Association of the Epstein-Barr Viruses with Hodgkin Lymphoma: An Analysis of Pediatric Cases in Thailand

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An investigation as to whether any association of pediatric HL in Thailand was likely to be EBV positive was performed on formalin-fixed paraffin embedded tissue sections using in situ hybridization for EBV encoded RNA (EBER) technique. The analysis was performed on 15 cases. They were 11 male and 4 female cases. The subtypes of HL according to WHO classification were nodular lymphocyte predominance in 1 (6.6%), nodular sclerosis in 4 (26.6%), mixed cellularity in 9 (60%) and lymphocyte depletion in 1 (6.6%). EBV encoded RNA by in situ hybridization was demonstrated in 92.8% of classic HL: 3 of 4 (75%) with nodular sclerosis; 9 of the 9 with mixed cellularity (100%) and 1 of 1 (100%) with lymphocyte depletion. Case of nodular lymphocyte predominance was negative for EBV, CD 15 CD 30 and positive for CD 20. CD 15 and CD 30 were positive in 78.6% and 85% respectively for classic HL.Our results suggest a strong association of EBV with pediatric classic HL (92.3%) particularly the mixed cellularity subtype (100%). The result confirms the male predominance in pediatric HL. Mixed cellularity is the most common subtype of HL in our series (60%).

Keywords: *Epstein-Barr viruses, Hodgkin lymphoma, Pediatric cases, Thailand*

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Epstein-Barr virus (EBV) is well known to be associated with a spectrum of diseases including endemic Burkitt's lymphoma, lymphoproliferative disorders in immunologically compromised individuals, Hodgkin lymphoma (HL), virus associated hemophagocytic syndrome, certain forms of T cell lymphoma, nasopharyngeal carcinoma and some gastric carcinoma[1]. EBV genomic DNA was first detected in HL in 1987[2] and subsequently the finding was confirmed by many groups of investigators[3-6]. Several studies have shown an increased incidence of EBV positivity in the pediatric HL[8-10]. The subtypes of pediatric and adult HL that are commonly associated with EBV are mixed cellularity[11-17] and interfollicular subset[14], inspite of the high frequency of nodular sclerosis or lymphocyte predominant subtypes in pediatric HL[10-17].

Frequency of EBV encoded RNA or latent membrane protein -1 in various subtypes of HL has been investigated in many countries[9,11,17]. The EBV is associated with HL in approximately one third of cases in developed countries and higher in developing countries[9,17]. To the authors' knowledge published data analized particulary in Thai children have not been documented. Therefore, the frequency of the association of the EBV and HL in Thai children is not known.

The aim of the present study was to investigate the association of EBV and various subtypes of pediatric HL in Thailand by in situ hybridization for EBV encoded RNAs (EBER) technique.

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Material and Method

All pediatric cases, (aged up to 15 years) recorded as HL in the files of the Institute of Pathology, Thailand, during the period 1992 through 2001 were retreived. Specimens were obtained from the patients admitted to Queen Sirikit National Institute of Child Health (formerly Children's Hospital) and 3 specimens were obtained from 3 provincial hospitals in Thailand. Sixteen cases were retrieved, one was

excluded because of the unavailability of the paraffin blocks. The morphologic criteria for the diagnosis and classification were those of WHO 2001 classification. The diagnosis of HL was confirmed in all 15 cases after a review of histological slides and immunohistochemical studies. Specimens were lymphnodes alone (12) lymph node and liver biopsy (1), lymphnode and skin biopsy (1) and spleen (1).

