have a higher risk of developing EBV-induced lymphoma, particularly pediatric patients [143, 144]. Literature suggest that EBV infection has a major pathogenic role in PTLDs, infecting 60%-80% of PTLD patients, including 100% of early-onset PTLD patients [28].

In most cases of PTLD, tumour cells express the latency III pattern, with some cells undergoing lytic replication [145]. However, there is variability between individuals once latency type I and II patterns have also been detected in PTLD biopsies. In these cases additional genetic or epigenetic changes are probably required for tumour outgrowth [146, 147].

2.4. Nasopharyngeal Carcinoma

NPC is a rare type of head and neck cancer that affects the upper part of oropharynx, the nasopharynx (Figure 9) [139]. NPC has been reported in almost all parts of the world, however, most cases are found in South East Asia, Southern China, North Africa and in the Eskimo population of Alaska (Figure 10) [148-150]. In 2012 it was reported 87 000 new cases of NPC at a global level, corresponding to 0.6% of all cases of cancer [138]. Because the nasopharynx is a deep-seated structure and the nasal and aural symptoms are nonspecific, a large portion of patients with NPC are only diagnosed when the tumour has reached advanced stages, III and IV (Appendix II) [139].

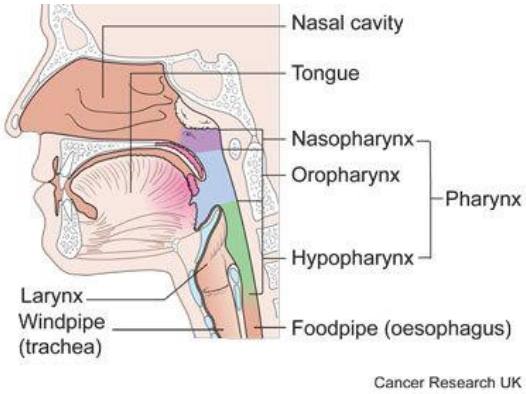

Figure 9: Nasopharynx location from cancer research UK

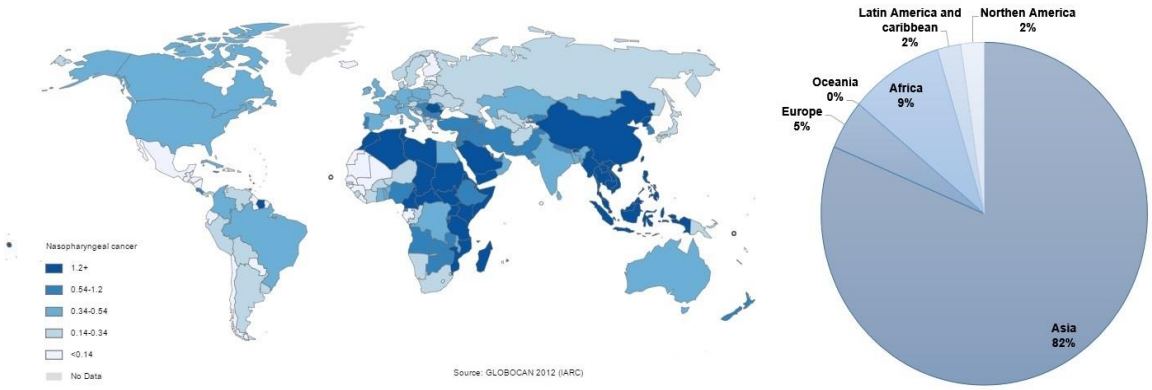

Figure 10: Nasopharyngeal carcinoma incidence worldwide, both sexes, all ages, Globocan 2012

Taking into account 2003 WHO classification NPC can be divided in three main types: keratinizing squamous cell carcinoma, non-keratinizing carcinoma, which can be divided diferentiated and undifferentiated, finally in basaloid squamous cell carcinoma respectively [151, 152]. NPC as several risk factors, such as EBV infection and classic head and neck aetiological factors including alcohol and tobacco [153].

Even though here is no doubt that NPC has a definite association with EBV, it is still not clear the specific pathogenic mechanism by which EBV causes NPC. A gap in the explanation of the EBV pathogenesis in the tumour is the fact that mature nasopharyngeal cells are not usually infected with EBV, though tumours have been shown to be infected before transformation [154]. It has been shown that the immature epithelial cells carry CD21 and can be infected by the virus. It is therefore postulated that EBV infects nasopharyngeal cells that have been stimulated by other environmental factors [155-157].

It appears that latency gene expression in NPC is intermediate between what is seen in latency I and latency II. The expression of EBNA1 and the EBERs are present in all EBV-positive NPC cases [158, 159]. It also appears that LMP2A can be detected in about 50% of NPC [160, 161]. In other hand, LMP1 is identified readily in only 35% of cases. LMP1 has been identified in all pre invasive lesions, suggesting that its expression is necessary in early lesions but may not be as essential in established carcinomas [162].

2.5. Gastric Carcinoma

GC is the sixth most common malignancy in both sexes worldwide with 952.000 new cases estimated in 2012, corresponding to 6.8% of all cases of cancer [138]. The incidence rates are almost twice higher in male (8.5%) than in female (4.8%) with about 631.000 and 320.000 new cases per year, respectively [138]. More than 70% of total cases occur in developing regions like Eastern Asia, Eastern Europe and Latin America. In contrast, the lowest incidence rates are observed in United States, Australia and some North European countries (figure 11) [138].

