

Original Article

Epstein-Barr Virus (EBV) Subtypes and Variants in Malignant Tissue from Malaysian Patients

Suat-Cheng Peh¹⁾, Lian-Hua Kim¹⁾, Kein-Seong Mun¹⁾,
Eng-Lai Tan²⁾, Choon-Kook Sam²⁾ and Sibrand Poppema³⁾

Epstein-Barr virus (EBV) is a ubiquitous virus that infects more than 90% of the world's population. In Malaysia this virus has been well documented in patients suffering from nasopharyngeal carcinoma, Hodgkin's lymphoma, and Burkitt's lymphoma. This study investigated the viral subtype and variant through the latent membrane protein-1 (LMP-1) gene taken from EBV-infected cancers in Malaysian patients. Nested polymerase chain reaction assays of EBV nuclear antigen-2 and single step polymerase chain reaction on the C-terminus of the LMP-1 gene were performed for virus typing and detection of a 30-bp deletion, respectively. Samples were obtained from 39 cases of nasopharyngeal carcinoma, 48 cases of Hodgkin's lymphoma, 19 cases of Burkitt's lymphoma and 19 cases of non-neoplastic reactive lymphoid tissues. All the tumors harbored EBV type A. Both the deleted and non-deleted variants were observed in the malignant tissues. In some cases, there was concurrent dual expression of both variants. Statistical analysis showed a significantly higher percentage of 30-bp deletions in cases of malignancies compared to reactive tissues. In conclusion, EBV type A predominates in Malaysian cancers, with a significant representation of the 30-bp deleted variant.

Key words Epstein-Barr virus, nasopharyngeal carcinoma, Hodgkin's lymphoma, Burkitt's lymphoma, latent membrane protein-1, 30-bp deletion

INTRODUCTION

Malignant diseases have always posed a serious problem to practitioners of the medical profession, and hold a morbid fascination and fear for the layman. In the course of exploring these diseases for cures and controls, various probable etiologies have been put forward for consideration, including sources from geographical and environmental causes. One etiological factor, which embraces both causes, is a virus. In 1964, Denis Burkitt and Anthony Epstein discovered a novel herpes virus, with a distinct serological pattern, while investigating endemic Burkitt's lymphoma in Africa. This virus was

named Epstein-Barr virus (EBV) and provided the first evidence of a link between a virus and a human malignancy^{1,2}.

EBV, a double-stranded, 170-kbp DNA virus packaged within an icosahedral capsid surrounded by an envelope, belongs to the human gamma-herpes virus subfamily (HHV-4). The viral genome exists in linear form in mature virions and in circular episomal form in latently infected cells, and encodes for more than 85 genes^{3,4}. This virus is ubiquitous, and infects more than 90% of the human population worldwide with a life-long, asymptomatic, latent infection⁵. Primary infection in children is often subclinical, but infection in later life can give rise to infectious mononucleosis^{1,4}. Its potent B-cell transforming and immortalizing characteristics^{5,6} have implicated this virus in the pathogenesis of many human lymphoproliferative disorders, notably Burkitt's lymphoma (BL)^{7,8}, classical Hodgkin's lymphoma (cHL)^{9,10,11,12}, nasal NK/T-cell lymphoma (NL)^{13,14}, post-transplantation lymphoproliferative disease (PTLD)^{2,15}, lymphomas associated with immunocompromised patients^{16,17}, in addi-

Received : Apr 9, 2003

Revised : Jun 18, 2003

Accepted : Jul 13, 2003

¹⁾Pathology Department, Faculty of Medicine, University of Malaya

²⁾Institute of Postgraduate Studies and Research, University of Malaya

³⁾University of Groningen, Netherlands

Address correspondence and reprint request to Suat-Cheng Peh, Department of Pathology, Faculty of Medicine, University Malaya, 50603 Kuala Lumpur, Malaysia.

tion to a known and close relationship with nasopharyngeal carcinoma (NPC)^{18,19}. Demonstration of replicating EBV in oral hairy leukoplakia is now regarded as pathognomonic for this premalignant condition, and is a prerequisite for confirmation of diagnosis²⁰.

The EBV expresses six nuclear proteins: EBV nuclear antigens (EBNA) -1, 2, 3A, 3B, 3C and leader protein (EBNA-LP); three latent membrane proteins (LMP) -1, 2A and 2B; and two small untranslated RNA species termed EBV-encoded RNA (EBER) -1 and 2^{21,22}. LMP-1 is a transmembrane protein with a 63-kDa phosphoprotein comprised of three domains, that are known to be required to transform Rat-1 fibroblasts²³. A virus with a 30-bp deletion near the C-terminus of LMP1 has an increased tumorigenic potential and is less immunogenic compared with the non-mutated B95.8 prototype²⁴. These characteristics are thought to increase its oncogenic potential.