Immunohistochemistry

Immunohistochemical studies were performed using formalin fixed paraffin embedded tissue sections. Paraffin sections were cut at 3 μm, mounted on aminopropyltriethoxysilane coated slides and dried at 50 C in an oven overnight. The panel of antibodies and dilutions used were as follows: anti CD3 (Dako, polyclonal) 1:100, CD20, L26 (Dako) 1:1500, CD30 (Novocastra) 1:150 and CD 15 (Becton Dickinson) 1: 25

After deparaffinization and rehydration, sections were blocked for endogenous peroxidase by 3% hydrogen peroxide in distilled water for 10 minutes. The sections were placed in a boiling solution of 0.01 M citrate buffer pH 6.0 in a domestic pressure cooker (Kuhnrikon, Switzerland) at full pressure for 2.30 minutes. After heating, sections were allowed to cool for 15 minutes and then were rinsed for 5 minutes with water and washing buffer (Tris - PBS - Tween 20 buffer). Sections were incubated with primary antibody in antibody diluent (DAKO) overnight at 4 C, rinsed three times in washing buffer and subjected to the detection system, using Envision method. Goat anti mouse or goat anti rabbit Envision-HRP enzyme conjugated was performed for 30 minutes at room temperature. The sections were then washed three times in buffer and incubated at room temperature with 3,3' diaminobenzidine tetrahydrochloride and hydrogen peroxide for 10 minutes. After the last wash in tap water, the slides were dipped in distilled water before counterstaining with Mayer's hematoxylin (45 second). Finally the slides were dehydrated through graded alcohols to xylene and mounted in Permount. (Fisher Scientifiic, USA).

In situ hybridization

Sections from paraffin embedded tissue, 3 μm thick were mounted on slides coated with 3-aminopropyltriethoxysilane (Sigma chemical Co., St Louis MO) and incubated at 50°C overnight. Deparaffinized and rehydrated sections were treated with 100 μ of proteinase K in 0.05 M Tris/HCI buffer pH 7.6 and incubated for 30 minutes at 37°C. The sections were then washed in distilled water, dehydrated through a graded series of ethanol and air dried. For hybridization, each section was covered with fluorescein labelled oligonucleotide cocktail probe for the detection of Epstein-Barr virus encodes RNA (EBER) sequences (Hybaid, UK). The slides were covered with coverslip and sealed with nail enamel. Hybridization was carried out at 37°C for 16-20 hours. After removal of the coverslip, the slides were washed in 50 mM Tris /HCI, 150 mM NaCI pH 7.6, containing 0.1% vivo Triton XI00 at room temperature for 3 times, 3 minutes, each.

For detection, the sections were incubated in 50 mM Tris /HCI, 150 mM NaCI containing 3% wlv BSA and 0.1% vivo Triton XI00 for 10 minutes. The blocking solution was blotted from the slides before sequential treatment with rabbit F (ab') anti FITC conjugated to alkaline phosphatase diluted 1:100 for 1 hour. The sections were then incubated overnight in a mixture of 5 bromo- 4 chloro-3 indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) in the dark at room temperature. The slides were rinsed in distilled water and counterstained in Mayer's hematoxylin for 10 seconds. The counterstained slides were washed in distilled water, air dried and mounted in Permount (Fisher Scientific).

Results

The ages of the 15 pediatric HL ranged from 11 months to 15 years with a peak at 4 to 6 years of age and declined towards adolescence. EBV was considered positive when EBER was identified in the RS cells. EBV was demonstrated in 100% of patients with the age of 4-9 years old (Fig. 1). There were 11 boys and 4 girls with a male to female ratio of 2.75:1. All the patients were Thai.

The clinical information, histologic subtypes, immunophenotypes and EBER results are shown in Table 1. The mixed cellularity (MC) was the most common subtype, accounting for 60% of the cases, followed by the nodular sclerosis (NS) subtype with 26.6%, nodular lymphocyte predominant (NLP) subtype with 6.6% and lymphocyte - depleted (LD) subtype with 6.6%.

The immunohistochemical results of the classical HL for Reed Sternberg cells (RS) and variants showed positivity of CD 15 in 11 of 14 cases (78.6%) and CD 30 in 12 of 14 cases (85.7%). None of the cases expressed CD3 and CD20. Only one case of NLPHL demonstrated CD20 positive, CD15 negative and CD30 negative RS.

