Analysis of the presence of Epstein-Barr virus in Hodgkin's lymphoma in Iranian children by EBER in situ hybridization

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Introduction:
Epstein-Barr virus is a tumorigenic herpes virus which belongs to DNA viruses, infects, and persists in B-lymphocytes of most human beings. This virus is associated with some cases of Hodgkin’s lymphoma. The aim of the present study was to investigate the presence of Epstein-Barr virus in Hodgkin’s lymphoma in children.

Materials & Methods:
In this study, 16 formalin-fixed paraffin embedded blocks of Hodgkin’s lymphoma tissue samples were collected from the archives of the Pathology Department of Fasa University of Medical Sciences. The presence of RNA transcripts encoded by the virus was examined with the EBERs in situ hybridization method.

Results:
The 16 formalin-fixed paraffin-embedded samples belonged to patients with Hodgkin’s lymphoma with the mean age of 8 years (range: 4 to 12 years). They were investigated using the EBERs in situ hybridization method. In 12 specimens (75%), including 9 male and 3 female samples, the Epstein-Barr virus was present in different subtypes of Hodgkin’s lymphoma. Eighty percent of mixed cellularity, 67% of nodular sclerosis and 100% of lymphocyte predominance subtypes proved positive. The presence of the Epstein-Barr virus in the age groups of 4-7 and 8-12 years was 71.5% and 77.8%, respectively.

Conclusion:
The results of the present study indicate a strong relationship between the Epstein-Barr virus and Hodgkin’s lymphoma in children. The relationship between the Epstein-Barr virus and Hodgkin’s lymphoma in Iranian children follows a pattern similar to that of other developing countries.

Keywords: Epstein-Barr Virus, Hodgkin's lymphoma, in situ hybridization, EBER
Predominant indicates a non-classical Hodgkin whereas mixed cellularity, nodular sclerosis, lymphocyte depleted and lymphocyte rich indicate a Classical Hodgkin lymphoma (2).

Although the pathogenesis of Hodgkin’s lymphoma is unknown as of yet, some studies have suggested the genetic predisposition of certain individuals to the disease. In addition, a linkage has been reported between Hodgkin’s lymphoma and specific Human Leukocyte Antigens (HLA), (3). Analysis of HLA class I polymorphism shows that defects in antigen expression are associated with the risk of EBV-positive Hodgkin’s lymphoma (4). Serological studies conducted in 1970 first demonstrated a strong association between Epstein-Barr virus infection and Hodgkin’s lymphoma and subsequently identified the presence of this virus in specimens of Hodgkin’s lymphoma using the Southern Blot method (5). Epstein-Barr virus infection spreads to other people through contact with the saliva of an infected person. The course of infection depends on socioeconomic status, genetic factors, geographical location and age at the time of first contact with the virus (6-7).

Seroepidemiological surveys on global scale are indicative of the high prevalence of the Epstein-Barr virus across different geographical regions of the world. Findings show that, in developing countries, the majority of children are infected with the Epstein-Barr virus by the age of 6 and possess an antibody titer against it, while in developed countries, more than 50% of the population remains susceptible to the virus until puberty. Primary infection with this virus is often latent in children, without clinical symptoms or else with non-specific symptoms; however, this virus causes infectious mononucleosis in 35-75% of adolescents and young adults (8). Studies have shown that the Epstein-Barr virus is associated with approximately one-third of the cases of Hodgkin’s lymphoma in developed countries, whereas a higher level of association with the virus is reported in developing countries. In these countries, the Epstein-Barr virus is often associated with Hodgkin's lymphoma of the mixed cellularity subtype compared to the nodular sclerosis subtype and is more prevalent among children and the elderly (9).

