J. Clin. Exp Hematopathol
Vol. 42, No. 2, Oct 2002

Original Article

The Pattern of Burkitt's Lymphoma in Malaysian Patients

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Endemic and sporadic Burkitt's lymphoma differ in site and age of presentation, and Epstein-Barr virus (EBV) association. This study aimed to describe the clinical presentation, EBV association and p53 expression of Burkitt's lymphoma in 59 Malaysian patients. Expression of Bcl-2, retinoblastoma and Ki67 proteins was also investigated. EBV was detected by EBER *in situ* hybridization. Expression of p53, Bcl-2, pRb and Ki67 proteins was detected by immunohistochemical staining. Polymerase chain reaction was employed for EBV subtyping and detection of translocation t (14 ; 18). The male to female ratio was 3.9 : 1. The most common age group was children younger than 15 years (72.9%), with a mean of 15.8 y. The disease is more common in ethnic Chinese. EBV association is 33.3% and all were infected with type-A virus. Expressions of p53, Bcl-2, pRb and Ki67 proteins were detected in 42/49 (85.7%), 9/54 (16.7%), 47/51 (92.2%) and 35/39 (89.7%) of the biopsies, respectively. Translocation t (14 ; 18) was not detected in cases expressing the Bcl-2 protein. Clinical presentation and EBV association of Burkitt's lymphoma in Malaysian patients corresponds with that of sporadic Burkitt's lymphoma. This study also reveals that a substantial proportion of cases express high levels of p53 and retinoblastoma proteins, in contrast to the Bcl-2 protein.

Key words Burkitt's lymphoma, Malaysia, Epstein-Barr virus, immunohistochemistry

INTRODUCTION

Burkitt's lymphoma, a highly aggressive B-cell malignancy, is a well-defined entity described by strict morphologic criteria of the proposed World Health Organization (WHO) classification scheme[1]. This lymphoma group was first described as a rare jaw sarcoma in African children in 1958 by a British surgeon, Dennis Burkitt[2]. It aroused much interest because jaw tumors of other histological types were rare in African children. A nations-wide survey in Africa countries revealed that the endemicity coincided with the distribution of rainfall, altitude, and malaria holoendemic or hyperendemic regions[3,4]. After the description of this African lymphoma by Burkitt, a tumor with similar histology was recognized in other parts of the world[5]. However, these sporadic cases occur at lower frequency compared to the endemic cases in Africa and Papua New Guinea[6].

Both endemic and sporadic Burkitt's lymphomas carry translocations involving the *c-myc* gene on chromosome 8 with one of the heavy chain or light chain genes of the immunoglobulin gene. However, they display large variation at the breakpoint on chromosome 8[7–9]. Endemic Burkitt's lymphoma almost always breaks at the far 5'-end upstream of the *c-myc* gene, while sporadic tumors contain breakpoints within or close to the first exon of the *c-myc* gene[9]. In addition, differences with regards to age and site of presentation, and rate of association to Epstein-Barr virus (EBV) are also observed in endemic and sporadic Burkitt's lymphoma[7].

The exact role of EBV in the pathogenesis of Burkitt's lymphoma remains uncertain due to the

Received : Jan 11, 2002
Revised : Mar 18, 2002
Accepted : May 20, 2002
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disparity in EBV association in endemic and sporadic Burkitt's lymphoma. Studies on Burkitt's lymphoma showed that EBV is detected in almost 100% of the patients in endemic areas, but only in approximately 15% to 30% of the patients in sporadic areas[7,8]. It has been postulated that EBV is involved in the first step of pathogenesis[10–12], where it immortalizes a group of B-lymphocytes. When these EBV-positive lymphocytes are stimulated by environmental agents, such as chronic holoendemic malaria, they then proliferate as a group of polyclonal pre-neoplastic cells. Continuous antigenic stimulation then increases the probability of recombinase error, which ultimately results in chromosomal translocation involving the *c-myc* gene, thence leading to malignancy. Subsequent aberrant events involving other oncogenes or tumor suppressor genes may occur, driving progression to a high-grade tumor. Studies showed that a tumor suppressor gene, *p53* is frequently mutated in Burkitt's lymphoma[13,14].

This current study describes Burkitt's lymphoma from patients in Malaysia with regard to clinical presentation, EBV association, EBV subtype, and expression of the p53 protein. In addition, the expression of Bcl-2 and retinoblastoma (pRb), and Ki67 proteins were also investigated.