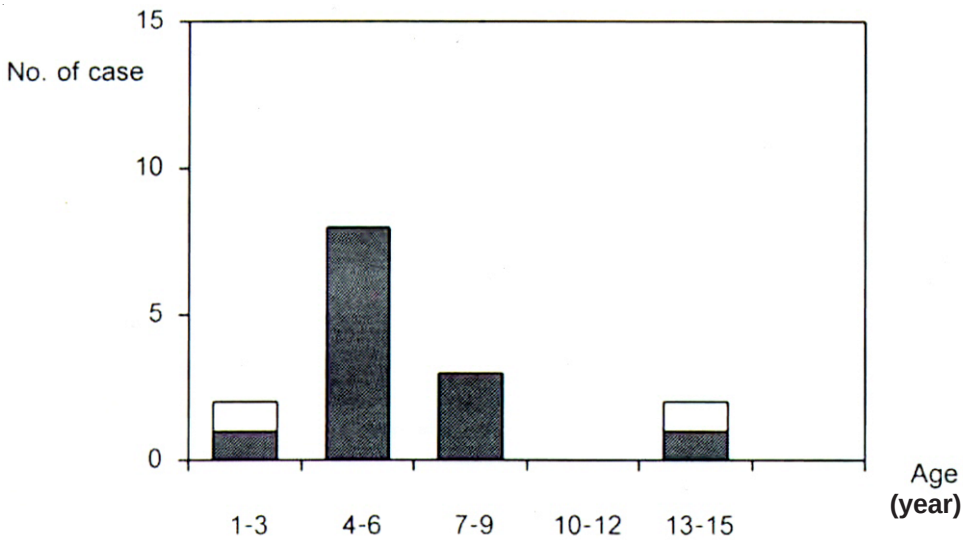

Fig. 1 Distribution of age and percentage of EBV positivity (shaded areas) in childhood Hodgkin lymphoma

All cases were tested for EBER by in situ hybridization (Fig. 2). The EBER positive rate for the pediatric classical HL was 13 of 14 cases (92.8%). The only negative case for EBER was a 13 year old boy with NSHL subtype that expressed CD15 and CD30 in the RS. The case of NLPHL was EBV negative.

Discussion

The incidence of HL varies among different countries. It accounts for 20% or more of all malignant lymphomas in Western countries but about 6-18% of those in Asian Countries[18-20]. The incidence of HL in Thailand is 7.9% (157/1983 cases) of all malignant lymphoma[21]. The present series comprised 15 cases of pediatric HL, 13 of the cases were from the Queen Sirikit National Institute of Child Health and accounted for 0.15% of surgical specimens and 23.5% of pediatric lymphoma. The incidence of HL in the present series was comparable to that of Korea[22].

A male preponderance of 73.3% or M:F 2.75:1 in the present series was similar to most of previous reports[14,17,23]. The age peak of the present

Table 1 : Clinical Information, Histologic subtypes, Immunophenotypes and EBER results

Age/ Sex	HL subtype	CD 15	CD 30	CD 20	CD 3	EBER
11/12/ F	NLPHL	-	-	+	-	-
4/ M	NSHL	+	+	-	-	+
5/ M	NSHL	-	-	-	-	+
6/ M	NSHL	+	+	-	-	+
13/ M	NSHL	+	+	-	-	-
3/ M	MCHL	+	+	-	-	+
4/ M	MCHL	+	+	-	-	+
4/ M	MCHL	+	+	-	-	+
5/ M	MCHL	-	+	-	-	+
6/ F	MCHL	+	+	-	-	+
7/ F	MCHL	+	+	-	-	+
8/ F	MCHL	+	+	-	-	+
9/ M	MCHL	-	+	-	-	+
15/ M	MCHL	+	+	-	-	+
5/ M	LDHL	+	-	-	-	+

NLPHL, Nodular lymphocyte-predominant HL; NSHL, Nodular sclerosis HL;
MCHL, Mixed cellularity HL; LPHL, Lymphocyte-depleted HL;
+, Positive staining of Reed Sternberg cells and variants; -, No staining of Reed Sternberg cells and variants