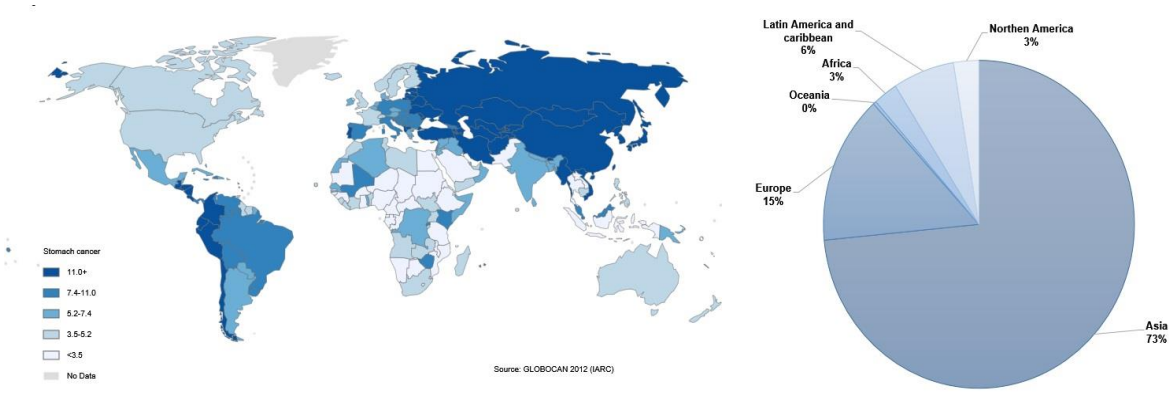

Figure 11: Gastric cancer incidence worldwide, both sexes, all ages, Globocan 2012

Adenocarcinomas are about 95% of all GC and the other cases include: Squamous cells cancers; lymphoma; gastrointestinal stromal tumours (GIST); and neuroendocrine tumours (NET). Histologically, GC can be classified by two different classifications, World Health Organization (WHO) and Lauren (Table 3) [163, 164]

Table 3: Comparison of Lauren's and WHO classification systems [153,154]

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

Anatomically, GC is divided into proximal and distal tumours depending on their localization of stomach. Proximal tumours are found in cardia region whereas distal carcinomas are often located in the antrum/pyloric region (Figure 12).

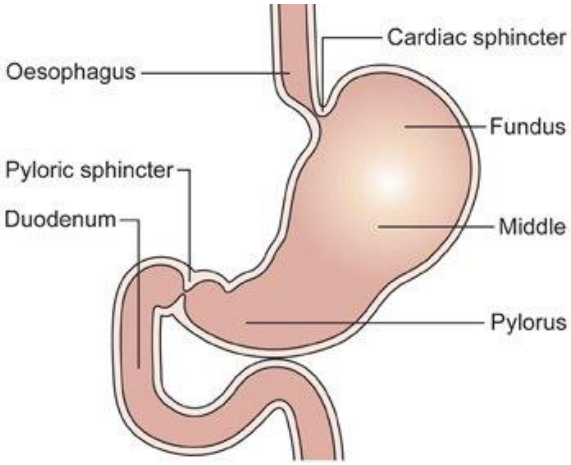

Figure 12: Illustrative scheme of the stomach regions from cancer research UK

Gastric carcinogenesis is a multistep process, where different factors are involved, including EBV [165]. EBV-positive GC is present in 10% of all GCs [166, 167]. EBV infection has been detected in different types of gastric cancer: gastric adenocarcinomas and lymphoepithelioma-like gastric carcinomas (LELC) [168]. LELC represents about 4% of all gastric carcinomas and more than approximately 80% of cases have EBV-infected cells [168]. Actually, scientific community is still debating the role of EBV infection on gastric carcinoma, and recent studies have suggested that EBVaGC is a distinct subgroup of gastric cancers with specific molecular features [169, 170]. The evidence for involvement of EBV in gastric carcinoma is based on the specific presence of viral gene products such as EBERs in tumour cells but not in the surrounding non-neoplastic epithelium [171], the presence of clonal EBV in tumour cells [172] and elevated EBV antibodies in prediagnostic sera of patients with EBV-associated gastric carcinoma [173].

EBVaGC has typically a latency I pattern in which only EBNA1, EBERs and BARTs are expressed. Even though, in some cases it can also express a small amount of LMP2A, while LMP1 is rarely expressed in EBVaGC. In these cases, the genes expressed do not correspond to any classification of latency patterns described in the literature [167].

3. EBV Latency

EBV infection can adopt mainly four different programs of latency, named type 0, I, II, and III latency (table 4) (Figure 13) [106]. The different latency programs adopted, are dependent on several cell-specific factors such as epigenetic events, which include DNA methylation, histone modifications and chromatin organization. EBV latency within B cells usually progresses from Latency III to Latency II to Latency I. [174, 175]. Moreover, latency programs have also been shown to correlate with the type of EBV associated disease, and they impose different phenotypic and functional properties on the viral genome carrying B cell [176, 177].

Table 4: EBV gene latency programmes

Latency type	Transcripts							EBV-associated disease
	EBERS	EBNA1	EBNA2	EBNA3 FAMILY	EBNALP	LMP1	LMP2	
Latency 0	+	-	-	-	-	-	-	Memory B cells in healthy individuals
Latency I	+	+	-	-	-	-	-	Burkitt lymphoma, Gastric Carcinoma
Latency II	+	+	-	-	-	+	+	Hodgkin lymphoma, Nasopharyngeal Carcinoma
Latency III	+	+	+	+	+	+	+	Post-transplant lymphoproliferative disease

3.1. Latency 0

Although EBV-infected proliferating B cells are eliminated by cytotoxic T cells in vivo, the virus is not totally eliminated from the body. Instead, EBV is able to establish in vivo latency, called latency 0 in memory B cells [2]. The switch from proliferation to latency 0 in memory B cells is still poorly understood. In this type of latency no viral gene expression is found [178].

3.2. Latency I

In type I latency only EBNA1 is expressed from the Qp promoter, while all the other latency-associated promoters remain silent [137]. Although the mechanism behind the switch in promoters is not completely understood, the repression of the Cp promoter during this period seems to be related to DNA methylation [174]. During type I latency, transcription of the viral transcripts EBERs and BARTs has also been described [30]. This type of latency is mainly associated with BL and GC.

3.3. Latency II

Another main type of latency, type II, is characterized by the expression of the following viral genes: EBNA1, LMP1 and LMP2, EBERs and BARTs. This type of expression was first detected in NPC in epithelial cells [179]. This type of latency can also be found in EBV positive Reed/Sternberg cells of HL of B cell origin [140, 180].

3.4. Latency III

Type latency III expresses all latent genes and can be found in PTLD. The EBV's role in cell growth is most evident in latency III, as EBNA2, EBNALP, EBNA3A and EBNA3C coordinately upregulate cMyc expression and cell proliferation, and EBV LMP1 enhances cell survival [76, 84, 181-184].

3.5. Other types of latency

Additionally to these thoroughly studied three latency programs, other types of EBV latency have also been described such as, a pattern which is found in some gastric and nasopharyngeal carcinomas in which EBNA1 and LMP2A are the only genes detected. This pattern is is described by the majority of authors as latency II-like, however, in some studies focused on only NPC the denomination latency IIb is also found [167, 185-189]. In a group of BL tumours, another pattern of latent gene expression has been described in which the expression of EBNA1, -3A, -3B, -3C and truncated EBNALP is observed, but not the expression of EBNA2 or LMPs. Since this latency program was found to be associated with the exclusive transcription from the Wp promoter, it was therefore called "Wp-restricted latency"[137, 190]. Another type of latency named latency IIb which lacks LMP1 but expresses EBNA2, was first seen in B-Chronic Lymphocytic Leukemia (B-CLL) cells infected with EBV in vitro [191].

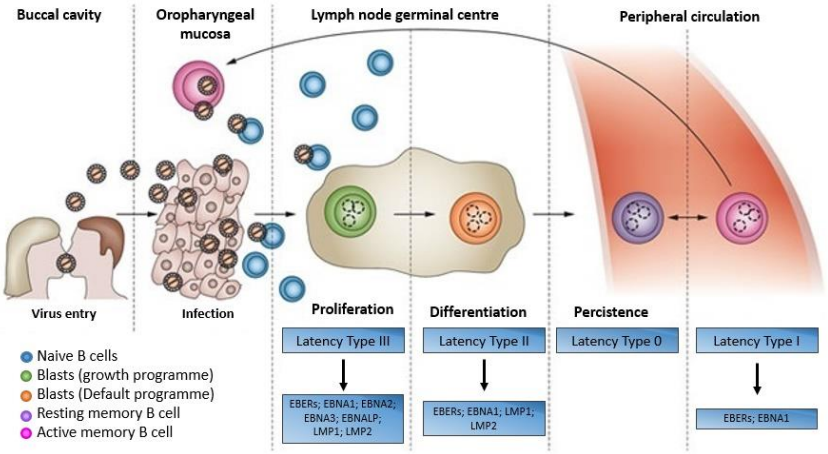

Figure 13: EBV cycle and latent states (Thorley-Lawson, 2008)

II. AIMS OF THE STUDY

Although there are many studies in this field, it is still not clear the latency pattern present in several malignancies, especially in gastric carcinoma. Therefore, it is necessary to clarify and understand which EBV proteins are expressed, in order to serve as a starting point for carcinogenesis studies.

The aim of this study is to characterize EBV latency in NPC and GC evaluating the expression of EBV proteins in different tumors' and try to establish a clinical correlation between the viral latency and the malignances.

III. MATERIAL AND METHODS

1. Population and Type of study

A retrospective cross-sectional study was performed using a total of 32 patients, with confirmed EBV-associated NPC and with EBVaGC attended at Portuguese Oncology Institute of Porto (IPO Porto).

Inclusion criteria: patients with histological confirmation of EBV associated cancer with representative tumour blocks for adequate evaluation of EBV presence. All tumours were submitted to histological examination by an experimented pathologist from our institution and classified according to the guidelines for each type of cancer.

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

The group of NPC cases, included 22 cases that were diagnosed between 2010 and 2014, and it consisted of 16 males and 6 females with median age of 55 years old (Table 5).

Table 5: Characterization of Nasopharingeal carcinoma cases

Variable	
Gender	n(%)
Male	17 (73.9)
Female	6 (26.1)
Age	
Median	55 ± 14,7
Minimum	20
Maximum	77
Histology (WHO 2003)	n(%)
Undifferentiated non-keratinizing carcinoma	23 (100)

The group of EBVaGC cases, included 9 cases that were diagnosed in 2011, and it consisted of 7 males and 2 females with median age of 69 years old (Table 6).

Table 6: Characterization of gastric carcinoma cases

Variable	
Gender	n(%)
Male	7 (77.8)
Female	2 (22.2)
Age	
Median	69 ± 10,994
Minimum	52
Maximum	82
Surgical Procedure	n(%)
Subtotal Gastrectomy	2 (22.2)
Total Gastrectomy	7 (78.8)
Histology	n(%)
WHO 2010	
Mixed Adenocarcinoma	2 (22.2)
Tubular Adenocarcinoma	5 (55.6)
Carcinoma with lymphoid stroma	2 (22.2)
Lauren (1965)	
Intestinal types	5 (55.6)
Indeterminate types	2 (22.2)
lymphoepithelioma-like carcinoma	2 (22.2)
Invasion pattern	n(%)
Expansive	6 (33.3)
Infiltrative	3 (67.7)

2. Sample collection and processing

Tumour tissues were collected from the institution archives and histological sections (3 µm slides) were obtained from formalin-fixed paraffin-embedded (FFPE) tissue blocks.