MATERIALS AND METHODS

Patients' material

Records of cases previously diagnosed as Burkitt's lymphoma were retrieved from the archives. Hematoxylin and eosin stained slides of 67 biopsies from 64 patients were reviewed for confirmation and classification according to the criteria from the WHO-proposed list of lymphoid neoplasms. Of these 67 biopsies, 5 (from 5 patients) were reclassified as diffuse large B cell lymphoma and hence excluded from further analysis. Seven cases (from 7 patients) from the remaining 59 patients were without tissue blocks for immunohistochemical staining. The demographic data of these patients was obtained from an information sheet volunteered by the clinicians. Serial, 3-μm sections of the remaining 55 biopsies from 52 patients with available tissue blocks were used for further analysis by immunohistochemical staining and *in situ* hybridization.

Immunohistochemical staining

Immunohistochemical staining was performed with a panel of antibodies, from Dako (Denmark), unless otherwise specified: CD3, CD20 (L26), Bcl-2 (clone 124), p53 (clone DO-7), pRb (clone G3-245, PharMingen), and Ki67. Antigen epitopes were retrieved for all of the antibodies by the microwave heat-inducing method. A standard, three-step immunoenzymatic staining method was used to localize the antigens, except for Bcl-2 and p53, which were localized using a two-step staining procedure (DAKO EnVision+ System).

In situ hybridization

The presence of EBV was detected by the in situ hybridization (ISH) technique, with fluorescein-conjugated EBV oligonucleotide probes (NCL-EBV, Novocastra, United Kingdom) for EBV early RNA (EBER). Alkaline phosphatase-conjugated rabbit anti-FITC was then added, followed by introduction of a substrate, 4-nitro-blue-tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP). The tissue sections were counterstained with Meyer hematoxylin. A known EBV-positive nasopharyngeal carcinoma was used as an external positive control for assessment of adequacy of each batch in the staining procedure.

DNA extraction

DNA was extracted from formalin-fixed, paraffin-embedded tissues using proteinase K (200 μg/mL) digestion. The supernatant containing DNA was used directly for PCR amplification.

EBV subtyping

EBV subtyping was carried out when the virus was detected by *in situ* hybridization. Amplification of the EBNA-2 gene was performed according to a nested PCR procedure, by using the two primer pairs: a) the outer primer pair[15] (sense: 5' TTT CAC CAA TAC ATG AAC C 3'; anti-sense: 5' TGG CAA AGT GCT GAG AGC AA 3'); and b) the inner primer pair (data in preparation for publication) (sense: 5' CAA TAC ATG AAC CRG AGT CC 3'; anti-sense: 5'

AAG TGC TGA GAG CAA CCG MC 3'). These primer pairs generated products of 368-bp for type A, and 473-bp for type B virus. Amplification was performed using 1 μl of DNA samples in a 50-μl reaction mixture containing 2.5 U of *Taq* DNA polymerase (Gibco BRL, USA), 1.5 mM $MgCl_2$, 0.2 mM dNTP mix (Biotools, Spain), and 0.5 μM of each primer in 1X PCR buffer (Gibco BRL, USA). *Taq* DNA polymerase was added after a hot-start step, followed by 30 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 final extension at 72°C for 5 min. Nested-PCR using the inner primer pair was carried out with 1 μl of the PCR product under the same conditions and annealing temperature at 60°C for 30 cycles.

Detection of t (14 ; 18) by PCR

Detection of t (14 ; 18) by PCR was performed on cases that expressed Bcl-2 by immunohistochemical staining. A nested-PCR previously described by Gribben *et al*.[16] were employed to amplify the major breakpoint region (mbr) of *bcl*-2 gene. The size of the PCR products ranges from 100 bp to 300 bp. A cell line with known mbr breakpoint, RL-7 (kindly provided by Dr John C. Chan of University of Nebraska Medical Center, USA) was used as a positive control. PCR products were run on 2% agarose gel (GIBCO BRL, USA) containing 5 μg/ml ethidium bromide (GIBCO BRL, USA), and was visualized on a UV illuminator.

RESULTS

There are 47 males and 12 females from a total of 59 patients, for a male to female ratio of 3.9 : 1. Ethnic Chinese formed the largest group constituting 52.9% (31/59) of the patients. This is followed by Malay (16/59, 27.1%), Indian (3/59, 5.1%), and others, which included the indigenous groups of Sabah and Sarawak (9/59, 11.9%). The majority are children less than 15 y old (43/59, 72.9%), with a mean age of 15.8 y (Fig. 1). The main sites of presentation are the lymph nodes (24/62, 38.7%) and abdominal viscera (19/62, 30.2%). Other common sites included the testes and ovary (4, 6.5%), gum and maxilla (4, 6.5%), tonsil (2, 3.2%), nose (2, 3.2%), and brain (2, 3.2%). The remaining 5 (8.1%) biopsies were taken, one each,

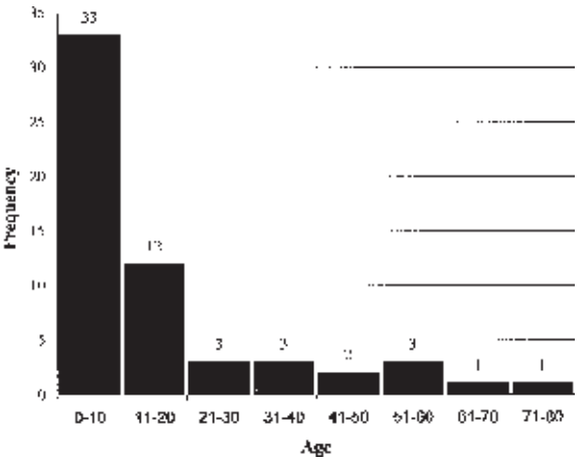

Fig. 1. Age distribution of Burkitt's lymphoma in Malaysian patients

TABLE 1. Association of EBV according to ethnic groups

ethnic group	EBV association			
	Total No. of patients	Positive (%)	Negative (%)	Association rate
Chinese	28	7	21	25%
Malay#	12	6	6	50%
Indian	3	0	3	0%
Others*	8	4	4	50%
Total	51	17(33%)	34(66%)	

#Does not include one non-reactive case
*Includes the indigenous groups in Sabah and Sarawak

from other sites (orbit, bone marrow, soft tissue of neck, upper thigh, and the infra-spinal region).

Seventeen (17/51, 33.3%) of the patients were infected with EBV (Table 1), and one case was not assessable by ISH for detection of EBER. Among the EBV-positive cases, 11 of 17 were children younger than 15 y, 5 of 17 were adults and one patient lacked information on age. There were 23 childhood and 11 adult tumors not infected by EBV. The ratio of children to adults in EBV-positive and EBV-negative patients was 2.2 : 1 and 2.1 : 1, respectively, with no correlation between the age and EBV-association in the tumors.

The rate of association with EBV in patients' material was found to be higher in Malay (50%) than in Chinese (25%) compared to zero among Indians. Burkitt's lymphoma from East Malaysia (Sabah and Sarawak) showed a higher association with EBV (4/8, 50%), than those from West Malaysia (13/43, 30%). Subtyping of EBV by PCR showed that all of the EBV-associated tumors were infected with type-A virus, irre-

spective of the ethnic origin of patients (Fig. 2).

Six of the EBV-positive biopsies were taken from abdominal viscera, 4 from lymph nodes, 3 from the gum and maxilla, and one each from the tonsil, brain, upper thigh, and infra-spinal region. The association rate of EBV and site of biopsy was 32% (6/19), 17% (4/24), and 75% (3/4) for abdominal viscera, lymph node, and gum and maxilla, respectively. The EBV-positive rate was significantly higher in biopsies from the gum and maxilla when compared to those from the abdominal viscera and lymph node.

Immunohistochemical staining was performed on 55 biopsies (from 52 patients) with available tissue blocks. The percentage of positive tumor cells for p53, pRb and Ki67 staining was performed by inspection. Staining for Bcl-2 was recorded as either positive or negative. Positive expression was scored when more than 10% of the tumor cells showed positive staining. Of the 55 biopsies analyzed, 7 cases showed negative p53 expression, and 6 cases were unreactive for p53 staining. The remaining 42/49 (85.7%) cases showed a high proportion of the tumor expressing p53 protein at various intensities (Table 2). In 24 (57.1%) of these 42 cases, more than 75% of the tumor cells expressed the p53 protein.

Bcl-2 protein was detected in 9/54 cases (16.7%), and one biopsy could not be assessed. PCR analysis did not detect the presence of translocation t (14 ; 18) involving mbr in any of these 9 cases, 3 (33.3%) were associated with EBV.

Four cases were considered negative for pRb expression since fewer than 10% of the tumor cells expressed pRb, while another 4 were did not react to pRb stain. Expression of pRb was detected in all of the remaining cases (47/51, 92.2%).

Ki67 expression was assessable in 39 (70.9%) of the cases because 16 (29.1%) were not immunoreactive. Among the 39 cases, 29 (74.4%) expressed Ki67 in more than 50% of the tumor cells, 6 (15.4%) expressed Ki67 in fewer than 50% of the tumor cells, and 4 (10.3%) expressed Ki67 in fewer than 10% of cells. Ki67 was detected in more than 50% of the tumor cells in all cases expressing Bcl-2. The expression of Ki67 also showed a positive correlation with the expression of p53.

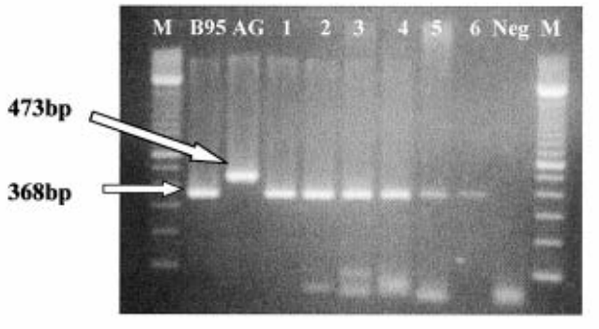

Fig. 2. Expression of Ki-67 antigen in MALT lymphomas
A, interfollicular area of grade 2 ; B, interfollicular area of grade 4 ; C, interfollicular area of grade 5 ; D, follicular colonization of grade 5.

TABLE 2. Staining pattern for p53 and pRb in Burkitt's lymphoma from Malaysian patients

Staining value	p53 expression		pRb expression	
	No.	%	No.	%
$<10\%$	7	13	4	7
$10\% \leq \chi < 25\%$	3	6	1	2
$25\% \leq \chi < 50\%$	8	15	6	11
$50\% \leq \chi < 75\%$	7	13	9	16
$75\% \leq \chi < 90\%$	19	35	19	35
$\geq 90\%$	5	9	12	22
Non-reactive	6	11	4	7
total	55	100	55	100

DISCUSSION

Endemic and sporadic Burkitt's lymphoma share a similar histological appearance, but differ in clinical presentations[3,4,7]. Both African and sporadic Burkitt's lymphoma frequently involve the abdominal viscera (50 to 60%), however, the jaw is rarely involved in sporadic Burkitt's lymphoma. African Burkitt's lymphoma peaks between the ages of 5 and 10 y, with a mean age of 7 ; while the sporadic type peaks at 17 y.

This series revealed only 4 cases (6.5%) with jaw and facial bone presentation. This rate agrees with experiences from the USA[4], Europe[17], Taiwan[18], and Hong Kong[19]. The common lymph node presentation (24/62, 38.7%) contrasts sharply with the rate in endemic areas, where fewer than 1% of cases present in the lymph nodes[8].

Children younger than 15 y old formed the largest group among our patients. This was similar to reports that Burkitt's lymphoma is one of the most common childhood lymphomas[17,20]. In Malaysia, Burkitt's lymphoma constitutes approximately 37.0% of childhood NHL (data in preparation for publication), and only about 5.0% of adult NHL[21]. The mean age at presentation

(15.8 y) in Malaysian Burkitt's lymphoma is intermediate between the pattern for cases from Africa (7 y) and USA (11 y)[4], and in other countries in Asia, such as Taiwan (33 y)[18], Hong Kong (35.5 y)[19], and Japan (28 y)[22].

Almost all endemic Burkitt's lymphomas are associated with EBV. Although more than 75% of patients with sporadic Burkitt's lymphoma are EBV seropositive, the association with EBV in the tumors is low, ranging from 15% to 30%[7]. Moreover, the association of EBV with sporadic Burkitt's lymphoma shows geographical variations. It has been reported to be as low as 5.0% in Europe and the USA[7,17], and as high as 73% in Egypt[23]. Asian countries, such as Taiwan[18], Hong Kong[19], and Japan[22], have reported association rates of 42.3%, 28.0%, and 13.0%, respectively. This study of Malaysians showed an association of 33.3% that fell within the Asian range.