In a study conducted by Alebouyeh et al. in 1994, the Epstein-Barr virus was reported to be present in 73% of the 139 specimens of Iranian children with Hodgkin’s lymphoma (10). In their study of 2003 on 55 specimens of Hodgkin’s lymphoma using the EBERs in situ hybridization method, Najafipour et al. reported the presence of the Epstein-Barr virus to be about 73% in cases with Hodgkin's disease (11), while in another study conducted in 2009 on 30 cases with Hodgkin's lymphoma using the immunohistochemistry method, Katebi et al. reported the presence of this virus to be 93% (12). In another study conducted in 2009 on 36 Iranian children with Hodgkin's lymphoma using the EBERs in situ hybridization method and the immunohistochemistry method, Djadali et al. reported the presence of the Epstein-Barr virus to be 44% and 58%, respectively (13).

The Epstein-Barr virus severely transforms Hodgkin and Reed-Sternberg (HRS) cells and expresses different types of latent proteins in these cells, including 6 nuclear antigens (EBNA-1LP, C3, B3, A3, EBNA-1 and EBNA-2), three latent membrane proteins (B2-LMP, A2-LMP, 1-LMP) and two untranslatable RNAs (EBER1 and EBER2), (14). Various methods exist for detecting the Epstein-Barr virus in cancerous cells, with the specific, sensitive method of EBERs in situ hybridization being superior to other methods and being capable of detecting EBERs latent gene products in malignant Reed-Sternberg cells. Since EBERs transcripts are expressed in as many as 1 million copies, this method is highly sensitive and
accurate (15). The purpose of the present study is to investigate the presence of the Epstein-Barr virus in specimens of children with Hodgkin’s lymphoma using the EBERs in situ hybridization method.

Materials and Methods
The present study was conducted over six months in 2012 and analyzed 24 tissue specimens of Hodgkin’s lymphoma in the form of formalin-fixed, paraffin-embedded blocks retrieved from the archives of the pathology department of Fasa University of Medical Sciences. Once the specimens were reviewed by the pathologist, 16 tissue specimens with confirmed Hodgkin’s lymphoma were selected for the study and the rest were excluded due to the unavailability of information on the patient and the lack of sufficient tissue. The age range of patients under study was between 4 and 12. Of the 16 cases of Hodgkin’s lymphoma, 12 cases were male (75%) and 4 female (25%). From the paraffin-embedded blocks, slides were prepared for Hematoxylin and Eosin (H&E) staining. This staining confirms Hodgkin’s lymphoma and its histologic subtypes. To prepare slides from the paraffin-embedded tissue specimens, 4-micrometer-thick slices were first cut and placed on coated slides using a microtome. Then, after an hour of exposure to 70°C temperature, the slides were deparaffinized and dehydrated in xylene and ethanol alcohol solutions and were then rinsed under running water. Subsequently, the slides were stained and then a pathologist examined them under a microscope for detecting Hodgkin’s lymphoma and its subtypes.

The EBERs in situ hybridization was performed using oligonucleotide probes marked by fluorescein (Novacastra, NCL-EBV-K) and forming a hybrid with the Epstein-Barr virus encoded RNA transcripts (EBERs). The slides were deparaffinized and dehydrated for 3 minutes in 99% V/V xylene solution and 95% ethanol alcohol. The tissue sections were then encircled by a Dako pen (Dako Denmark A/S, Germany) and proteinase K solution diluted in Tris buffer was then added to the tissues by a 100µl sampler. The slides were incubated for 30 minutes in a humidified chamber at 37 °C. After incubation, the slides were rinsed for 3 minutes in deionized water and dehydrated in 95% V/V and 99% ethanol alcohol. During the hybridization stage, 20µl of probe hybridization solution (NCL-EBV probe, Leica Co., UK) was added to the tissues and the slides were first incubated for 15 minutes at 65°C and then again for 2 hours at 37°C. After incubation was completed, the slides were washed in TBS containing V/V Triton x-100 0.1% for 3 minutes.