There are two EBV types, A and B, that are distinguished by genetic polymorphisms in EBNA 2, 3A, 3B and LP, whereby the alleles differ in predicted primary amino acid sequences by 47%, 16%, 20% and 28%, respectively^{3,25}. Previous studies have shown a different frequency of infections by the two EBV types, i.e. EBV-A and -B, as well as expression of the LMP-1 variant in different geographical locations^{7,21,26}. The possible influence these subtypes and variants may have in disease development is not yet clear. EBV-type A predominates in Europe, America, South America and Asia, and type B in Central Africa, New Guinea and in Alaskan Eskimos^{26,27}, as well as in lymphomas associated with immunocompromised patients^{16,27}.

In Malaysia, the presence of EBV has been documented well in patients suffering from NPC (almost 100% EBV-associated)^{4,19,28}, cHL (approximately 60% in adults and more than 90% in childhood cases)^{4,29} and BL (approximately 38%)⁴. This study had the aims of investigating the frequency of EBV types and the LMP-1 gene variant present in EBV-associated malignancies from Malaysian patients.

MATERIALS AND METHODS

Specimens

Archived tissue blocks of EBV-positive malignancies fixed in formalin and embedded in paraffin were retrieved for this study from the Department of Pathology, Faculty of Medicine, University of Malaya. These cases had been confirmed to be EBV-infected by *in situ* hybridization staining, using a probe for the EBV early RNA (EBER, Dako PNA probe, Denmark). Samples were 39 NPC, 19 BL, and 48 cHL (inclusive of 14 NPC and 20 cHL reported previously)^{4,30}. In addition, 19 reactive tonsils and lymph nodes (RLT) were selected for comparative study. Cell lines B95.8 (EBV type A positive, non-deleted LMP-1 variant) and AG876 (EBV type B positive, deleted LMP-1 variant) were used as positive controls for EBV typing and LMP-1 variants, respectively.

Before slicing the blocks the microtome (Leica RM2135, Germany) and work surfaces in the vicinity were cleaned carefully with 100% ethanol, followed by 70% ethanol and then DNA Away (GibcoBRL, USA). This cleaning procedure was adhered to strictly between the slicing of every paraffin block. A disposable blade was used to cut one 5- μ m-thick section from each block, followed by the cleaning procedure as described. The blade was then exchanged for a new one and new gloves were used before preparing the next block. A blank paraffin wax block was cut between every test case to serve as a negative control, and to check for absence of block-to-block contamination during the slicing procedure.

EBV subtyping and LMP-1 gene/30-bp deletion detection

a) DNA extraction

One 5- μ m-thick tissue section from each case was placed in a sterile, plastic 1.5 ml PCR tube, deparaffinized with xylene, rehydrated in decreasing concentrations of alcohol and then air-dried. The tissue was then resuspended and lysed overnight in 200 μ l of digestion buffer containing 1 \times PCR buffer (GibcoBRL) and 200 μ g/ml proteinase K (Boehringer Mannheim, Germany) at 55°C. Digestion was halted by inactivating proteinase K at 96°C for 10 min. Cell debris was pelleted by centrifuging at 10,000 rpm for 5

min. The supernatant was transferred to a new tube and used directly in the PCR procedure. Tubes containing blank block sections were subjected to a similar extraction procedure. DNA was extracted from cell lines using Tri-Reagent (Molecular Research Center Inc., USA) according to the manufacturer's recommendations.

b) Nested PCR for EBV subtyping

A hot-start, nested PCR was employed to enhance the sensitivity and specificity of detection. Consensus oligonucleotide primers were selected from conserved sequences in the U2 region encoding the EBNA-2 gene. A second set of inner primers was designed for the nested PCR. Amplification of EBNA-2 gene was carried using an automated PTC-2000 Peltier thermal cycler (DNA Engine; MJ Research, USA). All primers (Table 1) were synthesized by Genosys (Genosys Products, USA). The amplified product from EBNA-2A is 368-bp and EBNA-2B is 473-bp. First amplification was performed with 1 μ l of supernatant from centrifuged material of tissue samples and blanks, along with 0.5 μ l of DNA from cell lines, in a 50 μ l reaction mixture. This mixture contained 1.5 mM MgCl₂, 0.2 mM dNTP mix (GibcoBRL), 0.5 μ M of each primer and 2.5 U of HotStarTaq® DNA polymerase (Qiagen, Germany) in a standard PCR buffer (Qiagen). Samples were amplified with a first cycle of 95°C for 15 min to activate the HotStarTaq® DNA polymerase, then continued with 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min, with the last extension at 72°C for 5 min. The second, nested, PCR, was performed with 1 μ l of the product from the first PCR reaction using the second pair of primers in a similar reaction mixture, and amplified for 30 cycles with annealing temperature at 60°C. A non-template control, consisting of master mix only, in a similar volume of reaction mixture was included in each run of PCR. Cases not amplified after nested PCR were checked for DNA integrity by performing a PCR on the housekeeping β -globin gene. Products were analyzed by 3% agarose gel electrophoresis.