EBV can be classified into two subtypes based on the polymorphisms in their nuclear antigen (EBNA)-2 genes. It has been reported that type A predominates in Europe, US, and Asia, while type B is restricted to Central Africa, La Réunion, and Papua New Guinea[24,25] and in immunocompromised patients[26,27]. EBV subtyping in the current series showed that all of the tumors were infected with type A virus. It is unknown why Burkitt's lymphoma in other parts of the world is rarely associated with type B virus, although studies have indicated that type B virus is also found in healthy populations, with almost equal frequency in the USA[28], and less commonly in Japan[29]. It was suggested that prevalence of type A and type B virus may simply reflect the relative frequency of these viruses in the population[28].

Hence, taking into account all the clinical presentations: (a) low frequency of jaw tumors, but high frequency of lymph node and abdominal involvement; (b) mean age of presentation around 16 y old; and (c) intermediate EBV association rate (33.3%), we concluded that Malaysian Burkitt's lymphoma differs from African Burkitt's lymphoma, and conforms more closely to sporadic Burkitt's lymphoma, despite early EBV infection in the population[30,31] and the endemicity of malaria[32,33].

Wild-type p53 protein is present at low levels in cells and has a short half-life. However, mutation of the *p53* gene results in conformational changes of the protein that stabilize the protein and lead to a longer half-life. These mutant p53 proteins therefore will accumulate in the cells, making it possible for them to be detected by immunohistochemical staining[34,35]. Mutations in the *p53* gene occur frequently in Burkitt's lymphoma, *i. e.* 63% of Burkitt's lymphoma cell lines, and 33% of primary tumors[14]. Studies have shown that mutation of the *p53* gene are more commonly found in high-grade NHL, leading to the hypothesis that p53 is involved in the progression, rather than the initiation, of tumors[13,35]. This is in accord with the proposed form of pathogenesis, where the tumor suppressor gene is involved in the final step of the pathogenesis[10–12]. The p53 protein was detected frequently in this series (42/49, 76%). The explanation for the high rate of expression in Malaysian Burkitt's lymphoma awaits mutational analysis of *p53* gene.

The *bcl*-2 gene on chromosome 18 is involved in t (14 ; 18) translocation, which is present in a large majority of follicular lymphomas (85% to 90%) and 15% to 40% of diffuse large B-cell lymphomas[36,37]. Yano, *et al*.[38] and Karson, *et al*.[39] reported a subgroup of B-cell malignancy that carries both Burkitt-type translocations involving the *c-myc* gene with one of the immunoglobulin heavy or light chain genes, and translocations involving the *bcl*-2 gene, t (14 ; 18). In the current series, 9 of the 54 assessable cases (17%) expressed the Bcl-2 protein. However, PCR analysis showed that none of these cases carried the t (14 ; 18) translocation. Although translocation may occur in the minor cluster region (mcr), it is unlikely to account for our results because the involvement of mcr is far less common[37].

Functional loss of the translated protein from the retinoblastoma gene, another tumor suppressor gene first described for its involvement in the development of retinoblastoma, is related to a variety of other solid tumors[40]. Loss of pRb has been reported as one of the most frequently observed abnormalities in lymphoid malignancy[41]. Weide, *et al*.[42] reported loss of pRb expression in five out of eight (63%) cases of Burkitt's lymphoma. However, loss of pRb was found in only 4 cases (8%) in the current study. This finding is similar to the report by Martinez, *et al*.[43], where pRb expression was detected in 9/9 cases of Burkitt's lymphoma. Studies of other lymphomas, such as follicular lymphoma[44] and a cohort of 103 low grade and high grade

lymphomas[45], also gave similar results. Therefore, we concluded that loss of pRb is an uncommon phenomenon in lymphomas, and it probably does not play a significant role in the pathogenesis of Burkitt's lymphoma.

In conclusion, Burkitt's lymphoma in Malaysian patients expresses the same features as the sporadic type, *i. e.* a low rate of jaw involvement, mean age of 16 y old, and intermediate EBV association. Immunohistochemical staining of p53, Bcl-2 and pRb protein expressions showed no disparity from other reports, p53 and pRb proteins are expressed in a large majority of the cases, in contrast to the Bcl-2 protein.

Acknowledgements

This study was supported by the Malaysian Ministry of Science, Technology and an Environment IRPA research grant (06-02-03-0576). The authors are grateful to Dr John Chan, Department of Pathology and Microbiology, University of Nebraska Medical Center, USA for providing the cell line, RL-7.

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