In the detection phase, 100µl of blocker solution (diluted rabbit serum in 100 V/V Triton x-100 1.0% and W/V BSA3% TBS) was added to the tissues that were then incubated for 10 minutes at room temperature. Then the blocker solution was removed from the tissues and (Rabbit f(ab) anti-FITC/AP) Vial A with a dilution ratio of 1 to 180 in diluting solution (100 V/V Triton x-100 1.0% and W/V BSA3% TBS) was added to the slides that were then incubated for 30 minutes at room temperature.

Over the next stage, the slides were rinsed first in TBS and then in alkaline phosphatase substrate buffer. Slides were covered and incubated overnight at room temperature with a solution containing a substrate enzyme (Vial B) prepared with a dilution ratio of 1 to 50 in alkaline phosphatase substrate buffer and with 1µl of Levamisole hydrochloride inhibitor also added in. After an overnight incubation, the slides were rinsed with water and stained with Mayer’s hematoxylin. The slides were mounted and viewed under a microscope by a pathologist. Positive results often emerged in the form of brown dual-core cancer cells (Figure 1).

Statistical Analysis:
Data were analyzed in the statistical software SPSS 16 and using Fisher's exact
test. The statistical significance level was set at 0.05.

**Results**

In the present study, 16 children with Hodgkin’s lymphoma at an age range of 4 to 12 with a mean age of 8 were studied, out of whom 12 patients were male (75%) and 4 were female (25%). The patients were divided into two age groups, children between 4 and 7 years old (43.75%) and children between 8 and 12 years old (56.25%). Based on the histopathological examination, 5 patients (31.25%) belonged to the mixed cellularity subtype, 9 patients (56.25%) to the nodular sclerosis subtype and 2 patients (12.5%) to the lymphocyte-predominant subtype. The results of the study on the presence of the Epstein-Barr virus in Hodgkin's lymphoma samples using the EBERs in situ hybridization method are shown in Tables 1 and 2. Through this method, the presence of the virus in Reed-Sternberg cancer cells was identified as 75% (12 positive cases out of a total of 16). Based on gender, the presence of the Epstein-Barr virus was determined to be 75% in males (9 positive cases out of a total of 12) and 75% in females (3 positive cases out of a total of 4). The relationship between results of the EBERs in situ hybridization test in both female and male children with Hodgkin’s lymphoma were assessed through Fisher’s exact test and the statistical analysis showed no significant difference between the two genders (p-value=0.000). The presence of the Epstein-Barr virus in Hodgkin’s lymphoma histological subtypes was determined to be 80% in the mixed cellularity subtype (4 positive cases out of a total of 5), 67% in the nodular sclerosis subtype (6 positive cases out of a total of 9) and 100% in the lymphocyte-predominant subtype (2 positive cases out of a total of 2). Comparison of results of the EBERs in situ hybridization test for these subtypes using Fisher’s exact test showed no significant difference between the subtypes (p-value=0.826). Furthermore, the presence of the virus was more prevalent in the mixed cellularity subtype than in the nodular sclerosis subtype according to the present study. Moreover, 71.5% of the age group of 4-7 and 77.8% of the age group of 8-12 were EBV positive, and based on Fisher's exact test, no significant difference was observed between the two age groups (p-value=000). (Table 1).

**Discussion**

Hodgkin’s lymphoma is a worldwide disease with a complicated epidemiology possibly indicating multifactorial etiology such as interaction between genetic and environmental factors (16-17). The contribution of the Epstein-Barr virus to the etiology of Hodgkin’s lymphoma differs according to age groups and geographical regions so that age at the time of primary infection with the virus along with genetic background comprise a significant factor in developing clinical expressions of the infection (17).

Our results showed that, in Iranian children, Hodgkin's lymphoma is associated with the Epstein-Barr virus infection in Hodgkin and Reed-Sternberg malignant cells. Most studies conducted on Hodgkin’s lymphoma suggest epidemiological differences between developed and developing countries. Based on results of these studies, the age distribution of the lymphoma follows a double-hump model with two peak ages of 15-35 and above 50 (18-19) in developed countries, while in developing countries, the two peak ages are under 15 and above 50 (17-20).