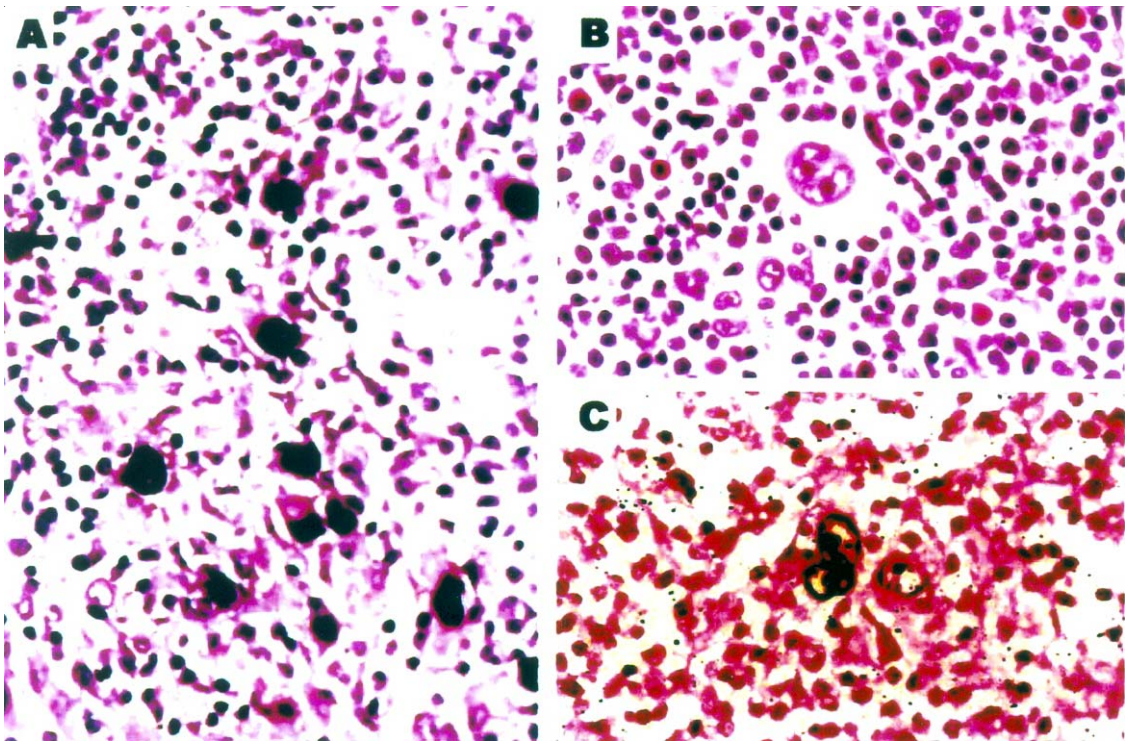

Fig. 2 A) In situ hybridization for EBER showing signal in the nuclei of Reed-Sternberg cells and variants (original magnification x 400)
B) Reed Sternberg cell (Hematoxylin-eosin stain; original magnification x 600)
C) Reed Sternberg cell shows nuclear labelling for EBER (in situ hybridization; original magnification x 600)

case occurred earlier between the age of 4-6 years; when compared to the first age peak at 7 and 12 years in developing countries and different from industrialized countries where the initial peak was delayed until adulthood[24-26]. EBV was present in all cases during the age peak (4-6 year) and all of them were either MCHL or NSHL.

MCHL was the most common subtype in the present series accounting for 60% followed by NS(26.6%). NLPHL and LDHL are the least common with one case in each type. When compared with a previous report of malignant lymphoma in Thailand, the predominant MCHL was also seen in 9 pediatric HL with 2 NSHL and 7 MCHL[27]. Among the Asian countries, the most prevalent MCHL in children was seen in Malaysia and Korea[28,29]. The results were different from those in Western countries where NSHL or NLPHL was the most common subtype[14-17].