c) PCR for Detection of LMP-1:

Amplification of the C-terminus of the LMP-1 gene was performed using a pair of primers flanking the characteristic 30-bp deletion region

(Table 1). First, 1 μ l of extracted DNA was used as a template in a reaction volume of 50 μ l, that contained 0.2 mM dNTP mix (Biotools, Spain), 0.5 μ M of each primer in a standard PCR buffer with 1.5 mM MgCl₂ (Qiagen, Germany) and 2.5 U 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. This was continued by 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 then analyzed by 3% agarose gel electrophoresis.

d) Agarose gel electrophoresis

Gel electrophoresis (E-C Electrophoretic Gel System, USA), using 3% agarose gel (GibcoBRL) containing 2 μ g/ml of ethidium bromide (GibcoBRL) in Tris-borate-EDTA (TBE) pH 8.0 buffer at 80 V was used to analyze the PCR products. The products were visualized under a UV light transilluminator (E-C Apparatus, USA). Cases exhibiting a PCR product length similar to B95.8 were interpreted as infected with EBV type A, non-deleted. Samples exhibiting longer PCR products compatible with being a fragment from AG876 were interpreted as infected with EBV type B, and to have a 30-bp deletion in the LMP-1 gene. Cell lines and five randomly selected cases were sent for DNA sequence analysis (Bio Basic Inc., Ontario, Canada, using the ABI Prism 377 sequencer) for confirmation of the specificity of the amplified products.

e) Southern Blot to detect the 30-bp deletion in LMP1

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 h in pre-hybridization buffer (50% formamide, 5 \times SCC, 5 \times Denhardt's solution, 0.02 M 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, but with 45% formamide) at 50°C. A set of 5'-biotin-labeled probes was used, one specific for the deleted region and another flanking the deleted region (Table 1). Visualization was achieved

Table 1. Sequence of primers for PCR

Primers/Probes	EBV B95.8 co-ordinates	Nucleotide sequence
<i>Primers for EBV subtyping by EBNA2 gene</i>		
Outer primers :		
Sense	2771-2789	5'-TTT CAC CAA TAC ATG ACC C-3'
Anti-sense	3149-3130	5'-TGG CAA AGT GCT GAG AGC AA-3'
Inner primers :		
Sense	2777-2796	5'-CAA TAC ATG AAC CRG AGT CC-3'
Anti-sense	3144-3127	5'-AAG TGC TGA GAG CAA GGC MC-3'
<i>Primers for LMP1 PCR</i>		
LMP-FUB2	168259-168278	5'-GAA GAG GTT GAA AAC AAA GGA-3'
LMP-FUE	168163-168183	5'-GTC ATA GTA GCT TAG CTG AAC-3'
<i>Probes for Southern Blot : 30-bp deletion</i>		
5'-biotin-labeled probe specific for deleted region	168259-168278	5'-GCC GTC ATG GCC GGA ATC AT-3'
5'-biotin-labeled probe flanking the deleted region	168311-168330	5'-GGC GGG CCC TGG TCA CCT CC-3'

using streptavidin conjugated with alkaline phosphatase as a secondary antibody (Kirkegaard & Perry Laboratories, USA), and NBT/BCIP (Boehringer Mannheim, Germany) as a substrate.

Statistical Analysis

The data were analyzed by Chi-square (Fisher's exact) test (EpiInfo version 6.04b, Atlanta: Centers for Disease Control and Prevention, USA; 1997). *p* values of less than 0.05 were accepted as statistically significant.

RESULTS

EBV subtyping

All the amplifiable adult (>15 y old) and child (≤15 y) reactive lymphoid tissues (12 out of 12), NPC (14 out of 14), BL (19 out of 19) and cHL (47 out of 48) specimens that underwent EBV subtyping yielded DNA products consistent with that of the 368-bp type-A EBV (Table 2).

LMP-1 30-bp deletion

In the samples comprised of reactive lymphoid tissues, all the amplifiable tissues from childhood patients showed the non-deleted variant, while 9 out of the 10 amplifiable adult reactive lymphoid tissue samples showed the deleted variant (Table 2). Both deleted and

non-deleted variants were observed in the malignant tissues. In some cases, concurrent dual expression of both deleted and non-deleted variants was detected. In child and adult BL all the samples showed either the presence of the deleted variant alone (8 of 12 children and 6 of 7 adults) or the presence of concurrent dual non-deleted and deleted variants (4 of 12 children and 1 of 7 adult cases). The results indicated that all child and adult BL harbor the 30-bp LMP-1 deleted virus. In the child and adult cHL cases the presence of the deleted or non-deleted variant alone was detected, as was concurrent dual expression of both variants (Table 2). An unusual feature of 30-bp LMP-1 deletion expression was noted in the cHL cases: while the presence of the deleted variant alone predominated in other malignancies and reactive lymphoid tissues (reactive lymphoid tissue 46.7%, NPC 84.6%, BL 73.7%), the concurrent presence of dual variants was most commonly observed in cHL (42.5%, Table 2). When the cHL cases were further subclassified, it was noted that all of the 9 cases of the nodular sclerosing type harbored the deleted variant (5 deleted alone and 4 dual variants). The concurrent presence of dual variants was noted in all cHL types, and the presence of a non-deleted variant alone was detected only in the mixed cellularity cHL (Table 3).