Epstein-Barr virus infection occurs across all human societies, but the age of initial infection is lower in poor and more highly populated communities so that in Africa, South East Asia and Latin America, Hodgkin’s disease usually occurs in early childhood (before the age of 5). However, in developed countries, the age of primary infection is higher and infectious mononucleosis is more common in youth.
Moreover, an underlying cause of immunosuppression has been suggested as the reason for the high prevalence of EBV positive Hodgkin's lymphoma in developing countries. Various infections, such as human immunodeficiency virus infection and malnutrition, count as possible causes for immunosuppression in these countries (22).

In developing countries, primary infection usually occurs during childhood and Hodgkin's lymphoma is associated with the Epstein-Barr virus and the mixed cellularity subtype is more prevalent in these countries. However, in developed and industrialized countries, Hodgkin's lymphoma is more prevalent in youth and is dominantly of the nodular sclerosis subtype (19-23). Hodgkin’s lymphoma tumor is composed of about 2% Hodgkin and Reed-Sternberg malignant cells and 98% non-cancerous infiltrating cells (24).

Several studies are being conducted across the world aiming to determine the relationship between the presence of the Epstein-Barr virus and various cancers such as Burkitt's lymphoma, B cell lymphoma in HIV positive and transplant patients, nasopharyngeal carcinoma and Hodgkin's lymphoma. Several studies have also been conducted in Iran about relationship between the presence of the virus and Hodgkin's lymphoma. The expansion of these studies presents a more thorough picture of the relationship between Hodgkin's lymphoma and the Epstein-Barr virus across various geographical regions and in different age groups.

The present study, which examined 16 paraffin-embedded specimens of children with Hodgkin's lymphoma and conducted in Fasa University of Medical Sciences, had certain limitations including the poor quality of certain paraffin-embedded specimens and the poor cooperation of pathology groups for using the specimens. The Epstein-Barr virus was observed in 12 cases out of a total of 16 children with Hodgkin's lymphoma aged 4-12 and a strong correlation was found between the lymphoma and the Epstein-Barr virus (75%), which nearly corresponds to previous studies conducted on children in other developing countries such as Peru (99%), South Africa (68%), China (72%), India (96%), Malaysia (93%), Thailand (92.8%), Jordan (47%) and Kuwait (79%), (20 & 25-26).

Hodgkin's lymphoma is reported to be more prevalent in males than in females – a difference that although not statistically significant, is consistent with findings of the present study (11, 27, 28, 29, 30, 31 & 32). The insignificance of this difference might owe to the small sample size of the present study. In addition, analysis of results of different Hodgkin’s lymphoma subtypes showed that the presence of the Epstein-Barr virus is higher in the mixed cellularity subtype than in the nodular sclerosis subtype, which also corresponds to other studies conducted in developing countries (11, 26, 27, 29, 32 & 33).

In developing countries, Hodgkin's lymphoma first occurs between the ages of 7 and 12, while in developed countries, primary incidence occurs with delay in early puberty or adulthood. Several reports suggest the role of the Epstein-Barr virus in a high percentage (sometimes up to a hundred percent) of Hodgkin's lymphoma specimens, particularly in developing countries. In addition, several factors such as genetic, environmental, geographical and economic conditions are involved in the pathogenesis of the disease. Similar to previous studies, the incidence of Hodgkin's lymphoma was observed in the present study in children belonging to both age groups of 4-7 and 8-12, though it was somewhat higher in the age group of 8-12 (32-34). Compared to similar studies conducted on the topic, the insignificance of the differences in parts of the present study might be attributed to its small sample size.
Conclusion

According to results obtained from the present study, the prevalence rate of the Epstein-Barr virus in specimens of children under the age of 14 with Hodgkin's lymphoma was 75%, thus indicating a strong relationship between the Epstein-Barr virus and Hodgkin's lymphoma in Iranian children just as in children of other developing countries.

References: