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The Extent of Genetic Diversity of Epstein-Barr Virus and its Geographic and Disease Patterns: A Need for Reappraisal

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1. Introduction

Epstein-Barr virus (EBV) is a lymphotrophic human gamma-1 herpesvirus transmitted primarily through saliva that infects over 90% of the world's population. EBV is implicated in several benign and malignant conditions. Typical to other gamma herpesviruses, EBV establishes latent infection in B lymphocytes. The virus persists lifelong in memory B cells as episomal (circular) DNA, undergoing episodic lytic replication in B cells and in epithelial cells, essential for spread from cell to cell and from host to host. EBV has a double-stranded DNA genome comprised of approximately 170-kilobases which encode more than 85 genes (Farrell, 2005; Rickinson and Kieff, 2007; Thompson and Kurzrock, 2004). During latent infection, EBV expresses a restricted set of genes, including two EBV-encoded RNAs (EBER-1 and EBER-2), six EBV nuclear antigens (EBNA-2, EBNA-2, EBNA-3A, −3B and −3C, leader protein (EBNA-LP), and three integral membrane proteins (LMP-1, LMP-2A and −2B) (Fig. 1). During lytic infection, a large number of structural genes and regulators are expressed, including immediate early gene BZLF-1 and its promoter Zp (Delecluse et al., 2007; Farrell, 2005; IARC, 1997; Rickinson and Kieff, 2007; Thompson and Kurzrock, 2004; Thorley-Lawson and Gross, 2004).

In developing countries, EBV typically occurs in early childhood and is asymptomatic; in developed countries, infection occurs in later childhood or young adulthood and can manifest as infectious mononucleosis (IM), which is self-limiting (Rickinson and Kieff, 2007). A small fraction of EBV-infected individuals develop malignancies, including Hodgkin lymphoma (HL), nasopharyngeal carcinoma (NPC), gastric adenocarcinoma, lymphoepithelioma-like carcinoma (LELC), nasal NK/T-cell lymphoma, and leiomyosarcoma (Hsu and Glaser, 2000).

Among individuals with primary immunodeficiencies (e.g, X-linked lymphoproliferative (XLP) syndrome), EBV infection frequently leads to fatal IM or malignant B cell lymphomas. Patients with acquired immunosuppression may also develop lymphoproliferative disorders. Transplant patients seronegative for EBV are at a markedly increased risk of post-transplant lymphoproliferative disease (PTLD) due to primary EBV infection (Cohen, 2003; Harrington et al., 1988; Purtilo, 1980; Purtilo, 1987). AIDS patients are at higher risk than the general population of developing both benign and malignant conditions, including oral hairy leukoplakia, immunoblastic lymphomas, Burkitt lymphomas, and Hodgkin lymphoma (Carbone, 2003; Carbone et al., 2009; Cohen, 2005).

A notable feature of EBV-associated malignancies is variation in incidence and the proportion of EBV-positive tumors in different geographic regions (Fig. 2). The incidence of NPC is higher in Southern China, Southeast Asia, and to a lesser extent the Maghrebi Arabic regions of North Africa and the Arctic, than in other regions of the world. However, the tight link between NPC and EBV is observed in all regions evaluated (Bray et al., 2008;Chang and Adami, 2006;Hsu and Glaser, 2000;IARC, 1997). Burkitt lymphoma (BL) occurs most commonly in equatorial Africa (BL in this region is recognized as the most common childhood tumor) than in other regions of the world. In contrast to NPC, the tight link between BL and EBV is only observed in Equatorial Africa, while in other regions only a fraction of BL cases are associated with EBV (Brady et al., 2008;Cardy et al., 2001;Chan et al., 1995;Gutierrez et al., 1992;Klumb et al., 2004;Mbulaiteye et al., 2009;Philip, 1985;Rochford et al., 2005). For HL, incidence is highest in Europe, in North America, and to a lesser extent in South America, and occurs less frequently in China or Africa. The fraction of HL cases that are associated with EBV have been shown to be high in Africa and S. America, but intermediate in China, N. America, and Europe (Dinand and Arya, 2006;Ferlay et al., 2004;Mueller and Grufferman, 2006;Weiss, 2000).

The reasons for the restricted geographic pattern of EBV-associated malignancies are poorly understood, but variation in human host genetic factors, environmental factors, or viral factors have been suspected. Substantial genetic sequence variation in EBV has been demonstrated among EBV isolates at multiple loci on the EBV genome, but the role of genetic variations has yet to be elucidated.

Here we review the literature on EBV genetic variation in healthy and diseased individuals from different geographical regions. First, we review the two main EBV genetic types (EBV type 1 and 2) and strains based on ebnotyping and restriction fragment length polymorphisms (RFLP). Second, we examine in more depth studies focused on DNA sequence variation within the LMP-1, EBNA-1 and BZLF-1 genes of EBV. Although understanding functional differences associated with genetic variations in EBV that affect disease risk will ultimately be important to fully understand the underlying molecular mechanisms for EBV-disease associations, these are not the topic of this review.

We summarize the state of the knowledge on EBV variation, identify gaps in our understanding, and suggest areas for further study. We hope our review will highlight the benefit of closer collaboration among virologists, molecular biologists, clinicians, epidemiologists, and others to untangle the mystery of how and why EBV, a ubiquitous virus, contributes to distinct malignancies in different populations.

2. EBV type 1 and 2

EBV is classified as EBV type 1 and 2, based on sequence variation in EBNA genes. Initial classification was based on differences in EBNA-2 identified in EBV isolates from lymphoblastoid cell lines (LCLs). EBV isolated from a North American IM case, B95-8 (GenBank: V01555.2), classified as a type 1, was found to have a slightly longer EBNA-2 reading frame than that of AG876, a type 2 virus isolated from a Burkitt lymphoma case in Central Africa (Dambaugh et al., 1984). Additional differences between type 1 and 2 have been reported, including mismatched base pairs in genes encoding EBNA3A, 3B and 3C, differing in 10%, 12%, and 19% of base pairs, respectively (Sample et al., 1990).

Previous studies examining the distribution of EBV subtypes indicated predominance of EBV type 1 strains (Zimber et al., 1986). More recent data, however, suggests that a notable proportion of individuals are infected with type 2 and that co-infection of both type 1 and 2 is also possible. Co-infection is more commonly detected in immunocompromised individuals, suggesting that superinfection with type 2 is likely acquired during immunocompromised states

or that immunity might differentially control the reactivation or persistence of EBV subtypes in an individual (Apolloni and Sculley, 1994; Yao et al., 1996b). The frequent detection of EBV type 2 in immunocompromised individuals is also reflected in the EBV subtypes associated with lymphomas in immunocompromised patients (Goldschmidts et al., 1992). The difference in the previous and recent results can be attributed to methodological changes in assessing EBV subtypes. Prior to the PCR-era, molecular epidemiological studies relied on EBV isolation by lymphocyte transformation, thereby introducing a selection bias towards transformation-efficient EBV subtypes (Rickinson et al., 1987). PCR has eliminated this bias through direct amplification and sequencing of EBV DNA.

While there is a greater appreciation that multiple EBV infections are common, EBV type 1 continues to be more frequently detected in Caucasian and Asian healthy individuals. Studies of Caucasian healthy populations, when combined, show that 74% of individuals tested are infected with type 1, 19% with type 2, and 7% are infected with both EBV types (Apolloni and Sculley, 1994; Correa et al., 2004; Klemenc et al., 2006; Sixbey et al., 1989; Trimeche et al., 2007). PCR studies of healthy Asian populations demonstrated an even stronger predominance of type 1: 85% of individuals tested are infected with type 1, 4% with type 2 and 11% with both types (Srivastava et al., 2000; Tiwawech et al., 2008; Zhou et al., 2001). Although the distribution of EBV type 1 and type 2 in other populations is less well understood, type 2 EBV was detected in 24% of individuals in Kenya, suggesting that this type might indeed be more prevalent in Africa (Young et al., 1987).

Although biological differences attributable to EBV types 1 and 2 provide rationale for classification of EBV into these two broad groups does, it is clear that these do not fully account for the natural diversity of EBV. First, intertypic recombinants, in which a type 1 EBNA-2 allele is paired with type 2 EBNA3A, −3B, and/or −3C alleles, have been found in both immunocompetent and immunocompromised populations in different geographic regions (Aguirre and Robertson, 1999; Burrows et al., 1996; Gorzer et al., 2006; Kim et al., 2006; Midgley et al., 2000; Yao et al., 1996a). Additionally, studies have shown that variations observed in the EBNA-2 and EBNA3A–C genes often do not correlate with variations observed in other regions of the EBV genome, including LMP-1 and EBNA-1 (Fassone et al., 2002; Gutierrez et al., 2000; Klemenc et al., 2006; Srivastava et al., 2000). Thus, a higher resolution of EBV strain classification based on sequence variation of multiple gene regions is likely to be informative.

3. RFLPs and Ebnotyping

Both restriction fragment length polymorphisms (RFLPs) and ebnotyping have been previously discussed in a review by Gratama and Ernberg (1995). RFLPs define variation due to loss or gain of restriction sites which are identified by comparing the size of EBV DNA fragment size of DNA digested by restriction enzymes. Ebnotyping involves assessing the variation in the molecular weight of EBNA-1, −2, −3a, −3b, and −3c proteins, related to the number of EBNA gene internal repeats or hydrophobicity/hydrophilicity, which may differ between strains (Gratama and Ernberg, 1995).

For example, one of the most extensively studied RFLP polymorphisms is the “f” variant that contains an extra BamHI site in the BamHI F region when compared with the F prototype (B95-8). Studies have reported a higher frequency of the “f” variant in NPC cases from Asia (142/209, 68%) compared with controls from this same region (14/49, 29%) (Abdel-Hamid et al., 1992; Lung and Chang, 1992; Lung et al., 1994; Lung et al., 1990; Lung et al., 1991; Tan et al., 2003; Wu et al., 1996). In contrast, the “f” variant is rarely observed in NPC (2.5% of 202 cases), NHL (0% of 31 cases), and healthy or nonmalignant controls (1.3% of 151 controls) from Europe, N. America, and Africa (Ayadi et al., 2007; Bouzid et al., 1994; Bouzid et al.,

1998; Henry et al., 2001; Higa et al., 2002; Khanim et al., 1996; Klemenc et al., 2006; Tamura et al., 1993). These findings suggest that the "f" variant is more common in Asia than other regions of the world. Results also suggest an association between the "f" variant and NPC risk, but the small number of individuals studied precludes any definitive conclusions. Another notable RFLP polymorphism, the XhoI loss, will be discussed in the next section due to its location within the LMP-1 gene.

Classification of EBV variants based on RFLP and ebnotyping have several limitations. First, RFLP-based tests would miss variation occurring outside of the restriction sites as well as miss single nucleotide substitutions that would not change fragment size. Similarly, ebnotyping-based tests are restricted to EBNA proteins as well as would miss changes that do not affect the overall size or polarity of the fragment being tested. Finally, ebnotyping is problematic because of its reliance on LCLs: there may be bias towards variants better able to transform LCLs and the in-vitro propagation of LCLs may introduce mutations that could be erroneously classified as real variants (Jenkins and Farrell, 1996; Munch, 1998; Walling et al., 1999).

4. DNA sequencing

DNA sequencing detects single base pair changes that permit the identification of a wider range of diversity. This review summarizes EBV genetic diversity identified in three gene regions: LMP-1, EBNA-1, and BZLF-1. These three regions are the focus of this review because they are the most frequently studied regions to date. In particular, LMP-1 has been studied extensively because it is thought to play a significant role in tumorigenesis; EBNA-1 has been the focus of previous evaluations because it is required for maintenance of EBV in its latent form and because it is expressed in all EBV-associated tumors; and BZLF-1 has been targeted for evaluation in the past because of the important role it plays in the switch from latent to lytic EBV infection.

4.1. LMP-1

LMP-1 consists of a 25 amino acid (AA) cytoplasmic amino-terminus (N-terminus), six predicted transmembrane domains (AA: 26–196), and a cytoplasmic carboxy-terminus (C-terminus) (AA: 197–386) (Fig. 1 & Fig. 3) (Baer et al., 1984;Fennewald et al., 1984). LMP-1 is an integral membrane protein that is expressed in NPC, HL, and nasal NK/T-cell lymphoma, but surprisingly absent in BL (Tao et al., 2006). The C-terminus interacts with cellular proteins through the C-Terminal Activation Region 1 (CTAR1) and CTAR2 and activates several signaling pathways, including the transcriptional nuclear factor-kB (NF-kB) whose activation is linked to the inhibition of apoptosis (Hatzivassiliou and Mosialos, 2002;Izumi and Kieff, 1997)

The two commonly reported LMP-1 gene polymorphisms include a 30 bp deletion in the C-terminus and the loss of restriction site XhoI in the N-terminus of the gene (Edwards et al., 1999; Nagamine et al., 2007; Saechan et al., 2006; Sung et al., 1998). The 30 bp deletion (168256–168287) causing the loss of 10 AA (343 to 352) was detected in EBV isolated from cell lines derived from NPC patients from Southern China (Miller et al., 1994). Hu et al. (1991) was the first to describe the loss of the XhoI restriction site from exon 1 of the LMP-1 gene in the CAO cell line derived from a Chinese NPC case (Hu et al., 1991). This change is caused by a single base substitution from G to T at the nucleotide position 169425. The 30 bp deletion and XhoI loss do not discriminate among variants defined by signature AA changes in the C-terminus of LMP-1 that are discussed in the next paragraph (Edwards et al., 1999).

Studies that have analyzed DNA-based variability in the C-terminus of LMP-1 have largely been evaluated based on AA changes rather than nucleotide changes. Therefore, we summarize findings from these studies at the AA level as well. At least two systems have been used to

classify LMP-1 C-terminus variants (Edwards et al., 1999; Walling et al., 1999). The first categorized variants into classes defined based on signature AA changes relative to the prototypic LMP-1 (B95-8) in the C-terminus (AA 189–377), and named these classes based on the geographic region from which the initial isolate was derived: Alaskan, China 1, China 2, China 3, Mediterranean + (Med +), Med -, and North Carolina (NC) (Table 1) (Edwards et al., 1999; Mainou and Raab-Traub, 2006; Miller et al., 1994; Sung et al., 1998). Using the Edwards classification system, phylogenetic tree analysis suggested that the China 1, Med and China 3 groups were closely related. The NC and Alaskan groups formed a second cluster with which the China 2 group was also related (Edwards et al., 1999). Subsequent to the initial report using this classification system, other studies (largely from Southeast Asian) have identified additional variants closely related to one or more of the 7 major LMP-1 C-terminus variant classes (Nguyen-Van et al., 2008; Saechan et al., 2006).

The second main classification system used to date characterized variants using a smaller number of AA changes in shorter segments on either side of the 33 bp repeat, while the LMP-1 variants described above were defined by changes across the entire span of the C-terminus region of LMP-1 (including the 33 bp repeat region - AA 250–298). Walling et al. (1999; 2003) identified 25 LMP-1 C-terminus variants including 14 variants with AA changes between AA 306 and 366 (Patterns 1, 2a, 2b, 2c, 3a, 3c, 4, 5, 6, 7, 8, 9) downstream of the 33 bp repeat region and 11 variants (Patterns A, B, C, D, E, F1, F2, F3, G, H) with changes between AA 209 and AA 245 or upstream of the 33 bp repeat region (Walling et al., 2003; Walling et al., 1999). The differences between the classification systems used by Edwards and Walling make comparisons across studies difficult at best and highlight the need for a consensus nomenclature so that future studies can be more directly compared.

The distributions of the 30 bp deletion, XhoI loss, and C-terminus sequence variants of LMP-1 based on the current literature are pooled in Table 2 and Table 3. These results must be interpreted cautiously due to the nonrandom sampling of individuals and the small number of individuals included in studies. It should be noted that studies published before 1995 were described in other reviews and were therefore not included here (Gratama et al., 1990;Jenkins and Farrell, 1996;Munch, 1998). Also, the geographic regions and health conditions included were those most commonly reported across studies. Finally, the varied types of specimens (e.g. blood, saliva, tissue) tested across studies were not considered when pooling results across studies.

The distribution of the 30 bp deletion by geography and disease is summarized in Table 2a. The prevalence of the 30 bp deletion among controls appears relatively higher in samples from China than in samples from other areas. Within geographic regions, the 30 bp deletion was relatively more frequently detected among cancer cases than among controls. However, the interpretation of these findings in the literature is limited, given the heterogeneity in study designs and the sparsity of data for many of the geographic and disease subgroups evaluated.

The distribution of XhoI loss by geography and disease is summarized in Table 2b. Although data from controls outside of Asia are limited, available data suggest that XhoI loss is more common in China than in other regions of the world. Within China and other Asian countries, XhoI loss was more frequently observed among cancer cases than controls. Additional studies are needed to fully evaluate geographic and disease patterns of XhoI loss.

The distribution of EBV variant classes defined based on AA differences in the C-terminus of LMP-1 across disease conditions and geographic regions is summarized in Table 3. Data from regions other than Asia are sparse. Within Asia, available data suggest that China1 is the most prevalent class. Additionally, the China1 variant group was slightly more frequently detected among NPC cases than controls in this region.

4.2. EBNA-1

EBNA-1 has 641 AA including a glycine-glycine-alanine (gly-gly-ala) repeat sequence thought to play a role in downregulating EBV mediated immune response by inhibiting antigen presentation (Fig. 1 & Fig. 4) (Levitskaya et al., 1995). EBNA-1 is the only latent protein that is consistently expressed in all EBV-positive malignancies including BL which express only EBNA-1 (Rowe et al., 1992). EBNA-1 is critical in maintaining EBV in the infected cells and facilitating episomal replication (Yates et al., 1984;Yates et al., 1996). In replicating latently infected EBV cells, EBNA-1 dictates equal partitioning of EBV genome, thus reducing the loss of EBV infected cells (Aiyar et al., 1998). EBNA-1 is also a transcriptional activator and activates expression of EBV transcripts via the latent C promoter (Sugden and Warren 1989).

EBNA-1 variants were first identified based on the C-terminus region of EBNA-1. Two broad EBV strains based on EBNA-1 were identified. The prototype (P) strain was similar to EBNA-1 from B95-8, differing from it by only 3 AA substitutions. The second strain differed from B95.8 by 15 AA substitutions and was therefore named the variant (V) strain. Because codon 487 was found to be a hot spot for variation, P and V subtypes were further classified based on the AA present at codon 487: P-alanine (P-ala), P-threonine (P-thr), V-proline (V-pro), V-leucine (V-leu), and V-valine (V-val) (Table 4). It is of note that V-alanine (V-ala), which contains the prototype amino acid (alanine) at residue 487, exhibits differences from the prototype in other codons, including codons 499, 500, 502, 517, 520, and 524. Subvariants of P-ala, P-thr, and V-val have more recently been characterized (listed in footnote to Table 4) (Sandvej et al., 2000;Wang et al., 2003).

In addition to changes in the C-terminus, EBNA-1 has variation in the N-terminus at codons 16, 24 and 27 (Gutierrez et al., 1997; Habeshaw et al., 1999). EBNA-1 N-terminus changes, though generally linked to variations in the C-terminus, have revealed additional variants that were not evident by evaluating the C-terminus alone (Gutierrez et al., 1997). The N-terminus changes reinforce the need to evaluate the EBV genome more comprehensively in order to characterize the full extent of EBV genetic heterogeneity. As an example of the degree of variability observed in EBNA-1 alone, 22 distinct DNA sequences (resulting in 19 different EBNA-1 protein sequences) based on N- and C-terminus base changes were identified in 26 healthy carriers and 17 IM patients from one study in Australia (Bell et al., 2008).

Table 5 summarizes the distribution of EBNA-1 sequence variants by disease condition and geography. In all geographic regions, P-ala or P-thr was detected either as a single strain or one of several strains in a large proportion of controls. Interestingly, V-val was detected in both cases and controls almost exclusively in China. Data from other regions and other disease conditions are sparse, precluding definitive conclusions regarding geographic and disease patterns of EBNA-1 variants.

4.3. BZLF-1

BZLF-1 consists of 3 exons: the first exon encodes a transactivating domain, the second encodes a strong basic domain that directs the ZEBRA to the nucleus, and the third exon encodes a domain thought to play a role in regulating the switch from latency to lytic infection (Fig. 1 & Fig. 5) (IARC, 1997). Antibodies for the product of the BZLF1 gene, transcription factor BamHI Z EBV Replication Activator (ZEBRA), have been frequently detected in NPC and BL cases, indicating an increased production of the protein (Joab et al., 1991;Tedeschi et al., 2007;Yoshizaki et al., 2000). ZEBRA (also known as Z, ZTA, EB1) activation triggers the virus to switch from latent to lytic infection. BZLF1 is a master regulator for expression of several early viral genes critical for productive replication of EBV and alone can activate the entire lytic cascade (Countryman and Miller, 1985;Rooney et al., 1989).

BZLF-1 variants have been defined using different regions of the gene, making comparisons across studies difficult (Chen et al., 1996a; Fassone et al., 2002; Grunewald et al., 1998; Ji et al., 2008; Sacaze et al., 2001). For example, in one study that compared the full BZLF-1 gene from 3 different cell lines, Packham et al. (1993) found that the BZLF-1 genes from the Akata and P3HR1 cell lines contained three copies of a 29 bp repeat (Fig. 5) in contrast to B95-8 which contained two copies of the repeat in the first intron (nucleotide position 102,647), an untranslated region of BZLF-1 (Packham et al., 1993). In another example, using the coding regions of BZLF-1 to define variations, Ji et al. (2008) identified five BZLF-1 variants based on the mRNA sequence changes when those were compared to the published sequence of BZLF-1 in B95-8 (Ji et al., 2008).

Based on the limited data on BZLF-1 variant distribution by geography and disease state (summarized in Table 6), there appear to be geographic differences in the distribution of variants defined based on the 29 bp repeat but no disease associations were apparent.

4.4. Other variants

The BZLF1 promoter, Zp, is a 220 bp element located immediately upstream of the BZLF1 coding sequence (Fig. 5) containing a variety of regulatory domains that control BZLF1 transcription in response to multiple signals (Shimizu and Takada, 1993). Several variants of Zp have been identified: Zp-P which resembles the B95-8 Zp sequence, Zp-V3, Zp-V4, and most recently Zp-PV (Gutierrez et al., 2002;Martini et al., 2007). Table 8 summarizes the distribution of Zp variants across geographic regions and health conditions (Gutierrez et al., 2002;Martini et al., 2007;Tong et al., 2003). Among controls studied, the prototype Zp-P was observed more commonly in the Americas and Europe than in China, where an almost even split was observed in the prevalence of the Zp-P and Zp-V3 variants. Zp-V3 was generally more commonly observed in cancer cases than in controls across geographic regions. Larger, systematic studies are needed before firm conclusions can be made regarding geographic and/or disease associations related to variants in Zp.

One of the first viral genes expressed after infection, the Epstein-Barr virus nuclear antigen-2 (EBNA2) plays an important role in B cell transformation. EBNA-2 initiates and maintains proliferation of transformed cells and prevents apoptosis of the transformed B cells. The structure of the EBNA-2 protein is characterized by a poly-proline (polyP) and poly-arginine-glycine (RG) stretch and 9 conserved regions throughout the gene region (Kempkes, 2005). A number of studies have identified heterogeneity in EBNA-2 of the type 1 virus (Aitken et al., 1994; Al-Homsi et al., 1998; Cohen et al., 1991; Schuster et al., 1996a; Schuster et al., 1996b; Sheng et al., 2004; Shim et al., 1998; Walling et al., 1994). Among the most notable nucleotide changes in EBNA-2 relative to B95-8, a triplet insertion of CTC (Leucine) at nucleotide 49136 was found in samples from German, US and African patients with a variety of non-malignant and malignant disorders (Aitken et al., 1994; Al-Homsi et al., 1998; Schuster et al., 1996a) and a 51 bp deletion at nucleotide 49102 was detected in LCLs from New Guinea as well as two German patients with fatal lymphoproliferative disease (Aitken et al., 1994; Schuster et al., 1996b). Additionally, the deletion of the entire EBNA-2 gene has been identified in a subset of African BL cell lines, but whether these recombined EBV genomes with gene-wide deletions/duplications occur in “normal” lymphocytes remains to be established (Kelly et al., 2002; Kelly et al., 2005). The findings for EBNA-2 are intriguing, but larger studies are needed to define sequence variation patterns and to evaluate associations with disease and geography.

5. Issues that should be considered when planning future studies to evaluate EBV genetic diversity and its geographic and disease patterns

5.1. Testing approach and variant classification

As summarized in the preceding sections, studies conducted to date have shown extensive diversity in EBV. Efforts that have targeted specific gene regions of the virus have identified substantial genetic heterogeneity in EBV and have demonstrated that as many as 80–100% of infected individuals carry multiple EBV variants (Bell et al., 2008; Bhatia et al., 1996; Edwards et al., 1999; Gutierrez et al., 2002; Gutierrez et al., 1997; Ji et al., 2008; Sitki-Green et al., 2003; Sitki-Green et al., 2002; Tierney et al., 2006; Walling et al., 2003; Walling et al., 1999).

Furthermore, the few studies that have evaluated diversity in more than a single region of the genome have suggested that there is incomplete linkage of genetic variation observed across these regions. As an example, Gutierrez et al. (2000) observed a larger number of unique variants when variability across EBNA-1, EBNA-2, and LMP-1 was examined concurrently than when any one of these regions was evaluated in isolation (Gutierrez et al., 2000). Thus, the full extent of EBV heterogeneity is likely to be greater than currently recognized.

Studies conducted to date have focused on different regions of EBV, making comparisons across studies difficult and limiting our ability to define the full spectrum of diversity existent within the EBV genome. Even within studies that have focused on the same gene regions, different approaches have often been used to group EBV variants, further complicating comparisons across studies. A more comprehensive exploration of EBV diversity across its entire genome might allow for the development of a consensus classification system of EBV variants into variant classes that could guide subsequent studies aimed at understanding EBV geographic and disease associations. McGeoch and Gatherer (2007) recently made genome-wide comparisons of nucleotide sequences among 3 different strains, B95-8, GD1 and AG876A (McGeoch and Gatherer, 2007). Such efforts will pave the way for future, larger studies aiming to assess EBV variation more comprehensively.

5.2. Study populations and specimen types

Studies evaluating EBV genetic diversity have often been small in size and have relied on convenience sampling of cases and controls, making estimates of the distribution of EBV variants by geography and disease status unstable and of questionable generalizability. For example, the majority of studies conducted to date have focused on China and on NPC with limited data available from other geographic regions or disease states. To address these limitations, future studies should use careful sampling techniques to ensure that the distribution of EBV variants observed in those studies is reflective of the distribution in the broader populations of interests. Studies should be large enough to provide robust epidemiologic estimates of EBV variant distribution and should include study groups representing various geographic regions and disease states (including healthy controls) to allow for a more comprehensive and systematic assessment of the distribution of EBV by geography and the possible link between specific EBV variants and disease.

The biological specimens in which EBV is detected need to be considered carefully in future studies. The wide variety of specimen types used in studies to date have included malignant and nonmalignant tissue, peripheral blood lymphocytes, and throat washings. Different EBV variants have been found in different specimen types from the same individual with indications that some variants preferentially infect certain sites or compartments (Chen et al., 1996a; Edwards et al., 2004; Gutierrez et al., 1997; Sacaze et al., 2001; Triantos et al., 1998; Walling et al., 2003). For example, in a study of EBNA-1 carboxyl sequence variants in matched blood

and saliva samples of 21 immunocompetent carriers, V-pro was detected in their peripheral blood lymphocytes only while V-val was detected only in their saliva (Gutierrez et al., 1997). These observations may reflect the adaptation of EBV to particular niches, resulting in the preponderance of some variants in certain compartments. Therefore, future studies should be mindful of the specimens used for testing, and at a minimum should ensure that the same specimen type is used for the case and control groups compared. More extensive evaluation of EBV variants at multiple sites within individuals is greatly needed to clarify differences in patterns by site.

6. Conclusions

Why EBV is associated with different malignancies in different geographic regions remains a mystery and may be related in part to EBV genotypic variability through specific disease and geographic associations. We have summarized the available literature on sequence variation in EBV genes including LMP-1, EBNA-1, and BZLF-1. Given the limitations of current studies, definitive statements regarding the link between EBV genotypes, disease and geography are not possible. We conclude that the true extent of EBV genetic diversity is likely to be greater than currently recognized. Future studies conducted in carefully selected populations, sufficiently powered to provide robust epidemiologic estimates and that utilize high-throughput testing approaches that permit full characterization of viral diversity, are needed to further our understanding of patterns of EBV genetic variation and their association with malignancies in different regions.

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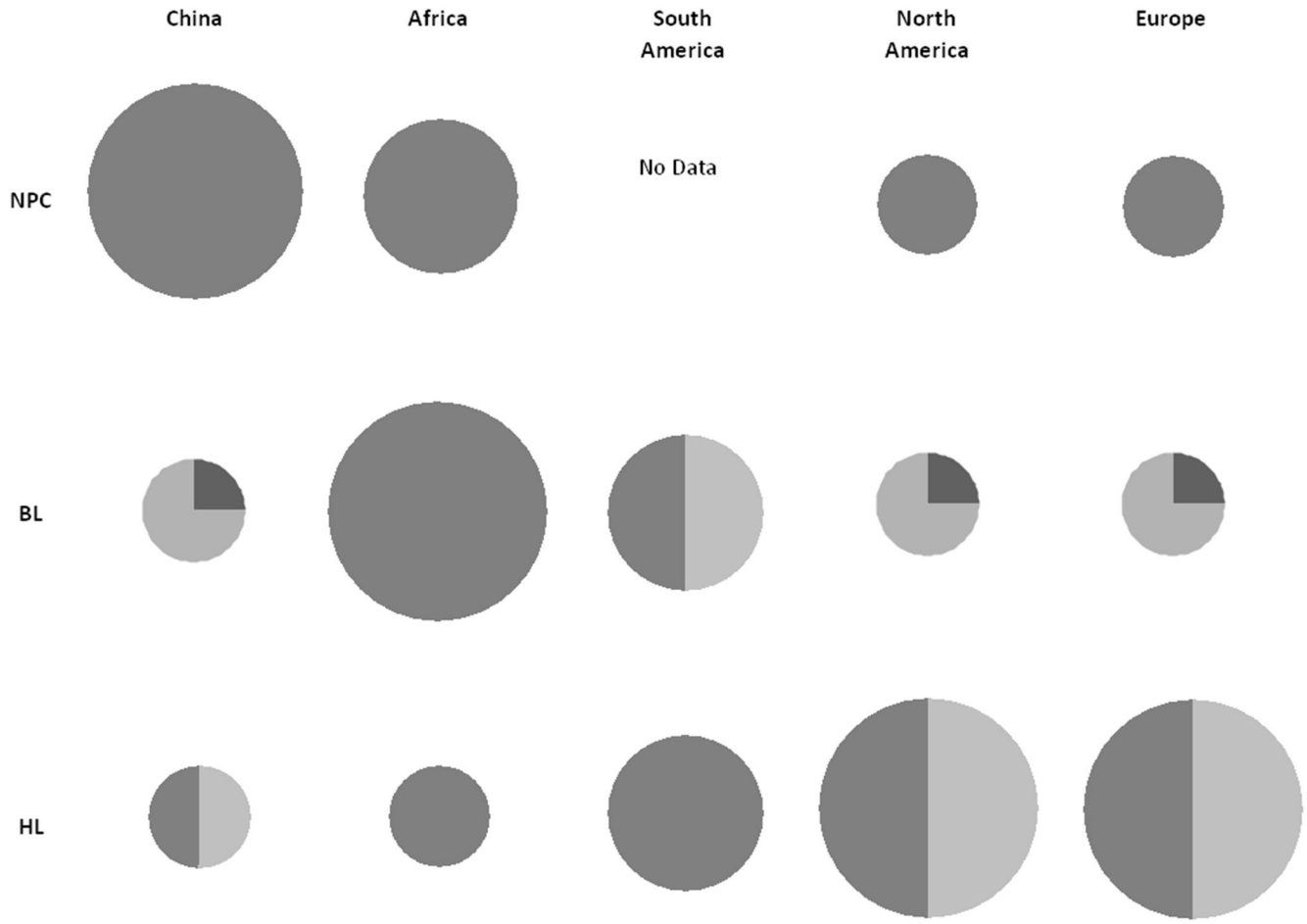

Figure 1.

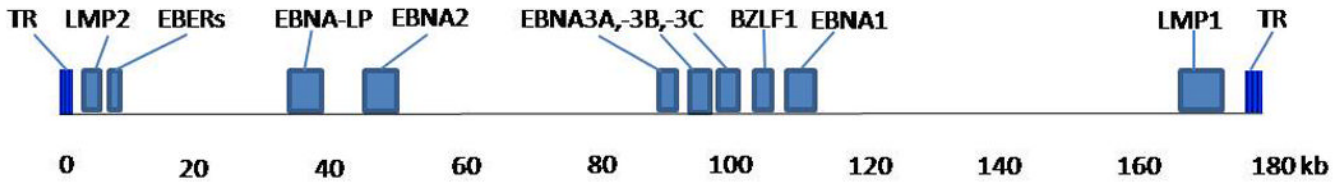

Figure 2.

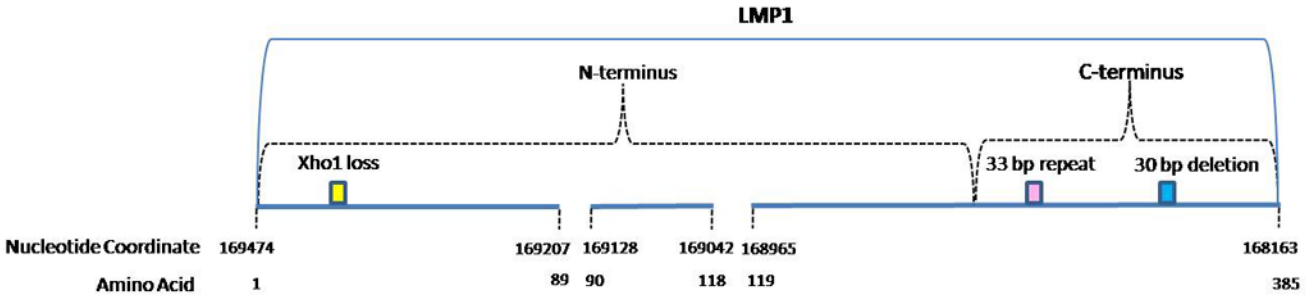

Figure 3.

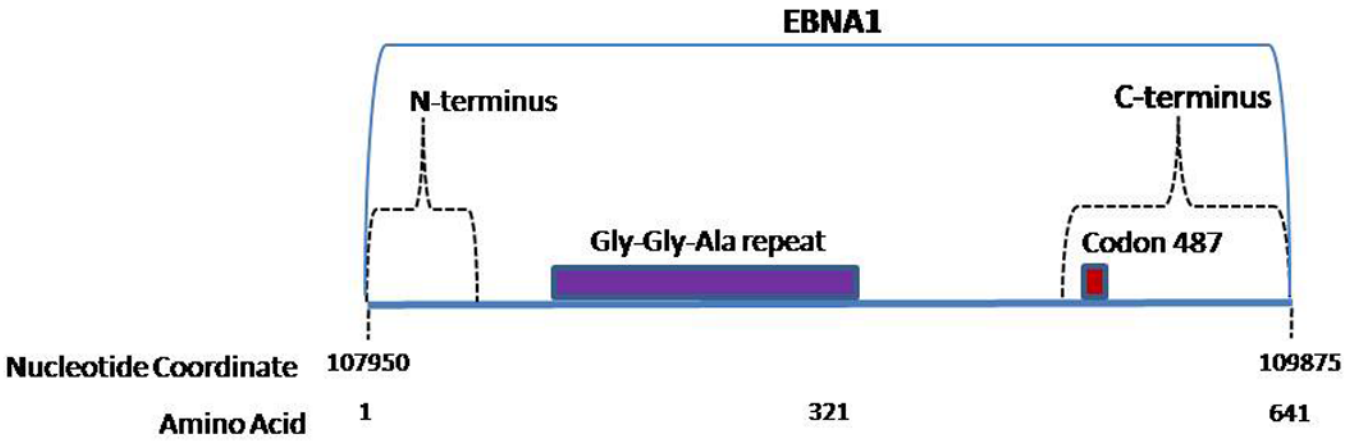

Figure 4.

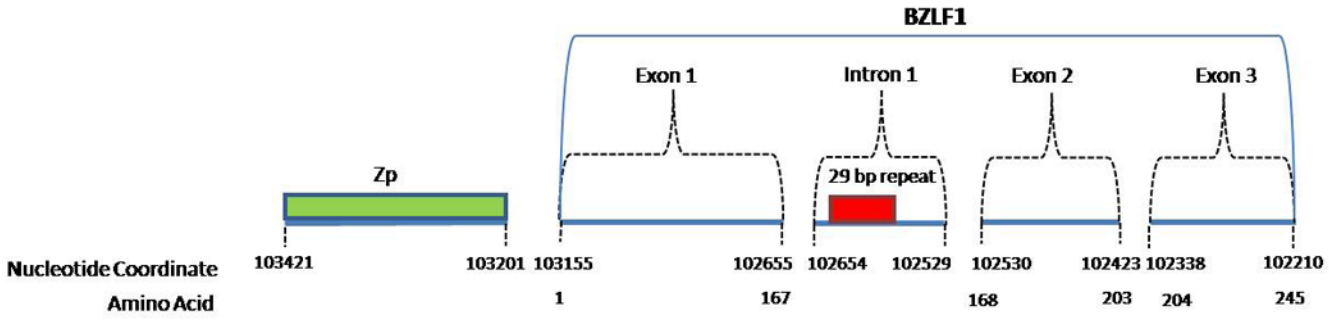

Figure 5.

Table 1

LMP-1 Carboxy Terminus Changes

Nucleotide Position	Nucleotide Change	Codon	AA Change	China 1	Med$^+$	Med	China 3	NC	Alaskan	China 2
168694	T to C	209	no change	x			x		x	x
168687	G to A	212	Gly to Ser	x			x		x	x
168640	C to T	227	no change						x	
168635	G to C	229	Ser to Thr		x	x				
168631	A to G	230	no change	x			x			
168626	G to C	232	Gly to Ala						x	
168587	C to A	245	Pro to His							x
	33 bp repeat	250–298								
168566	G to A	252	Gly to Asp							x
168404	T to A	306	Leu to Gln					x		
168395	G to A	309	Ser to Asn	x	x	x		x	x	x
168387	G to A	312	Asp to Asn						x	
168384	T to G	313	Ser to Ala						x	
168384	T to C	313	Ser to Pro					x		
168361	T to G	320	no change							x
168357	C to A	322	Gln to Asn/Lys	x				x	x	
168357	C to G	322	Gln to Lys		x	x				
168356	A to C	322	Gln to Thr					x		
168355	A to T	322	Gln to Lys	x						
168330	G to C	331	Gly to Gln					x		x
168329	G to A	331	Gly to Gln					x		x
168329	G to C	331	Gly to Ala						x	
168320	A to G	334	Gln to Arg	x	x	x				
168309	T to C	338	Leu to Pro					x	x	x
168308	T to C	338	Leu to Ser	x	x	x	x	x	x	x
168295	A to T	342	no change	x	x	x	x	x	x	x
168290	G to A	344	Gly to Asp							x
168288	G to A	345	Gly to Ser						x	
168287-168256	30 bp del	343–352		x	x		x			
168267	C to A	352	His to Asn					x		

Nucleotide Position	Nucleotide Change	Codon	AA Change	China 1	Med+	Med	China 3	NC	Alaskan	China 2
168266	A to G	352	His to Arg		x	x				
168260	G to A	354	Gly to Asp						x	
168257	G to T	355	Gly to Val						x	x
168248	A to C	358	His to Pro					x		
168238	G to A/C	361	no change		x	x				
168225	T to A/G	366	Ser to Thr	x	x	x	x			x

Table 2

Table 2a. Prevalence (%) of 30 Base Pair Deletion in the LMP-1 gene by Clinical Condition and Geographic Region (total cases)

Clinical condition	China	Other Asian	Africa	South America	North America	Europe
NPC	83.8% (857)	55.1% (301)			11.1% (18)	27.8% (18)
BL			38.1% (21)	80.0% (15)		
HL	83.1% (65)			60.2% (88)	48.1% (27)	33.9% (277)
AIDS lymphoma					47.4% (19)	53.9% (167)
Nonmalignant/healthy	75.1% (189)	34.0% (47)	25.6% (39)	17.1% (175)	47.9% (48)	33.6% (295)

Table 2b. Prevalence (%) of XhoI Loss in the LMP-1 Gene by Clinical Condition and Geographic Region (total cases)

Clinical condition	China	Other Asian	Africa	South America	North America	Europe
NPC	94.8% (269)	74.4% (117)				0% (9)
BL			0% (11)			
HL	89.6% (67)				20.0% (15)	6.9% (29)
AIDS lymphoma						
Nonmalignant/healthy	79.6% (162)	0% (22)	5.1% (39)			46.2% (68)

(Berger et al., 1999; Chang et al., 2006; Chang et al., 1995; Chen et al., 1996b; Cheung et al., 1998; Cheung et al., 1996; Chiang et al., 1999; Correa et al., 2004; D'Addario and Chauvin, 2000; Dolcetti et al., 1997; Fassone et al., 2002; Guiretti et al., 2007; Gutierrez et al., 2000; Hayashi et al., 1997; Itakura et al., 1996; Jeon et al., 2004; Khanim et al., 1996; Lin et al., 2004; Lindsey et al., 2008; Nguyen-Van et al., 2008; Plaza et al., 2003; Sandvej et al., 1997; Santon and Bellas, 2001; Santon et al., 1995; See et al., 2008; Sung et al., 1998; Tan et al., 2003; Tang et al., 2008; Tao et al., 1998; Tiwawech et al., 2008; Trimeche et al., 2007; Vallat-Decouvelaere et al., 2002; Zhang et al., 2002; Zhou et al., 2001)

(Barozzi et al., 1999; Berger et al., 1999; Chang et al., 1995; Cheung et al., 1996; Khanim et al., 1996; Lin et al., 2004; Lin et al., 1995; Lin et al., 2003; Santon and Bellas, 2001; See et al., 2008; Shu and Tu, 2000; Tan et al., 2003; Tang et al., 2008; Zhou et al., 2001)

Table 3

Prevalence (%) of Variants Based on the Carboxy Terminus of LMP-1 by Clinical Condition and Geographic Region

Clinical Condition	LMP-1 Genotype	Asia	Africa	North America	Europe
NPC	China 1	82.5%		66.0%	0.0%
	China 2	9.7%		0.0%	0.0%
	China 3	9.0%		0.0%	0.0%
	Med	0.0%		0.0%	89.0%
	Alaskan	0.0%		33.0%	0.0%
	NC	0.0%		0.0%	0.0%
	B95-8	5.1%		0.0%	11.0%
	Other	1.8%[a]		0.0%	0.0%
total cases		217	0	3	9
BL	China 1		0.0%	0.0%	
	China 2		0.0%	0.0%	
	China 3		0.0%	0.0%	
	Med		100.0%	100.0%	
	Alaskan		0.0%	0.0%	
	NC		0.0%	0.0%	
	B95-8		0.0%	0.0%	
total cases		0	1	1	0
HL	China 1	100.0%			56.0%
	China 2	0.0%			0.0%
	China 3	0.0%			0.0%
	Med	0.0%			12.0%
	Alaskan	0.0%			0.0%
	NC	0.0%			3.0%
	B95-8	0.0%			29.0%
total cases		1	0	0	34

Clinical Condition	LMP-1 Genotype	Asia	Africa	North America	Europe
Nonmalignant/healthy	China 1	70.0%		33.0%	27.0%
	China 2	0.0%		0.0%	0.0%
	China 3	0.0%		0.0%	0.0%
	Med	13.3%		33.0%	12.0%
	Alaskan	0.0%		0.0%	0.0%
	NC	0.0%		0.0%	18.0%
	B95-8	10.0%		33.0%	42.0%
	Other	6.7%[a]		0.0%	0.0%
total cases		60	0	3	33

(Edwards et al., 1999; Saechan et al., 2006; Tiwawech et al., 2008)

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Table 4

EBNA-1 Carboxy Terminus Changes

	Prototype (B95-8)		Prototype-like	Variant			
	P-ala[a]		P-thr[b]	V-val[c]	V-pro	V-leu	V-ala
AA #	Codon	AA					
471	CAA	Gln	nc	nc	Glu	Glu (GAA)	nc
475	AAC	Asn	nc	nc	nc	Ser (AGC)	nc
476	CCG	Pro	nc	nc	Gln	Gln (CAG)	nc
487	GCT	Ala	Thr (ACT)	Val (GTT)	Pro (CCT)	Leu (CTT)	nc
492	AGT	Ser	nc	nc	Cys (TGT)	Cys (TGT)	nc
499	GAC	Asp	nc	Glu (GAG)	Glu (GAG)	Glu (GAG)	Glu (GAG)
500	GAA	Glu	nc		Asp (GAT)	Asp (GAT)	Asp (GAT)
502	ACT	Thr	nc	Asn (AAT)	Asn (AAT)	Asn (AAT)	Asn (AAT)
517	CTT	Leu	nc	nc	nc	nc	Leu (TTA)
520	CTA	Leu	nc	Leu (CTC)	nc	Leu (CTC)	Leu (CTC)
524	ACT	Thr	Ile (ATT)	Ile (ATT)	Ile (ATT)	Ile (ATT)	Ile (ATT)
525	GCC	Ala	Gly (GGC)	nc	Gly (GGC)	Gly (GGC)	nc
533	CTT	Leu	nc	Ile (ATT)	nc	nc	nc

[a] Subvariants of P-ala have AA substitutions at 499, 524, and 533 (Sandvej et al., 2000)

[b] Subvariant of P-thr has a Glu (GAG) instead of Asp (GAT) at AA 499 (Sandvej et al., 2000)

[c] Subvariants of V-val have AA substitutions at 528 and 585- (Do et al., 2008; Wang et al., 2003) nc: no change in codon

Table 5

Prevalence (%) of Variants Based on the Carboxy Terminus of EBNA-1 by Clinical Condition and Geographic Region

Clinical Condition	EBNA-1 Genotype	China	Africa	South America	North America	Europe
NPC	P-ala	0.0%				18.2%
	P-thr	4.0%				72.7%
	V-val	95.2%				0.0%
	V-leu	0.0%				0.0%
	V-pro	0.0%				0.0%
	Multiple/Other	0.8%[a]				9.1%[d]
	Total Cases	125	0	0	0	11
BL	P-ala		2.5%	0.0%		60.0%
	P-thr		41.8%	8.3%		40.0%
	V-val		0.0%	0.0%		0.0%
	V-leu		54.4%	75.0%		0.0%
	V-pro		1.3%	0.0%		0.0%
	Multiple/Other		0.0%	16.7%[c]		0.0%
	Total Cases	0	79	12	0	2
HL	P-ala	0.0%		0.0%	16.7%	13.6%
	P-thr	0.0%		25.0%	50.0%	81.8%
	V-val	100%		0.0%	0.0%	0.0%
	V-leu	0.0%		75.0%	33.3%	4.5%
	V-pro	0.0%		0.0%	0.0%	0.0%
	Multiple/Other	0.0%		0.0%	0.0%	0.0%
	Total Cases	2	0	20	12	22
AIDS lymphoma	P-ala			20.0%	15.8%	36.7%
	P-thr			40.0%	31.6%	38.8%
	V-val			0.0%	0.0%	2.0%

Clinical Condition	EBNA-1 Genotype	China	Africa	South America	North America	Europe
	V-leu			20.0%	26.3%	8.2%
	V-pro			0.0%	0.0%	0.0%
	Multiple/Other			20.0%[3]	26.3%[d]	14.3%[f]
	Total Cases	0	0	5	19	49
Healthy/nonmalignant	P-ala	16.2%	5.3%	16.7%	46.4%	40.0%
	P-thr	2.7%	36.8%	33.3%	17.9%	55.0%
	V-val	48.6%	0.0%	0.0%	0.0%	0.0%
	V-leu	0.0%	47.4%	50%	0.0%	2.5%
	V-pro	0.0%	0.0%	0.0%	0.0%	0.0%
	Multiple/Other	32.4%[a]	10.5%[b]	0.0%	35.7%[e]	2.5%[d]
	Total Cases	37	38	6	28	8

(Bhatia et al., 1996; Chang et al., 1999; Do et al., 2008; Fassone et al., 2002; Gutierrez et al., 2000; Gutierrez et al., 1997; MacKenzie et al., 1999; Sandvej et al., 2000; Wang et al., 2003; Wang et al., 2002; Zhang et al., 2004)

[a] V-val + P-ala;

[b] P-ala/thr + V-pro;

[c] V-ala;

[d] Not specified;

[e] P-ala + P-thr/V-val

[f] P-ala + P-thr

Table 6

Prevalence (%) of Variants Based on the 29 Base Pair Repeat in the 1st Intron of BZLF-1 by Clinical Condition and Geographic Region

Clinical condition	BZLF-1	China	North Africa and Europe
NPC	B95-8 (2 copies)	18.7%	93.5%
	P3HRI/Akata (3 copies)	81.3%	6.5%
	total	75	35
Healthy	B95-8 (2 copies)	21.2%	80.0%
	P3HRI/Akata (3 copies)	81.8%	20.0%
	total	33	75

(Chen et al., 1996a; Grunewald et al., 1998)

Table 7

BZLF-1 (Zp) Promoter Base Changes

Nucleotide Position	Change	Zp-V3	Zp-V4	Zp-PV
103322	T to G	x	x	
103316	A to G	x	x	
103281	A to G	x	x	
103233	C to T			x
103226	T to C		x	

(Gutierrez et al., 2002; Martini et al., 2007; Tong et al., 2003)

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Table 8

Prevalence (%) of Variants Based on the BZLF-1 Promoter (Zp) by Clinical Condition and Geographic Region

Health condition	Zp	China	Africa	N./ S. America	Europe
NPC	Zp-P	13.0%			
	Zp-V3	82.6%			
	Zp-V4	0.0%			
	Zp-PV	0.0%			
	Multiple/Other	4.3%			
	Total	46	0	0	0
NHL	Zp-P	100%	71.4%	45.8%	
	Zp-V3	0.0%	28.6%	54.2%	
	Zp-V4	0.0%	0.0%	0.0%	
	Zp-PV	0.0%	0.0%	0.0%	
	Multiple/Other	0.0%	0.0%	0.0%	
	Total	3	7	24	0
AIDS lymphoma	Zp-P				54.0%
	Zp-V3				30.2%
	Zp-V4				0.0%
	Zp-PV				11.1%
	Multiple/Other				4.8%
	Total	0	0	0	63
Non-malignant/healthy	Zp-P	47.5%		81.3%	70.0%
	Zp-V3	50.0%		0.0%	0.0%
	Zp-V4	0.0%		18.8%	0.0%
	Zp-PV	0.0%		0.0%	0.0%
	Multiple/Other	2.5%		0.0%	30.0%[a]
	Total	40	0	16	20

Health condition	Zp	China	Africa	N./ S. America	Europe

(Gutierrez et al., 2002; Martini et al., 2007; Tong et al., 2003)

[a] Zp-P + Zp-V3