Representations of gel analysis results for EBV typing in BL and detection of the 30-bp LMP-1 gene deletion in Malaysian patients with

Table 2. Pattern of EBV subtype and 30-bp LMP-1 deletion variants in Malaysian EBV-associated malignancies and reactive lymphoid tissues

Disease type	Total No. of cases	EBV subtypes		30-bp LMP-1 deletion pattern			
		Amplified cases (%)	Subtype	No. of amplifiable cases (%)	Deleted cases (%)	Dual (%)	Non-deleted (%)
Reactive tissue	19	#12/12(100)	A	15/19(78.9)	7(46.7)	2(13.3)	6(40.0)
Child	6	3/3	A	5/6	0	0	5
Adult	13	9/9	A	10/13	7	2	1
NPC*	39	#14/14(100)	A	39/39(100)	33(84.6)	5(12.8)	1(2.6)
BL	19	19/19(100)	A	19/19(100)	14(73.7)	5(26.3)	0
Child	12	12/12	A	12/12	8	4	0
Adult	7	7/7	A	7/7	6	1	0
cHL	48	47/48(97.9)	A	+40/43(93.0)	12(30.0)	17(42.5)	11(27.5)
Child	13	12/13	A	12/13	3	7	2
Adult	33	33/33	A	27/28	9	10	8
Unknown	2	2/2	A	1/2	0	0	1

Childhood : age < 16 years ; Adult : age(16 years *All cases were from the adult age group ; #, only 12 cases of reactive lymphoid tissues and 14 cases of NPC specimens were analyzed for EBV subtyping ; +, only 43 cHL specimens were analyzed for LMP-1 30-bp deletion ; NPC, nasopharyngeal carcinoma ; BL, Burkitt's lymphoma ; cHL, classical Hodgkin's lymphoma ; dual variant, concurrent presence of deleted and non-deleted LMP-1.

Table 3. Pattern of EBV subtyping and 30-bp LMP-1 deletion variants in classical Hodgkin's lymphoma

Disease type	Total No. of cases	EBV subtypes		30-bp LMP-1 deletion pattern			
		Amplified cases (%)	subtype	No. of amplifiable cases (%)	Deleted cases (%)	Dual (%)	Non-deleted (%)
cHL	48	47/48(97.9)	A	+40/43(93.0)	12(30.0)	17(42.5)	11(27.5)
Mixed cellularity	31	31/31	A	28/29	7	10	11
Nodular sclerosing	13	12/13	A	9/11	5	4	0
Lymphocyte rich	2	2/2	A	2/2	0	2	0
Lymphocyte depleted	1	1/1	A	00	0	0	0
Unclassified	1	1/1	A	1/1	0	1	0

+, only 43 cHL specimens were analyzed for LMP-1 30-bp deletion ; cHL, classical Hodgkin's lymphoma ; dual variant, concurrent presence of deleted and non-deleted LMP-1.

BL, NPC and cHL are shown in Figures 1 and 2, respectively.

Although the statistical analysis showed an overall significantly higher percentage of the 30-bp deleted variant (deleted and dual variant) in malignancies compared with reactive lymphoid tissues ($p=0.014$), significant difference actually involved only BL and NPC ($p=0.004$ and $p=0.001$, respectively), but not cHL ($p=0.513$).

DISCUSSION AND CONCLUSION

EBV is associated with a wide range of human malignancies. However, the contribution

of EBV strains to variation in the pathogenesis of these malignancies is not clear. This study revealed that in both reactive and tumor tissues, all the amplifiable DNA material consistently showed EBV type A. This result is in keeping with our previous observation³⁰, and the findings of other studies in Asia that showed a predominance of EBV type A in normal and neoplastic lesions^{4,14,29}. The clinical implications of this finding in terms of disease progression and prognosis between EBV-type A and B virus infection are still uncertain.