EBV was demonstrated in 92.3% of classical HL in the present series and involving all subtypes. The percentage of EBV positivity in Thai children in the present report was much higher than a previous report from Thailand (64%) which included all age groups. Pediatric and adult cases were not separately studied in this previous report[30]. In recent reports, cases of pediatric HL from Malaysia, Peru, Kenya, Honduras, China, Greece and Nagasaki, Japan showed positivity in 90-100%[9,28,31-35]. Among other Asian countries, high percentage of EBV positivity in pediatric HL was also noted in Hong Kong (4 of 5 cases, 80%) and Korea (12 of 16 cases, 75%). The results were different from Taiwan (4 of 6 cases, 66.7%) and Philippines (4 of 8 cases, 50%)[29,36-38]. A recent report on the role of EBV in pediatric HL from 10 geographical area, the proportion of latent membrane protein - l positive case varied, being 50% of case from the United Kingdom (38 of 75 cases), South Africa (9 of 18 cases), Egypt (7 of 14 cases) and Jordan (8 of 16 cases); 60% from the United Arab Emirates (6 of 10 cases); 70% from Australia (11 of 16 cases); 81% from Costa Rica (34 of 42 cases); 88% from Iran (7 of 8 cases); 90% from Greece (20 of 22 cases) and 100% of 56 cases from Kenya[34]. EBV has been demonstrated in 36% -54% of pediatric HL in Western countries with the highest proportion in children younger than 9 -10 years of age and a strong

association of MCHL[9,17,23]. Thus EBV shows an association with pediatric HL but the involvement varies according to the geographical, socio-economic, environmental or genetic conditions.

Two pathogenic mechanisms probably influenced by the presence of EBV in the Reed-Sternberg cells and variants are: Resistance of the RS cells to apoptosis; and escape of RS cells from a cytotoxic T lymphocytes mediated immune response. Latent membrane protein-l is implicated as the most likely EBV encoded protein responsible for this EBV mediated pathogenic effect[39]. The present study supported that EBV played an important role in the pathogenesis of pediatric classical HL in Thailand.

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ความสัมพันธ์ระหว่างเอ็บสไตน์ บาร์ ไวรัส และมะเร็งต่อมน้ำเหลือง ชนิดฮอดชกิน: การวิเคราะห์ในผู้ป่วยเด็กในประเทศไทย

วิจิตรา เหมศรีชาติ, จารุพรรณ ปิ่นทอง

โครงการนี้เป็นการศึกษาความสัมพันธ์ระหว่างมะเร็งต่อมน้ำเหลือง ชนิดฮอดชกิน ในผู้ป่วยเด็กในประเทศไทย และเอ็บสไตน์ บาร์ ไวรัส โดยการใช้ชิ้นเนื้อที่แช่ในฟอร์มาลิน และฝังในพารัฟฟิน โดยใช้วิธีทางโมเลกุลพยาธิวิทยา การวิจัยพบว่ามีผู้ป่วยทั้งหมด 15 ราย เป็นชาย 11 คน และหญิง 4 คน ชนิดของฮอดชกิน แบ่งเป็นมีลิมโฟซัยด์มาก 6.6% ชนิดโนดูลา สเคลอโรซีส 26.6% มิกซ์เซลลูลาลิตี 60% และลิมโฟซัยด์น้อย 6.6% ผลการศึกษาพบมีไวรัสอาร์เอ็นเอ 92.8% ในคลาสสิคฮอดชกิน 75% ในโนดูลาสเคลอโรซีส 100% ในชนิดมิกซ์เซลลูลาลิตี และ 100% ในชนิดลิมโฟซัยด์น้อย ในการทดลองนี้พบว่าฮอดชกิน ชนิดมีลิมโฟซัยด์มาก ไม่มีไวรัสอาร์เอ็นเอ การศึกษาซีดี 15 และ ซีดี 30 พบผลบวก 78.6% และ 85% ตามลำดับ ในคลาสสิคฮอดชกิน

ผลการทดลองบ่งชี้ว่ามีความสัมพันธ์อย่างสูง ระหว่างเอ็บสไตน์ บาร์ ไวรัส และคลาสิคฮอดชกิน ในเด็กไทย (92.3%) โดยเฉพาะชนิดมิกซ์เซลลูลาลิตี (100%) เด็กชายเป็นมากกว่าเด็กหญิง และชนิดมิกซ์เซลลูลาลิตีพบบ่อยกว่าชนิดอื่น (60%)