3. EBV detection (EBER-ISH)

Tissue samples were dewaxed in xylene for 2 x 3 minutes. After dewaxing, slides were sequentially hydrated in 100% v/v ethanol for 2 x 3 minutes, 96% v/v ethanol for 3 minutes and distilled water for 2 x 3 minutes. Proteolytic treatment was performed by addition of 10

mM proteinase K and incubation at 37ºC during 30 minutes. Finished the incubation time, endogenous peroxidase activity was blocked by incubating the slides in 3% hydrogen peroxide (H_2O_2) for 10 minutes at room temperature, then solution the slides were immersed in distilled water for 2 x 3 minutes and then dehydrated in 96% v/v ethanol followed 100% v/v ethanol for 3 minutes to facilitate air drying.

Epstein-Barr virus was identified by in situ hybridization (ISH) for detection of EBV-encoded small RNA (EBER). Hybridization results in duplex formation of sequence present in EBV infected cells (EBERs) and specific probe. The *Bond™ Ready-to-use ISH EBER Probe* (Leica, Newcastle upon Tyne, UK) was used with a volume of 20 µl for each slide. Slides were covered with coverslip, and then incubated at 37ºC for 2 hours. Nonspecific antibody binding was block using *UltraVision Large Volume Detection System Anti-Polyvalent, HRP* (THERMO SCIENTIFIC, Fremont, USA). It was incubated for 10 minutes at room temperature and washing was performed with TBS, 0.1% v/v Trinton X-100 (TBS-T) 2x 5 minutes.

EBERs detection was performed *with Bond™ Anti-Fluorescein Antibody* (Leica, Newcastle upon Tyne, UK) diluted 1:150 in TBS, 3% m/v BSA, 0.1% v/v Trinton X-100 with incubation at room temperature for 30 minutes. After washing 2 x 3 minutes with TBS, the revelation of hybrids was performed with the *UltraVision Large Volume Detection System Anti-Polyvalent, HRP* (THERMO SCIENTIFIC, Fremont, USA). Briefly, the *Biotinylated Goat Anti-Polyvalent Antibody* (THERMO SCIENTIFIC, Fremont, USA) was added at room temperature for 10 minutes, washed with TBS-T 2 x 5 minutes followed by the addition of *Streptavidin Peroxidase* (THERMO SCIENTIFIC, Fremont, USA) with incubation for 10 minutes at room temperature. Streptavidin shows high affinity with several secondary antibody-conjugated biotin molecules providing a good revelation signal. Detection of hybrids is achieved by enzymatic reaction using a specific substrate to peroxidase. *ImmPACTTM DAB, Peroxidase Substrate* (VECTOR, Burlingame, CA USA) was used during 4 minutes at room temperature and diluted 3:100. The final washing was performed with distillated water 2 x 5 minutes.

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

4. EBV proteins expression analysis

Epstein-Barr virus latent proteins were identified by immunohistochemistry (IHC) technique, which detects them in FFPE tissue, blocks using specific antibodies for each protein (table 7). IHC is a method for localizing specific antigens in FFPE tissues based on antigen–antibody interaction. The technique is widely used in dermatologic diagnostics and research, and its applications continue to be extended because of its ease of use, reliability, and versatility. In IHC an antigen–antibody construct is visualized through light microscopy by means of a colour signal.

Table 7: Antibodies and conditions used for the detection of different EBV proteins

Protein	Primary Antibody	Dilution	Incubation	Positive expression in carcinoma cells
LMP1	NCL-EBV-CS1-4, Leica, Newcastle upon Tyne, UK	1:100	3h, room temperature	Cytoplasm
LMP2A	15F9, THERMO SCIENTIFIC, Fremont, USA	1:250	Overnight, 4ºC	Cytoplasm and membrane

Tissue samples were dewaxed in xylene for 2 x 4 minutes. After dewaxing, slides were sequentially hydrated in 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. Antigen retrieval was performed using a heat induced epitope retrieval method, where the slides were submersed a citrate-based antigen unmasking solution (VECTOR, Burlingame, CA 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 20 minutes and then washed in Tris-buffer saline containing 0.02% Tween 20 (TBS-T), and the endogenous peroxidase was blocked with 3% hydrogen peroxide (H_2O_2) for 10 minutes. Subsequently, the slides were washed in TBS-T 2x 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 with the specific EBV latent protein antibodies (the specific conditions of each antibody are described in table 6). After being rinsed in TBS-T, Biotinylated Goat Anti-Polyvalent Antibody (THERMO SCIENTIFIC, Fremont, USA) was added and incubated in a humid chamber at room temperature for 10 minutes. The next step was washing with TBS-T 2 x 5 minutes following the addition of Streptavidin Peroxidase (THERMO SCIENTIFIC, Fremont, USA) with

incubation for 10 minutes at room temperature. Detection of hybrids is achieved by enzymatic reaction using a specific substrate to peroxidase, 3, 3'-diaminobenzidine (DAB) ImmPACTTM DAB (VECTOR, Burlingame, CA USA) was used during 4 minutes at room temperature and diluted 3:100. The final washing was performed with water 2 x 5 minutes.

Mayer's hemalum solution (Millipore, Darmstadt, Germany) was used as counterstain for 10-30 seconds, depending of dye's use. After coloration, slides were washed in running water for 5 minutes and the following step was sequential dehydration in 70% v/v ethanol for 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).

5. Quality control

Positive and negative tissues controls were used in order to ensure the quality of the protocol. As a positive control for EBV proteins, PTLD FFPE tissue samples were used, once this malignancy expresses all EBV proteins. As for negative control, it was used GC or NPC FFPE tissue samples that lacked the specific antigen.

6. Data analysis

The clinicopathological characteristics of the tumour were compared to the expression of EBV latency proteins and consequently to the EBV latency pattern in GC and NPC pacients.

IV. RESULTS

1. EBER-ISH

In all 9 cases of GC and 23 cases of NPC included in this study, a reconfirmation of the presence of EBV was preformed using the EBER-ISH technique. Of the 23 EBV positive NPC cases, a case has shown to have insufficient tumour tissue for performing the remaining tests and therefore, this case is going to be excluded from other tests, in this manner NPC cases were reduced to 22. In figure 14 it is represented the results of EBER-ISH in tumor samples.

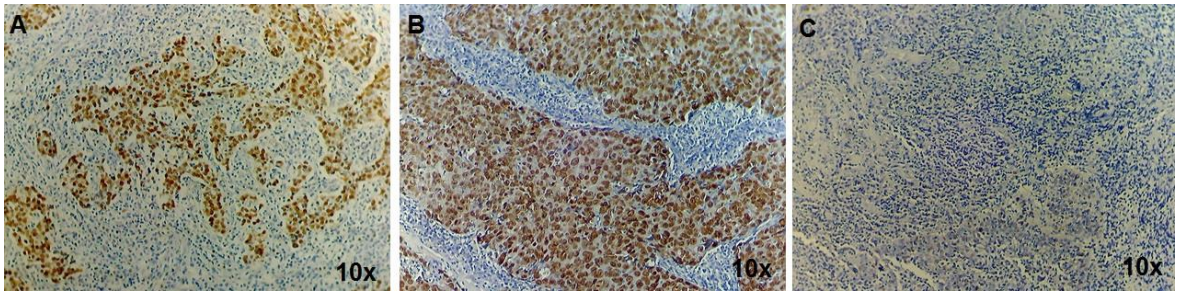

Figure 14: EBER-ISH Results **A)** GC EBV positive; **B)** NPC EBV positive; **C)** Negative control

2. Protein expression

2.1. LMP1

None of the cases of GC showed to be positive for LMP1. As for NPC, 21 (95.5%) of the examined cases have showed to be positive for LMP1 (Figure 15). In figure 16 it is represented LPM1 immunohistochemestry results in tumour samples.

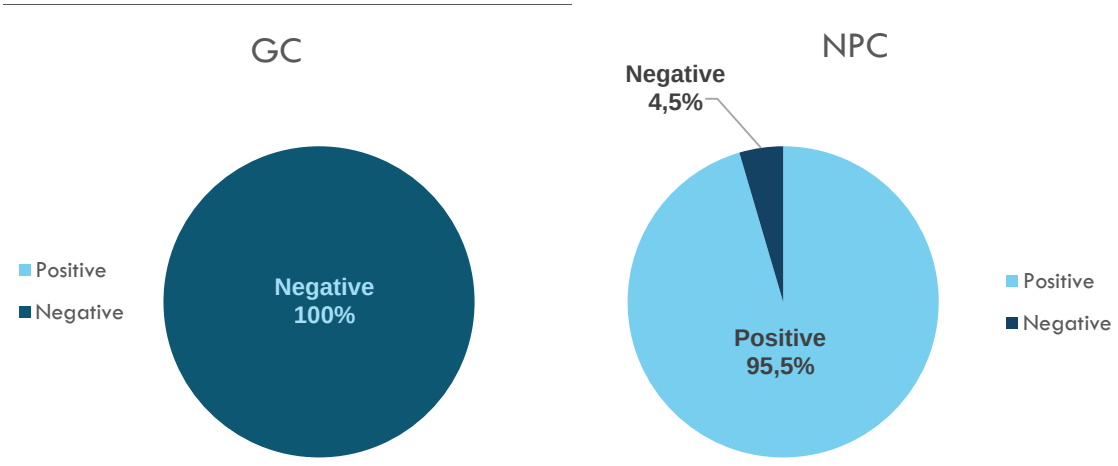

Figure 15: LMP1 results

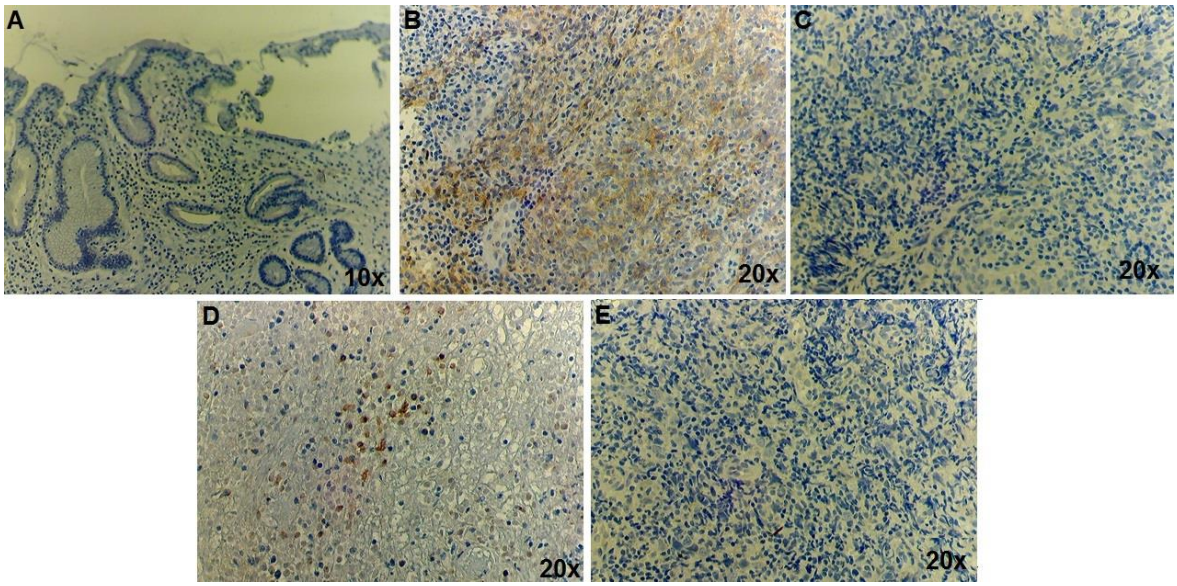

Figure 16: IHC LMP1 results **A)** GC case negative for LMP1; **B)** NPC case positive for LMP1; **C)** NPC case negative for LMP1; **D)** Positive Control; **E)** Negative control

2.2. LMP2A

Of all cases of GC, only 4 proved to be positive for LMP2a. As for NPC, all 22 cases examined proved to be positive for LMP2a (Figure 17). In figure 18 it is represented LPM1 immunohistochemestry results in tumour samples.

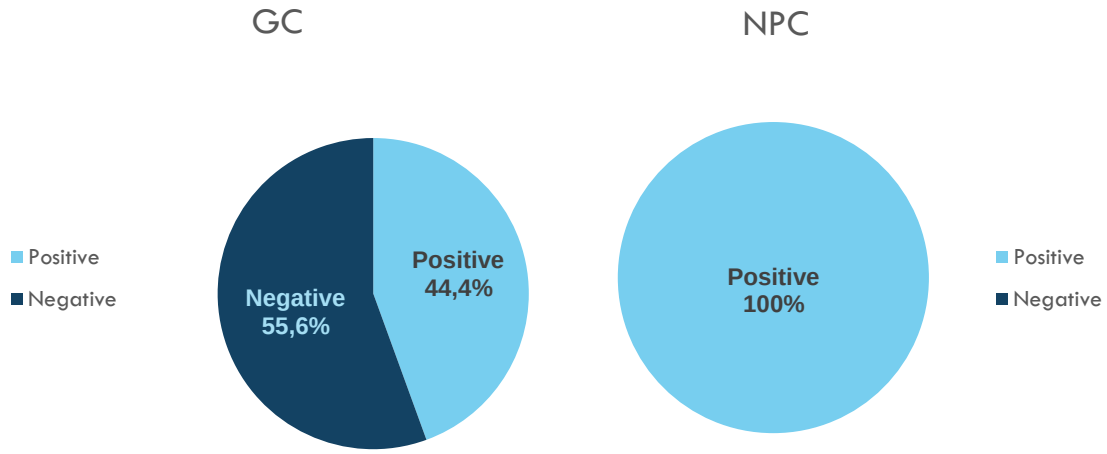

Figure 17: LMP2a results

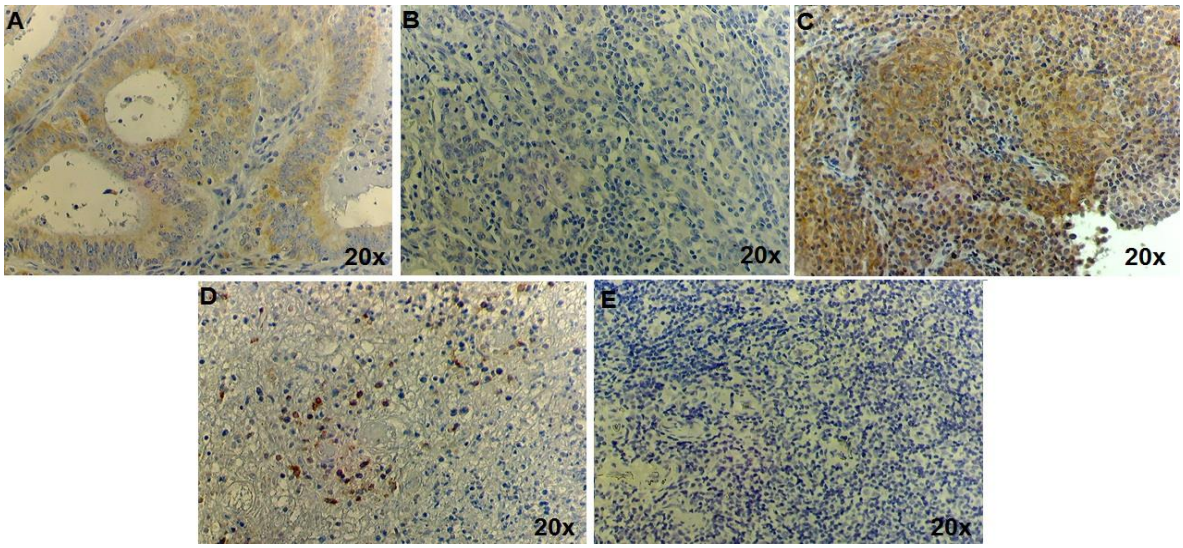

Figure 18: IHC LMP2a results **A)** GC case positive for LMP2a; **B)** GC case negative for LMP2a; **C)** NPC case positive for LMP2a; **D)** Positive control; **E)** Negative control

3. EBV latency

Assuming that EBNA1 is expressed in all EBV associated tumours, it was given to each of the cases used in this study an EBV latency pattern based on the expression of LMP1 and LMP2a.

3.1. NPC

Table 8 describes the principal baseline characteristics of each NPC case used in this study, including sex, age, histological type and expressed proteins. The majority of cases, 21 (95.5%), had the expression of both LMP1 and LMP2a, which corresponds to a latency II pattern. Only 1 case (4.5%) expressed a distinct latency pattern similar to latency II, but without the expression of LMP1. This pattern is going to be referred as “latency II-like”.

3.2. EBVaGC

Table 9 describes the principal baseline characteristics of each EBVaGC case used in this study, including sex, age, tumour location, histological type and expressed proteins. From all 9 cases of GC, 5 (55.6%) had no expression of LMP1 and LMP2a. In these, a latency I pattern is observed. The remaining 4 cases, had the expression of LMP2a, but not LMP1. These cases presented a “latency II-like” pattern.

Table 8: Description of nasopharyngeal cancer cases

ID	Gender	Age	Histological Type (WHO 2003)	EBER-ISH	LMP1	LMP2a	Latency Pattern
1	F	44	Undifferentiated non-keratinizing carcinoma	+	+	+	II
2	F	76	Undifferentiated	+	+	+	II
3	F	54	non-keratinizing carcinoma	+	+	+	II
4	M	45	Undifferentiated	+	+	+	II
5	M	61	non-keratinizing carcinoma	+	+	+	II
6	M	20	Undifferentiated	+	+	+	II
7	M	51	non-keratinizing carcinoma	+	+	+	II
8	M	74	Undifferentiated	+	+	+	II
9	M	58	non-keratinizing carcinoma	+	+	+	II
10	F	56	Undifferentiated	+	+	+	II
11	M	34	Undifferentiated	+	+	+	II
12	M	53	non-keratinizing carcinoma	+	+	+	II
13	M	66	Undifferentiated	+	+	+	II
14	M	57	non-keratinizing carcinoma	+	+	+	II
15	M	71	Undifferentiated	+	+	+	II
16	M	41	non-keratinizing carcinoma	+	+	+	II
17	M	71	Undifferentiated	+	+	+	II
18	M	77	non-keratinizing carcinoma	+	+	+	II
19	M	57	Undifferentiated	+	+	+	II
20	F	35	non-keratinizing carcinoma	+	+	+	II
21	M	54	Undifferentiated	+	+	+	II
22	M	64	non-keratinizing carcinoma	+	-	+	II like

ND – Not Determinated

Table 9: Description of gastric cancer cases

ID	gender	Age	Localization	Histological Type (WHO 2010)	EBER-ISH	LMP1	LMP2a	Latency Pattern
1	M	52	Cardia, Fundus and Cardia/Fundus	Lymphoepithelioma	+	-	-	I
2	M	75	Corpus	Tubular adenocarcinoma	+	-	-	I
3	M	80	corpus/antrum	Tubular adenocarcinoma	+	-	-	I
4	F	68	Corpus	Lymphoepithelioma	+	-	-	I
5	M	80	Corpus	Tubular adenocarcinoma	+	-	-	I
6	M	82	Cardia, Fundus and Cardia/Fundus	Tubular adenocarcinoma	+	-	-	II like
7	M	55	corpus/antrum	Mixed adenocarcinoma	+	-	-	II like
8	M	64	Corpus	Tubular adenocarcinoma	+	-	+	II like
9	F	66	Antrum, Pylorus e Antrum/Pylorus	Mixed adenocarcinoma	+	-	+	II like

V. DISCUSSION

EBV is a human cancer-associated virus that infects about 90% of the global population, without causing major symptoms in the majority of lifelong carriers [29, 192]. EBV is currently associated with many lymphoid and epithelial malignancies in both immunocompetent and immunocompromised individuals [52]. Even though the association of this virus with lymphoid malignancies is clear and relatively well understood, the same is not true for epithelial ones [52, 192]. Lately, there has been an increasing interest on the EBV-associated epithelial cancers that represent 80% of all EBV-associated malignancies [193]. Among these, NPC and EBVaGCs are the most common, with 78 000 and 84 000 new cases, respectively, reported annually worldwide [193].

According to literature, EBV infection can adopt four different patterns of latency, named type 0, I, II, and III [106]. In order correctly assign a latency pattern it must be taken into account the expression of at least four latent proteins, EBNA1, EBNA2, LMP1 and LMP2a [174, 175]. These specific latency EBV-transcription programmes have been associated with many human tumours and arise from specific stages in the EBV life cycle [176, 177, 194]. Nevertheless, there are several studies showing controversial information regarding the differential expression of EBV in some malignancies, hence we performed a retrospective study, in order to analyze the expression of EBV latency proteins and consequently the EBV latency pattern in NPC and EBVaGC.

NPC pathogenesis is highly associated with EBV, with almost 100% of the undifferentiated types having evidence of EBV presence [195]. Literature suggests that NPC shows typically a Latency II pattern, were the expression of the EBERs and EBNA1 is accompanied by the expression of LMP1 and LMP2A/B [195], nevertheless some suggest that LMP1 expression is very variable, with only approximately 35% of cases being unequivocally positive for LMP1 [196]. Our results showed a higher percentage of LMP1 positivity (95.5%), which is in agreement with literature confirming the latency II pattern for the majority of our cases. Despite that, one case showed a pattern of viral protein expression similar to latency II presented in the other cases, but with no expression of LMP1 (latency II-like). Failure to detect the LMP1 in one case may reflect biological heterogeneity or may be due to limitations of the paraffin-embedded IHC method as has been reported by others [197, 198]. Some researchers showed that the invasion and metastasis of NPC is promoted by LMP1, and a few studies have also shown a worse prognosis and survival [199] [200, 201] even though others studies argued that LMP1 is not associated with NPC prognosis [202, 203]. Through our results, it was not possible to draw any conclusions regarding the role of LMP1 on the prognosis in NPC cases, once the only case that had no expression of LMP1 had very similar clinicopathological characteristics to many of the ones that expressed LMP1.

EBVaGC is a “new” entity that has been described as a distinct subtype that accounts for nearly 10% of gastric carcinomas with specific clinicopathological features, including predominance among males and a proximal location in the stomach. [204]. Since EBVAGC has been subject to controversial information regarding EBV association and protein expression, we have performed a systematic review on the expression of different latent EBV proteins described in literature. The literature review showed that, EBNA1 is expressed in 98%, while LMP2a is only expressed in 52% of cases, while there was no evidence of EBNA2 and LMP1 expression (Table 10). Hence, this information results in three different types of latency patterns (Table 11). Interestingly, the majority of the cases showed a pattern of viral protein expression, where only EBNA1 and LMP2a were expressed. This type of latency is a midterm between the latency I and latency II patterns, described as Latency II-like pattern. The second most frequent latency, present in 46% of cases was latency I, which is the type of latency manly attributed to EBVaCGs. Nevertheless, we have also observed one case showing a latency 0 pattern that is usually only seen in memory B cells.

Table 10: Frequency of EBV latent proteins observed in different studies

Study	n	EBNA1 n (%)	EBNA2 n (%)	LMP1 n (%)	LMP2A n (%)	LMP2B n (%)
Cheng N, *et al*. 2014 [189]	7	7 (100)	0 (0)	0 (0)	5 (71.4)	----
Lee JM. *et al*. 2011 [205]	4	4 (100)	0 (0)	0 (0)	1 (25.0)	----
Chen J. *et al*. 2011 [185]	8	7 (87.5)	0 (0)	0 (0)	5 (62.5)	----
Luo B, *et al*. 2005 [188]	11	11 (100)	0 (0)	0 (0)	4 (36.4)	0 (0)
Hoshikawa Y, *et al*. 2002 [186]	3	3 (100)	0 (0)	0 (0)	2 (66.7)	----
zur Hausen A, *et al*. 2000 [187]	10	10 (100)	0 (0)	0 (0)	6 (60.0)	----
Sugiura M, *et al.* 1996 [206]	7	7 (100)	0 (0)	0 (0)	3 (42.9)	0 (0)
TOTAL n(%)	50	49/50 (98.0)	0/50 (0)	0/50 (0)	26/50 (52.0)	0/19 (0)

Table 11: Frequency of EBV latency patterns observed in different studies

Study	n	Lat 0 n(%)	Lat I n(%)	Lat II n(%)	Lat III n(%)	Lat “II-like” n(%)
Cheng N, *et al*. 2014 [189]	7	-	2 (28.6)	-	-	5 (71.4)
Lee JM. *et al*. 2011 [205]	4	-	3 (75.0)	-	-	1 (25.0)
Chen J. *et al*. 2011 [185]	8	1 (12.5)	2 (25.0)	-	-	5 (62.5)
Luo B, *et al*. 2005 [188]	11	-	7 (63.6)	-	-	4 (36.4)
Hoshikawa Y, *et al*. 2002 [186]	3	-	1 (33.3)	-	-	2 (66.7)
zur Hausen A, *et al*. 2000 [187]	10	-	4 (40.0)	-	-	6 (60.0)
Sugiura M, *et al.* 1996 [206]	7	-	4 (57.1%)	-	-	3 (42.9%)
TOTAL n(%)	50	1/50 (2.0)	23/50 (46.0)	0/50 (0)	0/50 (0)	26/50 (52.0)

In our study, the results were congruent to those described from this literature review, since both of the most frequent latency patterns were also observed in our study. The only difference was that latency I, was the most frequent with 55.6% of cases, while latency II-like was observed in 44.4% of the cases. It is suggested by literature that, the lack of LMP1 expression in EBVaGC, indicates that LMP1 may not be necessary for maintaining the tumour malignant state, but it might participate in an earlier stage of the tumour development, and is down-regulated afterwards [206]. Alternatively, the lack of LMP1 may result of a clonal selection of LMP1-negative tumour cells by immunological pressure, once EBV-specific cytotoxic T cells are potentially directed against the viral latent proteins other than EBNA1 [206]. Recently, it was also demonstrated that LMP2A up-regulates surviving gene expression through the NF-kB pathway and that it up-regulates cellular DNA methyltransferase 1 through the phosphorylation of STAT3, causing promoter hypermethylation of a tumour suppressor gene, PTEN, in EBVaGC [185]. Since LMP2A has demonstrated to play important roles in the oncogenic processes of EBVaGC, and it is expressed in about half of the cases, it is crucial to not be restricted to three types of latency patterns that are described and accepted by literature, and thereby new ones, with different tyes of protein expression, should be taken into consideration.

Even though our results are important and this is the first study to describe latency patterns in our population, it would be important to complement this work with some extra information. For example, compare GC llymphoepithelioma with NPC undifferentiated non-keratinizing carcinoma, once they have similar histology, but it seems that they have different latency patter, for this it would be necessary to increase the number of cases of GC. It would be also important to complement our results with information from other proteins, such as EBNA1, that in this study is assumed to be expressed in very tumour, but it should be done in order to eliminate possible doubts of the existence of latency 0; EBNA2; and finally BARF1, once it is suggested by literature that it might act as a viral oncogene in the development of EBVaGC [187].

It is very important correctly identify all latency patterns and which carcinogenic processes are taking place in each of them, because once we know it, it is much easier to have an accurate prognosis and by consequence a proper treatment for each individual.

VI. CONCLUSION

EBV infection can adopt different patterns of latency, and these have a close correlation with the different types of EBV associated diseases. With this study it was possible to confirm that in our population, NPC is characterized by a latency II pattern, while EBVaGC shows a latency I pattern. We were also able to identify a novel, and yet not accepted, latency pattern associated mainly associated with EBVaGC, but also described in one case of NPC. We described that latency pattern as latency II-like, and it is characterized by the expression of EBNA1 and LMP2a.

This is the first study regarding latency patterns of NPC and EBVaGC in Portugal and further studies are required to confirm these evidences, especially with more cases and ideally from different regions of Portugal. It also important to increase the panel of proteins studied so we can correctly describe the different latency patterns, and in order to better understand the role of each protein in tumour carcinogenesis, functional assays would also be important.

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VIII. APPENDIX

Appendix I

Parecer CES IPO: 74REAV/2015
Assunto: Reavaliação de Estudo de Investigação **Epstein-Barr virus latency in different malignances**

Investigadora: **Cláudia Oliveira**
Data: 04 de Junho de 2015

PARECER

É parecer desta CES não existir impedimento de natureza ética ao desenvolvimento do referido estudo de investigação.

Dr. Artur Lima Bastos
(Presidente da CES – IPO Porto EPE)

Appendix II

Apendix II – Sistematic review flow Diagram