The characteristics of the 30-bp deletion region between amino acids 322-366 near the

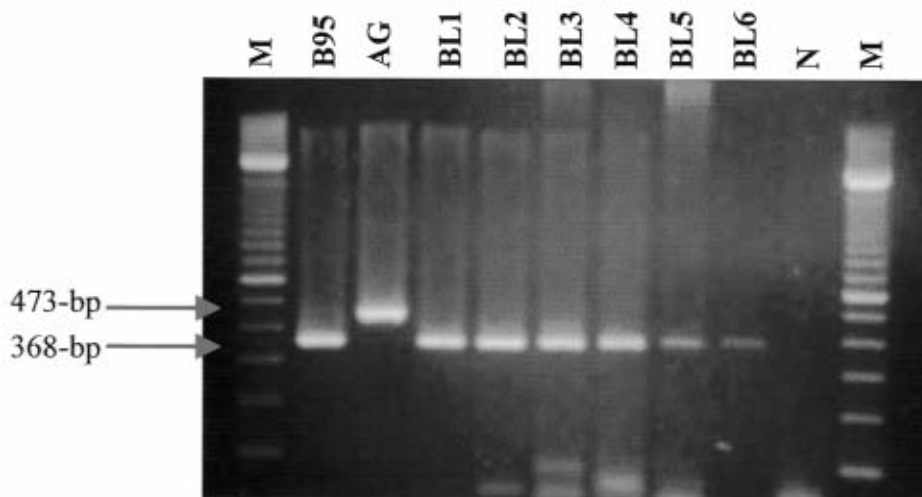


Fig. 1. Representative electrophoresis gel of EBV subtyping in Burkitt's lymphoma M, 100-bp DNA ladder ; B95, B95.8 positive cell lines ; AG, AG876 positive cell line ; BL, Burkitt's lymphoma ; N, Negative control

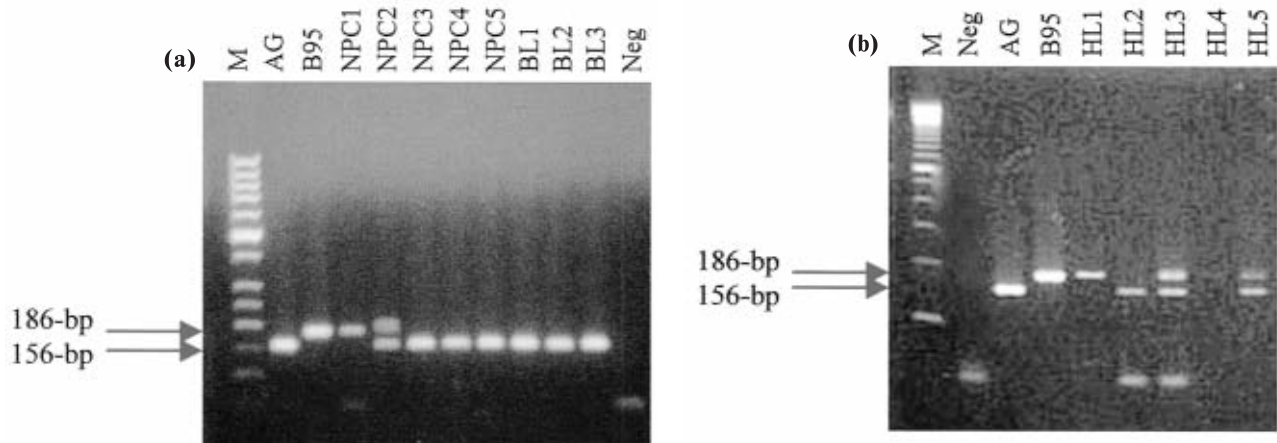


Fig. 2. Representative electrophoresis gel of LMP-1 30-bp deletion in EBV-associated malignancies (a) in nasopharyngeal carcinomas and Burkitt's lymphomas ; (b) in classical Hodgkin's lymphomas M, 100-bp DNA ladder ; B95, B95.8 positive cell lines for non-deleted variant ; AG, AG876 positive cell line for deleted variant ; BL, Burkitt's lymphoma ; NPC, nasopharyngeal carcinoma ; HL, Hodgkin's lymphoma ; Neg, negative control

C-terminus of the LMP-1 gene were first identified in virus isolates derived from Chinese NPC biopsies by Hu and Chen, *et al*²⁴. The three domains of LMP-1 are the short 23-amino acid intracytoplasmic N-terminal domain, six transmembrane domains of the 162-amino acid and a long 200-amino acid C-terminal cytoplasmic tail domain^{31,32}. As with genes in other viruses, LMP-1 shows considerable variation in different EBV isolates and these variants have different oncogenic potentials^{7,24,31}. In addition to EBV possessing potent B cell transforming and immortalizing features^{5,6,24}, studies have shown that

expression of the EBV LMP-1 protein in the established rodent fibroblasts cell line Rat-1 leads to loss of contact and growth inhibition, anchorage independence and tumorigenicity in nude mice³³. LMP-1 can deregulate growth and maturation of human keratinocytes^{24,28}, and can induce expression of epidermal growth factor receptor (EGFR), which is associated with human epithelial tumors, such as NPC³⁴. The LMP-1 C-terminus region can interact with tumor necrosis factor receptor (TNFR) family-associated protein³⁵. Various studies^{5,36,37} have also revealed that LMP-1 increases homotypic adhesion and

up-regulation of various adhesion molecules, such as lymphocyte function-associated antigens (LFA-1 and LFA-3), intracellular adhesion molecule (ICAM-1) and B lymphocyte activation markers (CD23 and CD40), as well as inducing expression of the anti-apoptotic bcl-2 gene³⁸. The characteristics of the 30-bp deletion region between amino acids 322-366, near the C-terminus, confer increased oncogenic potential. Several studies have shown that the deleted LMP-1 variant in EBV-positive HL is associated with increased aggressiveness in tumors^{39,40}.

In the current study, we confirmed results of our earlier study, by Kim and Peh³⁰, that a high proportion of 30-bp LMP-1 deletion variants was observed in neoplastic (NPC, 97.4% ; BL, 100% ; cHL, 72.5%) and reactive lymphoid tissues (60%) than in the non-deleted variant. This picture agrees with reports from other parts of Asia^{13,21}, where the 30-bp LMP-1 deleted variant predominates. This finding may reflect the high prevalence of the deleted variant in local populations. However, the significantly more frequent observation of the deleted variant in malignancies, compared to the reactive tissues ($p=0.014$), suggests a role for the deleted variant in malignant transformation. It was also noteworthy that in the reactive lymphoid tissues the deleted variant was not detected in any of the 5 specimens from children, whereas 9 out of 10 adult cases contained the deleted variant. This suggests that deleted variant, due to its inherently reduced immunogenic capacity, may possess a survival advantage and hence is more frequently involved in the later process of malignant transformation.

Previous reports have indicated that deleted LMP-1 is associated with histologically more aggressive cHL and with the presence of giant atypical Hodgkin/Reed-Sternberg cells^{21,39}. However, there are also reports suggesting that the presence of the deleted variant simply reflects the frequency of the deleted variant in the general population^{41,42}. The relevance of the deleted variant in the pathogenesis of cHL is not supported in the current study because there was no statistically significant difference in the presence of deleted variant in cHL compared to the reactive lymphoid tissues ($p=0.513$). The presence of the deleted variant in cHL may be an epiphenomenon, reflecting a high incidence of this variant in the population. On the other hand, the concurrent presence of both deleted and non-

deleted variants was especially high in Malaysian cHL (16 out of 38 cases), which initially seemed to contradict the theory of monoclonality of EBV in cHL⁹. However, PCR amplification of the entire tissue sections did not point to different cellular origins of the EBV genome. In tumor tissue sections, EBV-positive cHL cells are admixed with bystander, non-neoplastic B-cells, and both these cell populations may harbor different virus variants. On the other hand, the existence of two different variants within the neoplastic cellular population, such as the Reed-Sternberg cells, cannot be excluded totally. The exact origin of the two variants can only be resolved by using precise methods in specimen sampling, such as microdissection techniques, which can isolate individual cells for analysis.

The 4 cases of reactive tissues, which were not amplifiable, were most likely due to insufficient DNA or degradation of DNA material. This loss of DNA material can be caused by the fixation procedure and processing of tissue blocks⁴³.

In conclusion, EBV subtype A dominates in Malaysian cancers, and a significantly higher percentage of these cases showed the presence of the 30-bp LMP1 deleted variant compared to non-neoplastic lymphoid tissue. The latter possibly suggests that selection of the deleted variant occurs in the process of cancer development.

ACKNOWLEDGMENT

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

REFERENCES

- 1 Rickinson AB, Kieff E : Epstein-Barr virus. In : Knipe DM, Howley PM (eds) : Fields Virology. 4th ed. Philadelphia, Lippincott Williams & Wilkins Publishers, 2001, pp 2576-2615.
- 2 Lyons SF, Liebowitz DN : The roles of human viruses in the pathogenesis of lymphoma. *Semin Oncol* 25 : 461-475, 1998.
- 3 Munch M : Epstein-Barr virus strain characterization. *APMIS* 106 : 425-433, 1998.
- 4 Peh SC, Kim LH, Poppema S : Frequent presence of subtype A virus in Epstein-Barr virus-associated malignancies. *Pathology* 34 : 446-450,

- 2002.
- 5 Ring CJA : The B-cell immortalizing functions of Epstein-Barr virus. *J Gen Virol* 75 : 1-13, 1994.
 - 6 Rickinson AB, Young LS, Rowe M : Influence of the Epstein-Barr virus nuclear antigen EBNA2 on the growth phenotype of virus-transformed B cells. *J Virol* 61 : 1310-1317, 1987.
 - 7 Chen WG, Chen YY, Bacchi MM, Bacchi CE, Alvarenga M, Weiss LM : Genotyping of Epstein-Barr virus in Brazilian Burkitt's lymphoma and reactive lymphoid tissue. *Am J Pathol* 148 : 17-23, 1996.
 - 8 Anwar N, Kingma DW, Bloch AR, list all authors : The investigation of Epstein-Barr viral sequences in 41 Cases of Burkitt's lymphoma from Egypt. *Cancer* 76 : 1245-1252, 1995.
 - 9 Vasef MA, Kamel OW, Chen YY, Chen YY, Medeiros J, Weiss LM : Detection of Epstein-Barr virus in multiple sites involved by Hodgkin's disease. *Am J Pathol* 147 : 1408-1415, 1995.
 - 10 Peh SC, Looi LM, Pallesen G : Epstein-Barr virus (EBV) and Hodgkin's disease in a multi-ethnic population in Malaysia. *Histopathol* 30 : 227-233, 1997.
 - 11 Preciado MV, De Matteo E, Diez B, Menarguez J, Grinstein S : Presence of Epstein-Barr virus and strain type assignment in Argentine childhood Hodgkin's disease. *Blood* 86 : 3922-3929, 1995.
 - 12 Ambinder RF, Browning PJ, Lorenzana I, describe all authors : Epstein-Barr virus and childhood Hodgkin's disease in Honduras and the United States. *Blood* 81 : 462-467, 1993.
 - 13 Chang YS, Su IJ, Chung PJ, Shu CH, Ng CK, Wu SJ, Liu ST : Detection of An Epstein-Barr virus variant In T-cell lymphoma tissues identical to the distinct strain observed in nasopharyngeal carcinoma in the Taiwanese population. *Int J Cancer* 62 : 673-677, 1995.
 - 14 Sidagis J, Ueno K, Tokunaga M, Ohyama M, Eizuru Y : Molecular epidemiology of Epstein-Barr virus (EBV) in EBV-related malignancies. *Int J Cancer* 72 : 72-76, 1997.
 - 15 Gratama JW, Zutter MM, Minarovits J, Oosterveer MAP, Thomas ED, Klein G, Ernberg I : Expression of Epstein-Barr virus encoded growth transformation-associated proteins in lymphoproliferations of bone marrow transplant recipients. *Int J Cancer* 47 : 188-192, 1991.
 - 16 Sculley TB, Appoloni A, Hurren L, Moss DJ, Cooper DA : Coinfection with A- and B-type Epstein-Barr virus in human immunodeficiency virus-positive subjects. *J Infect Dis* 162 : 643-648, 1990.
 - 17 DeMario MD, Liebowitz DN : Lymphomas in the immunocompromised patient. *Semin Oncol* 25 : 492-502, 1998.
 - 18 Lung ML, Chang GC, Miller TR, Wara WM, Phillips TL : Genotypic analysis of Epstein-Barr virus isolates associated with nasopharyngeal carcinoma in Chinese immigrants to the United States. *Int J Cancer* 59 : 743-746, 1994.
 - 19 Pathmanathan R, Prasad U, Sadler R, Flynn K, Raab-Traub N : Clonal proliferations of cells infected with Epstein-Barr virus preinvasive lesion related to nasopharyngeal carcinoma. *N Engl J Med* 333 : 693-698, 1995.
 - 20 Walling DM, Perkins AG, Webster-Cyriaque J, Resnick L, Raab-Traub N : The Epstein-Barr virus EBNA-2 gene in oral hairy leukoplakia : Strain variation, genetic recombination, and transcriptional expression. *J Virol* 68 : 7918-7926, 1994.
 - 21 Khanim F, Yao QY, Niedobitek G, Sihota S, Rickinson AB, Young LS : Analysis of Epstein-Barr virus gene polymorphism in normal donors and in virus-associated tumors from different geographic locations. *Blood* 88 : 3491-3501, 1996.
 - 22 Rowe M, Lear AL, Croom-Carter D, Davies AH, Rickinson AB : Three pathways of Epstein-Barr virus gene activation from EBNA1-positive latency in B lymphocytes. *J Virol* 66 : 122-131, 1992.
 - 23 Moorthy RK, Thorley-Lawson DA : All three domains of the Epstein-Barr virus-encoded latent membrane protein LMP-1 are required for transformation of Rat-1 fibroblasts. *J Virol* 67 : 1638-1646, 1993.
 - 24 Hu LF, Chen F, Zheng X, Ernberg I, Cao SL, Christensson B, Klein G, Winberg G : Clonability and tumorigenicity of human epithelial cells expressing the EBV encoded membrane protein LMP1. *Oncogene* 8 : 1575-1583, 1993.
 - 25 Sample J, Young L, Martin B, Chatman T, Kieff E, Rickinson A, Kieff E : Epstein-Barr virus types 1 and 2 differ in their EBNA-3A, EBNA-3B, and EBNA-3C genes. *J Virol* 64 : 4084-4092, 1990.
 - 26 Abdel-Hamid M, Chen JJ, Constantine N, Mas-soud M, Raab-Traub N : EBV strain variation : geographical distribution and relation to disease state. *Virology* 190 : 168-175, 1992.
 - 27 Boyle MJ, Vasak M, Tschuchnigg M, Turner JJ, Sculley T, Penny R, Cooper DA, Tindall B, Sewell WA : Subtypes of Epstein-Barr virus (EBV) in Hodgkin's disease : Association of B-Type EBV and immunocompromise. *Blood* 81 : 468-474, 1993.
 - 28 Dawson CW, Rickinson AB, Young LS : Epstein-Barr virus latent membrane protein inhibits

- human epithelial cell differentiation. *Nature* 344 : 777-780, 1990. 29.
- 29 Peh SC, Sandvej K, Pallesen G : Epstein-Barr Virus (EBV) in Malaysian upper-aerodigestive-tract lymphoma : Incidence and type. *Int J Cancer* 61 : 327-332, 1995.
- 30 Kim LH, Peh SC : Epstein-Barr virus-associated lymphomas in Malaysia : High frequency of a 30-bp deletion in the viral latent membrane protein-1 (LMP-1) oncogene. *J Clin Exp Hematopathol* 43 : 11-19, 2002.
- 31 Leung SY, Yuen ST, Chung LP, Chan ASY, Wong MP : Prevalence of mutations and 30-bp deletion in the C-terminal region of Epstein-Barr virus latent membrane protein-1 oncogene in reactive lymphoid tissue and non-nasopharyngeal EBV-associated carcinomas in Hong Kong Chinese. *Int J Cancer* 72 : 225-230, 1997.
- 32 Eliopoulos AG, Blake SMS, Floettmann JE, Rowe M, Young LS : Epstein-Barr virus-encoded latent membrane protein 1 activates the JNK pathway through its extreme C-terminus via a mechanism involving TRADD and TRAF2. *J Virol* 73 : 1023-1035, 1999.
- 33 Wang D, Liebowitz D, Kieff E : An EBV membrane protein expressed in immortalized lymphocytes transforms established rodent cells (Part 2). *Cell* 43 : 831-840, 1985.
- 34 Miller WE, Earp HS, Raab-Traub N : The Epstein-Barr virus latent membrane protein 1 induces expression of the epidermal growth factor receptor. *J Virol* 69 : 4390-4398, 1995.
- 35 Mosialos G, Birkenbach M, Yalamanchill R, VanArsdale T, Ware C, Kieff E : The Epstein-Barr virus transforming protein LMP1 engages signaling proteins for the tumor necrosis factor receptor family. *Cell* 80 : 389-399, 1995.
- 36 Wang F, Gregory C, Sample C, Rowe M, Liebowitz D, Murray R, Rickinson A, Kieff E : Epstein-Barr virus latent membrane protein 1 (LMP1) and nuclear proteins 2 and 3C are effectors of phenotypic changes in B lymphocytes : EBNA-2 and LMP1 cooperatively induce CD23. *J Virol* 64 : 2309-2318, 1990.
- 37 Wang D, Liebowitz D, Wang F, Gregory C, Rickinson A, Larson R, Springer T, Kieff E : Epstein-Barr virus infection membrane protein alters the human B-lymphocyte phenotype : Deletion of the amino terminus abolishes activity. *J Virol* 62 : 4173-4184, 1988.
- 38 Finke J, Fritzen R, Ternes P, Trivedi P, Bross KJ, Lange W, Mertelsmann R, Dolken G : Expression of bcl-2 in Burkitt's lymphoma cell lines : Induction by latent Epstein-Barr virus genes. *Blood* 80 : 459-469, 1992.
- 39 Knecht H, Bachmann E, Brousset P, Sandvej K, Nadal D, Bachmann F, Odermatt BF, Delsol G, Pallesen G : Deletions within the LMP1 oncogene of Epstein-Barr virus are clustered in Hodgkin's disease and identical to those observed in nasopharyngeal carcinoma. *Blood* 82 : 2937-2942, 1993.
- 40 Knecht H, Bachmann E, Brousset P, describe all authors : Mutational hot spots within the carboxy terminal region of the LMP1 oncogene of Epstein-Barr virus are frequent in lymphoproliferative disorders. *Oncogene* 10 : 523-528, 1995.
- 41 Hayashi K, Chen WG, Chen YY, describe all authors. Deletion of Epstein-Barr virus latent membrane protein 1 gene in United States and Brazilian Hodgkin's disease and reactive lymphoid tissue : High frequency of a 30-bp deletion. *Hum Pathol* 28 : 1408-1414, 1997.
- 42 Essop MF, Engel M, Close P, Sinclair-Smith, C Pallesen. Epstein-Barr virus in Hodgkin's disease : Frequency of a 30-bp deletion in the latent membrane protein 1 (LMP-1) oncogene in South African patients. *Int J Cancer* 84 : 449-451, 1999.
- 43 Pan LX, Diss TC, Isaacson PG : The polymerase chain reaction in histopathology. *Histopathol* 26 : 201-217, 1995.