J. Clin. Exp Hematopathol
Vol. 43, No. 1, Mar 2003

Original Article

Epstein-Barr virus-Associated Lymphomas in Malaysia : High Frequency of a 30-bp Deletion in the Viral Latent Membrane Protein-1 (LMP-1) Oncogene

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A variant of Epstein-Barr virus (EBV) with a 30-base pair (bp) deletion in the latent membrane protein-1 (LMP-1) gene has been reported in many EBV-associated malignancies at different frequencies in different geographical locations. This study aims to determine the frequency of this variant in Asian EBV-associated lymphomas. We analyzed a total of 81 cases of known EBV-associated lymphomas [20 classical Hodgkin's, 19 Burkitt's, 33 T- and natural killer (NK)/T-cell, and 9 diffuse large B-cell lymphomas (DLBCL)] and 19 reactive lymphoid tissues from the archives. The presence of the 30-bp deletion at the 3' end of the LMP-1 gene was demonstrated by hybridization of polymerase chain reaction (PCR) products after amplification with a pair of primers flanking the deletion region. The DNA from 96.3% (78/81) of the lymphomas and 78.9% (15/19) of the reactive lymphoid tissues were amplified by PCR. The lymphomas showed infection by the deleted variant in 91.0% (71/78) of the cases, including 33.3% (26/78) cases with dual variant infection. The deleted variant was observed in 100% (19/19) of the Burkitt's lymphoma, 89.5% (17/19) of the classical Hodgkin's lymphomas, 90.6% (29/32) of the T- and NK/T-cell lymphomas and 75.0% (6/8) of DLBCL, whereas it was detected in 60.0% (9/15) of the reactive lymphoid tissues. The difference in the frequency of deleted LMP-1 variants observed between the lymphomas and the reactive lymphoid tissues was statistically significant ($p=0.006$). A significant difference was also observed between childhood and male lymphoma patients and reactive lymphoid controls of comparable age or gender group ($p<0.001$ and $p=0.012$ respectively). In conclusion, a high frequency of the 30-bp deletion in the LMP-1 gene was observed in the Malaysian population. The significantly higher percentage of deleted variant observed in EBV-associated lymphomas suggests the possibility of a selection mechanism during malignant transformation.

Key words Epstein-Barr virus (EBV), latent membrane protein-1 (LMP-1), lymphomas, dual LMP-1 variants

INTRODUCTION

The Epstein-Barr virus (EBV) is one of the viruses that infect humans most effectively. More than 90% of the world's population carries this virus as a life long asymptomatic infection[1].

This ubiquitous B-cell lymphotrophic human gamma-herpes virus is strongly associated with several human malignancies, notably nasopharyngeal carcinoma (NPC), Burkitt's lymphoma (BL), classical Hodgkin's lymphoma (HL), natural killer (NK)/T-cell lymphoma and lymphomas in immuno-compromised patients[1-4]. EBV is present as an episome (latency) in these neoplasms and only a limited number of viral genes are expressed, producing six EBV nuclear antigens (EBNA 1-6), three latent membrane proteins (LMP-1, 2A and 2B) and two small RNA transcripts (EBER1 and EBER2)[1-4]. Some of these gene products are involved in latent viral

Received : Jul 8, 2002
Revised : Aug 23, 2002
Accepted : Aug 28, 2002
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DNA replication and activation of the transcription of EBV genes, as well as in the transformation and the immortalization of B-lymphocytes[2–4].

BNLF1, which encodes for LMP-1, is not only essential for B-cell transformation; it is also the only EBV gene that has transforming effects in non-lymphoid cells[5,6]. It is considered to be a viral oncogene due to its ability to transform rodent fibroblasts in vitro and render them tumorigenic in nude mice[5,6]. In B lymphoblasts, LMP-1 induces DNA synthesis and plays an important role in the initiation and/or the maintenance of the immortalized state[6,7]. LMP-1 induces many of the phenotypic changes associated with EBV infection, including increased homotypic adhesion and up-regulation of adhesion molecules such as lymphocyte function-associated antigens (LFA-1 and LFA-3), intracellular adhesion molecule (ICAM-1)[8], B-cell activation markers (CD23 and CD40)[8] and the anti-apoptotic bcl-2 gene[7]. It alters cell growth and NF-kB activity through signaling via tumor necrosis factor receptors-associated factors (TRAFs)[9].

LMP-1 is an integral membrane protein of 386 amino acids, with a short cytoplasmic amino-terminal domain (25 amino acids), a 6-transmembrane domain (161 amino acids) and a long cytoplasmic domain at the carboxy-terminus (C-terminus). The C-terminus is important for transforming activity[5,8]. Sequence analysis of the LMP-1 C-terminal domain showed differences between the prototype EBV B95.8 strain and the Chinese NPC-derived isolates[10,11], as well as various EBV-associated disorders and lymphomas[3,4,12–15], which include 7 single-base mutations, 30- and 69-base-pair (bp) deletions, and 33-bp repeats in this region. Studies using murine transfectants and *in vitro* cell line models showed that EBV with the characteristic 30-bp deletion between amino acids 322 and 366 at the C-terminus of the LMP-1 gene has a higher tumorigenic potential and are less immunogenic than the non-mutated prototype (B95.8)[6,16]. The higher tumorigenic potential is thought to be due to the reduced turnover of the LMP-1 protein, thus increasing its oncogenicity[5]. Khanim *et al*.[4] and Zhang *et al*.[11] studied materials from different geographical regions and reported comparable frequencies of viral gene polymorphism in normal donors and EBV-associated lymphoproliferative disorders from similar geographical regions. Wild-type is predominant over the 30-bp deleted variant in European HL, African and New Guinean BL, whereas an inverse pattern with a predominance of deleted LMP-1 was observed in Chinese NPC and Chinese normal donors.

EBV-associated malignancies of various types are commonly seen in Malaysian patients[17–21] and a higher prevalence of the type A virus was reported previously[22]. However, the frequency of the 30-bp deletion in LMP-1 gene of our EBV-associated lymphomas has not been well studied, although Sandvej *et al*.[17] previously reported the presence of a 30-bp deletion in a small series of Malaysian peripheral T-cell lymphoma (PTCL). This study aims to investigate the distribution pattern of the 30-bp LMP-1 deletion and non-deletion variants in larger numbers and a wider spectrum of lymphoid malignancies from Asian patients.

MATERIALS AND METHODS

Samples

We analyzed a total of 81 cases of EBV-associated lymphomas, including 20 classical HLs (12 mixed cellularity, 6 nodular sclerosing and 2 lymphocyte rich), 19 BLs, 33 T-cell non-Hodgkin's lymphomas (T-NHLs) [11 PTCL-unspecified, 2-angioimmunoblastic (AILD) T and 20 NK/T] and 9 diffuse large B-cell lymphomas (DLBCLs). Nineteen cases of reactive tonsils and lymph nodes from non-neoplastic conditions were included for a comparative study. Paraffin-embedded tissue blocks and stained slides of these cases were retrieved from the archives for review and confirmation of diagnosis as well as the presence of EBV by the EBER *in situ* hybridization technique[18,19]. All the confirmed cases were subjected to polymerase chain reaction (PCR) amplification of the C-terminus of the LMP-1 gene to determine the presence or the absence of the 30-bp deletion.

DNA Extraction

DNA extraction was performed on one 5-μm thick section as described previously[22]. Briefly, the 5-μm thick section from each case was cut and placed in a sterile 1.5 ml tube and subjected

to a series of deparaffinization and washing in xylene and alcohol. The tissue pellet was lysed overnight at 55°C in a digestion buffer containing 1X PCR buffer (Gibco BRL, USA) and 200 μg/ml proteinase K (Boehringer Mannheim, Germany). The digestion was stopped by heating at 96°C for 10 min, and the cell debris was spun down. The supernatant containing the DNA was transferred to a new tube, and was used directly in PCR. One blank block made of paraffin wax only was cut between each test case, to check for block-to-block contamination during the cutting step. The tubes containing the blank blocks were subjected to similar treatments, along with those containing the tissue sections. DNA extractions from EBV infected cell lines, B95.8 (wild type LMP-1) and AG 876 (30-bp deleted LMP-1), were performed using Tri-Reagent™ (Molecular Research Centre Inc. USA), according to the manufacturer's recommendation.

Detection of the 30-bp deletion in the LMP-1 gene by PCR

Amplification of the C-terminus of the LMP-1 gene was performed using a pair of primers flanking the characteristic 30-bp deletion region[10] (Table 1). Cell lines B95.8 and AG876 were used as positive controls respectively for wild type, which gives a 186-bp PCR product (B95.8), and the deleted variant, which produces a 156-bp product (AG876).

A quantity of 1μl of extracted DNA was used as the template in a reaction volume of 50μl, containing 0.2 mM dNTPs mix (Biotools, Spain), 0.5μM of each primer in a standard PCR buffer with 1.5 mM $MgCl_2$ (Qiagen, Germany) and 2.5U of HotStarTaq® DNA polymerase (Qiagen, Germany). Amplification was performed with a first cycle of 95°C for 15 min to activate the HotStarTaq® DNA polymerase as described by manufacturer, continued with 45 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min, with the last extension at 72°C for 3 min to complete the reaction. PCR products were analyzed by 3% agarose gel eletrophoresis.

The cases in which the length of the PCR product coincided with that of B95.8 were concluded as being non-deleted, whereas samples exhibiting a shorter PCR product similar to AG876 were considered to contain a 30-bp deletion in the LMP-1 gene. Cell lines and five randomly selected cases were sent for DNA sequencing analysis (Bio Basic Inc., Ontario, Canada, using the ABI Prism 377 sequencer) for confirmation of the specificity of the amplified products.

Southern Blotting

PCR products resolved in 3% agarose gel were transferred overnight to a TotalBlot+™ (Amersco, USA) positively charged nylon membrane following denaturation in 0.5 M NaOH. The membrane was pre-hybridized at 50°C for 4 hours in pre-hybridization buffer (50% formamide, 5X SSC, 5X Denhardt's solution, 0.02M sodium phosphate, 0.5 mg/ml denatured salmon sperm DNA and 0.5% SDS) followed by overnight hybridization with 100 ng/ml of probe in hybridization buffer (similar to pre-hybridization buffer, except that 45% formamide was used) at 50°C. A set of 5'-biotin-labeled probes, one specific for the deleted region and another flanking the deletion region, was used[23] (Table 1). Visualization was achieved by using streptavidin conjugated with alkaline phosphatase as secondary antibody (Kirkegaard & Perry Laboratories,

Table 1. Sequence of PCR primers and hybridization probes

Primers/Probes	EBV B95.8 co-ordinate	Sequence
Primers for PCR		
LMP-FUB2	168326-168348	5'-GAAGAGGTTGAAAACAAAGGA-3'
LMP-FUE	168163-168183	5'-GTCATAGTAGCTTAGCTGAAC-3'
Probes for Southern blotting		
5'-Biotin-labelled probe specific for the deleted region	168259-168278	5'-GCCGTCATGGCCGGAATCAT-3'
5'-Biotin-labelled probe flanking the deleted region	168311-168330	5'-GGCGGGCCCTGGTCACCTCC-3'

Table 2. Distribution of LMP-1 30-bp deletion variant in lymphomas and reactive lymphoid tissues by ethnic groups and gender

Cases	PCR No. (%)	LMP-1 30-bp deletion		
		Deleted variant alone No. (%)	Dual variant* No. (%)	Non-deleted variant No. (%)
Lymphomas	78/81 (96.3)	45/78 (57.7)	26/78 (33.3)	7/78 (9.0)
Ethnic Groups				
Malay	25/27 (92.6)	12/25 (48.0)	10/25 (40.0)	3/25 (12.0)
Chinese	28/28 (100)	22/28 (78.6)	6/ (21.4)	0
Indian	11/11 (100)	3/11 (27.3)	5/11 (45.4)	3/11 (27.3)
Sarawak Indigenous tribes#	4/5 (80.0)	2/4 (50.0)	1/4 (25.0)	1/4 (25.0)
Sabah Indigenous tribes#	10/10 (100)	6/10 (60.0)	4/10 (40.0)	0
Gender				
Male	54/57 (94.7)	32/54 (59.2)	17/54 (31.5)	5/54 (9.3)
Female	24/24 (100)	13/24 (54.2)	9/24 (37.5)	2/24 (8.3)
Reactive lymphoid hyperplasia	15/19 (78.9)	7/15 (46.7)	2/15 (13.3)	6/15 (40.0)
Ethnic Groups				
Malay	11/14 (78.6)	4/11 (36.4)	2/11 (18.2)	5/11 (45.4)
Chinese	1/1 (100)	0	0	1 (100)
Indian	3/4 (75.0)	3/3 (100)	0	0
Gender				
Male	8/9 (88.9)	3/8 (37.5)	1/8 (12.5)	4/8 (50.0)
Female	7/10 (70.0)	4/7 (57.1)	1/7 (14.3)	2/7 (12.6)

* Dual variant : Cases with both deleted and non-deleted variants
#Sarawak and Sabah : States in East Malaysia

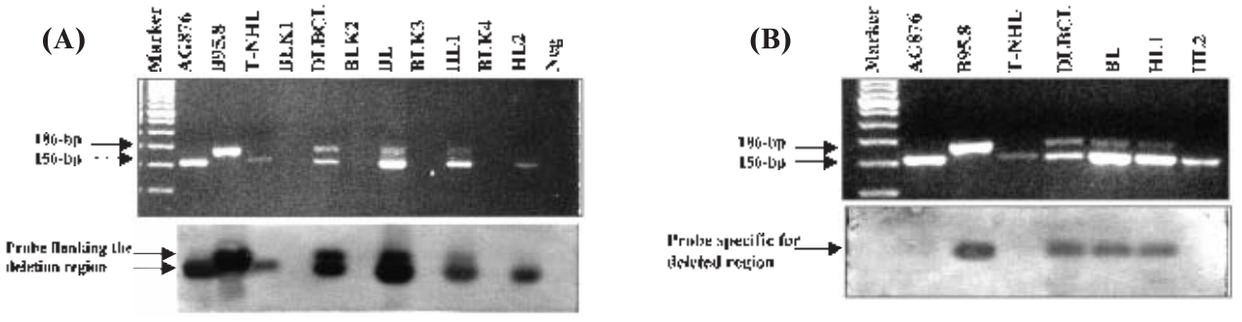

Fig. 1. Representative gel electrophoresis and corresponding Southern blot for LMP-1 gene deletion in lymphomas.
(A) No block-to-block cross-over contamination as shown in gel and Southern blot membrane. Both the 186-bp and the 156-bp PCR products hybridized with the probe flanking the deleted region. **(B)** Only the 186-bp fragment showed hybridization with the probe specific for the deleted region. The absence of hybridization with the 156-bp products is consistent with the occurrence of deletion in these cases.
Marker, 50-bp DNA ladder ; AG876, positive cell line (deleted variant) ; B95. 8, positive cell line (non-deleted variant) ; T-NHL, T-cell non-Hodgkin's lymphoma ; DLBCL, diffuse large B-cell lymphoma ; BL, Burkitt's

USA) and NBT/BCIP (Boehringer Mannheim, Germany) as substrate.

Statistical Analysis

Statistical analysis of the data was performed using the Chi-square test (EpiInfo version 6. 04b, Atlanta : Centers for Disease Control and Prevention, USA ; 1997). Yates-corrected p values <0.05 were accepted as statistically significant.

RESULTS

PCR amplification of the C'-terminal of LMP-1 gene flanking the characteristic 30-bp deletion was successful in 96.3% (78/81) of the lymphoma cases and 78.9% (15/19) of the reactive lymphoid tissues (Table 2). There was no block-to-block carry-over contamination as observed in gel electrophoresis analysis (Figure 1A). The 30-bp LMP-1 gene-deleted viral variant was observed in 91.0% (71/78) of the lymphomas, where

57.7% (45/78) showed deleted variant alone and 33.3% (26/78) had dual variants (deleted and non-deleted together). Reactive lymphoid tissues showed that 60.0% (9/15) of the cases was infected with the deleted variant (Table 2). When considering only the number of cases with the dual variant, we found a three-fold difference between lymphomas (33.3%, 26/78) and normal reactive tissues (13.3%, 2/15). A reverse pattern was observed in these two groups of cases for the non-deleted variant (9.0%, 7/78 and 40.0%, 6/15 respectively). This difference is statistically significant ($p=0.017$). Likewise, the difference observed between these two groups of diseases is significant, both in the total number of deleted cases (sum of deleted and dual variants, $p=0.006$) and the number of deleted variants alone ($p=0.016$).

The gender and ethnic distribution of the 30-bp LMP-1 gene deletion is presented in Table 2. All ten lymphomas from indigenous tribes (8 Kadazan, 1 Bisaya and 1 Sungai) of Sabah (a State in East Malaysia) were associated with the presence of deleted variants (6 deleted variants alone and 4 dual variants). However, statistical analysis did not reveal any significant difference between the different ethnic groups. More cases of lymphoma from male patients showed deleted variants (90.8%) when compared to reactive lymphoid hyperplasia from the same gender (50.0%), with $p=0.012$. All the 5 amplifiable childhood reactive lymphoid cases (<15 years old) harbored the non-deleted variant of the LMP-1 gene, whilst 95.6% (22/23) of the childhood lymphomas harbored the deleted variant (Table 3). This difference was statistically significant ($p<0.001$). These 23 cases of childhood lymphoma with the LMP-1 deleted variant consisted of 12 BLs (52.2%), 9 HLs (39.2%) and one DLBCL (4.3%).

High frequency of deleted variant was observed across the different types of lymphomas (Table 3), the highest being in BL (100%). Regardless of the age group, a higher proportion of dual variant infection (73.7%, 14/19) was observed in the cases of HL compared to other subtypes of lymphoma. The NK/T-cell lymphoma showed the highest proportion of deleted variant alone (85.0%, 17/20) when compared to the other T-NHL subtypes. Statisti-

Table 3. Pattern of 30-bp LMP-1 deletion variant in Malaysian EBV-associated lymphomas and reactive lymphoid tissues by age group

Cases	Lymphomas (%)									Reactive lymphoid tissues (%)
	Total	*cHL*			*BL*	*T-NHL*			*DLBCL*	
		MC	*NS*	*LR*						
Total										
Deleted variant	45/78 (57.7)	3/19(15.8)			14/19 (73.7)	23/32(71.9)			5/8 (62.5)	7/15 (46.7)
Dual variant*	26/78 (33.3)	14/19(73.7)			5/19 (26.3)	6/32(18.7)			1/8 (12.5)	2/15 (13.3)
Non-deleted variant	7/78 (9.0)	2/19(10.5)			0/19 (0)	3/32(9.4)			2/8 (25.0)	6/15 (40.0)
Childhood										
Deleted variant	11/23 (47.8)	1	1	0	8	0	0	0	1	0
Dual variant*	11/23 (47.8)	6	0	1	4	0	0	0	0	0
Non-deleted variant	1/23 (4.4)	1	0	0	0	0	0	0	0	5
Adult										
Deleted variant	34/55 (61.8)	0	1	0	6	5	1	17	4	7
Dual variant*	15/55 (27.3)	3	3	1	1	4	0	2	1	2
Non-deleted variant	6/55 (10.9)	1	0	0	0	1	1	1	2	1

*Dual variant : cases with both the deleted and the non-deleted variants

EBV, Epstein-Barr virus ; PCR, polymerase chain reaction ; cHL, classical Hodgkin's lymphoma ; MC, mixed cellularity ; NS, nodular sclerosis ; LR, lymphocyte rich ; BL, Burkitt's lymphoma ; T-NHL, T-cell non-Hodgkin's lymphoma consisting of peripheral T-cell lymphoma, angioimmunoblastic T and NK/T-cell lymphoma ; DLBCL, diffuse large B-cell lymphoma

cal analysis showed a significant difference between the cases containing the deleted variants (deleted and dual variants) in BL and T-NHL, and those in reactive lymphoid tissues ($p=0.010$ and $p=0.021$ respectively).

Southern blot

PCR products subjected to Southern hybridization analysis showed that all the bands coinciding with the 186-bp PCR products hybridized with the probe specific for the region flanking the deleted fragment, as well as the probe specific for the deleted region. In contrast, the PCR products coinciding with the 156-bp band only hybridized with the probe flanking the deleted fragment but not with the probe specific for the deleted region, confirming the presence of a specific 30-bp deletion in the lower molecular weight products. The cases with the dual variant showed two bands hybridized with the probe flanking the deleted region (Figure 1A), but only the 186-bp fragment hybridized with probe specific for the deleted region (Figure 1B).

DNA Sequencing

The DNA sequence analysis confirmed that all the PCR fragments were in agreement with the prototype cell line, B95.8. The shorter fragments showed a 30-bp deletion in the 168253-168282 region.

DISCUSSION

Studies on the etiological role of EBV in many malignancies from different populations and geographical locations reveal a variable pattern of viral strains and variants in the diseases[3,4,10,11,24,25]. A variant of LMP-1 gene with a 30-bp deletion at the C-terminus has previously been detected, predominantly in Asian NPC[4,10,11], acquired immune-deficiency syndrome (AIDS) and AIDS-related lymphomas[3]. It is also associated with histologically more aggressive lymphomas, such as HL with giant atypical Hodgkin/Reed-Sternberg (H/RS) cells[12], NK/T-cell lymphomas[26,27], and in immunocompromised conditions, e.g., post-transplant lymphoproliferative disorders[14]. The 30-bp deleted LMP-1 variant can confer growth advantage to the infected cells owing to its reduced turnover and enhanced NF-kB-mediated signaling[5,7,9]. It is also less immunogenic, allowing the infected cells to escape host immune surveillance[16]

Our study shows that the 30-bp deletion in the LMP-1 gene is frequently present in our patients-derived materials, irrespective of the disease types. This may reflect the higher prevalence of the deleted LMP-1 variant in the population, a situation similar to other reports from Asia[4,11,20,26–28]. However, a significantly higher frequency ($p=0.006$) of the 30-bp deletion in the LMP-1 gene was observed in lymphomas (91.0%) when compared to non-neoplastic reactive lymphoid tissues (60%). Likewise, in a study by Tan *et al.*[20], a higher frequency of the deleted LMP-1 variant was detected in Malaysian NPC (100%) when compared to the normal healthy donors (8%). These differences observed between normal and tumor suggest the possibility of a variant selection during malignant transformation[3,6,20,26,29].

We did not observe any difference in the incidence of the deleted LMP-1 variant between different ethnic groups, even though EBV-associated lymphomas are more common in ethnic Indians in Malaysia[18], with the exception of lymphoma of the upper aerodigestive tract[19]. This further supports the notion that the deleted LMP-1 variant is prevalent, and the higher rate of EBV association with some diseases in certain ethnic group is more likely to be due to the host factor. However, the reasons for the higher incidence of deleted variant in lymphoma from male patients when compared to reactive lymphoid hyperplasia from the same gender are not immediately apparent.

Childhood lymphomas in this study, consisting mainly of BLs (52.2%, 12/23) and HLs (43.5%, 10/23), showed the presence of the deleted LMP-1 variant in 95.6% (22/23) of these cases (100% in BLs and 90% in HLs), in contrast to 0% in childhood reactive lymphoid cases ($p<0.001$). There is no immediate explanation why there should be a higher selection of deleted variant in children. However, one may speculate that deleted variant selection in children could be one of the major contributing factors in high incidence of EBV-induced childhood malignancies in developing countries[24]. Our findings of the deleted LMP-1 variant in childhood BLs and HLs are in agreement with reports by Chen *et al.*[23], who showed a high frequency (80%) of the 30-bp LMP-1 deletion

in Brazilian childhood BLs, and Santon *et al*.[30], who reported a frequency of 79.2% of LMP-1 deletion in childhood HLs in Spain.

One hundred percent of the BLs in this study harbored the deleted LMP-1 variant while the LMP-1 protein is not known to be expressed in BLs (latency I)[2,23], raising doubts as to whether the presence of the LMP-1 deletion could be of any importance in the development of BLs. Hence, the high frequency of LMP-1 deletion observed may be directly related to the general observation of higher prevalence of the deleted variant in malignancies. Constitutive expressions of c-myc and EBNA-1 in EBV-associated BL are thought to be more potent transformers, hence the LMP-1 oncoprotein may not be as critical to oncogenesis[3]. T-cell lymphomas in our series showed an overall frequency of 90.6% (29/32) harboring the 30-bp deletion, with 95.0% (19/20) in NK/T-cell lymphomas (Table 3). These findings are also in agreement with reports from Hong Kong (91%)[26], Taiwan (86%)[28] and Japan (96.4%)[27].

One of the most interesting observations of our study is the high prevalence of dual variant infection in our classical HLs (73.7%) : 77.8% in adult and 70.0% in childhood HLs, compared to the reactive lymphoid controls (13.3%). Santon *et al*.[30], likewise reported that 12.8% of adult HLs and up to 50% of their pediatric HLs were harboring EBV of both the 30-bp deleted and the non-deleted types, compared to their healthy adult (26.5%) and children (27.3%). There were no reports of dual LMP-1 variant infection in HL from studies in countries such as Europe[4,12], the United States of America[25,31], Brazil[25], and Italy[32]. However, dual infections were reported in normal donors (5.6%) and HIV-positive patients (11.0%) from Italy[32], normal nasopharynx biopsies (27.0%) and nasal NK/T-cell lymphoma (4.0%) from Hong Kong[26]. All the 30-bp deletion studies were performed using sensitive PCR techniques[4,12,25,30-32]. The observed higher dual variant infection in HL from Malaysia is probably an epi-phenomenon since there is no immediate explanation that can be offered at this point in time.

Our findings together with those of Santon *et al*.[30] appear to contradict monoclonality of EBV in HL[31], and in EBV-associated tumors in general[21] ; however, it must be noted that PCR amplification of whole tissue section cannot differentiate the cellular localization of EBV. HL tissues contain EBER positive bystander B-cells in addition to EBV infected H/RS cells. Hence, the two variants detected in these cases may in fact be originating from tumor cells and bystander B-cells. This hypothesis appears to be supported by Meggeto *et al*.[33] and Faumont *et al*.[34], who showed that the H/RS cells and the bystander cells are infected by two different strains of EBV in their SCID mice and single cell isolation studies, respectively. On the other hand, both Hummel *et al*.[35] and Küppers *et al*.[36] also reported the existence of HL with polyclonal populations of H/RS cells at presentation, which subsequently evolved into clonal population. Hence, the other possible explanation of our observation in HL could be that two distinct variants of virus exist in polyclonal population of H/RS cells, in support of Hummel *et al*.[35]and Küppers *et al*.[36].

Based on the background knowledge of clonal proliferation of cells infected with EBV[21,31] and the 'slipped mispairing' mechanism in the hot-spots of LMP-1 C-terminus[17], we speculate that both variants are probably derived from a single virus strain, although we could not exclude the possibility of a second infection. Hence, the accumulation of mutations, deletions and recombination at the hot-spots of LMP-1 C-terminus take place after repeating cycles of proliferation over a period of time, with deleted variants exceeding non-deleted variants due to selection pressure. This hypothesis can explain the spectrum and varying proportion of these two variants occurring in different groups of diseases. For example, it can explain the facts that classical HLs, which contain large numbers of reactive lymphocytes in the tumor tissue, show higher proportion of dual variant, and diagnostic BL tissues, which tend to contain predominantly tumor cells, show relatively lower proportion of dual variant, or the absence of non-deleted variant. Similar explanation may also be offered to the different patterns observed in the various subtypes of T-NHL.

In conclusion, the 30-bp deletion in the LMP-1 gene is frequently present in Malaysia. The significantly higher percentage of deleted variant observed in EBV-associated lymphomas suggests the possibility of a selection mechanism during malignant transformation.

ACKNOWLEDGMENT

We would like to express our special acknowledgement to Mr. Tan Eng Lai for his technical advice.

This study was supported by the Malaysian Ministry of Science, Technology and Environment R&D research grant, Top-Down Biotechnology, 26-02-03-0586.

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