ASSOCIATION OF EPSTEIN- BARR VIRUS AND HODGKIN'S DISEASE

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Abstract- We have analyzed paraffin sections from 55 patients with histologically confirmed Hodgkin's disease (HD) for the presence of Epstein-Barr virus (EBV) markers using in situ hybridization to detect the EBV-encoded RNAs (EBERs) and immunohistochemistry to identify latent membrane protein-1 (LMP1) expression. Tissue specimens from 55 cases of Hodgkin's disease included 22 mixed cellularity (MC), 27 nodular sclerosis (NS), one lymphocyte depleted (LD) and 5 lymphocyte predominance (LP). All of the confirmed EBV associated cases were examined and subtyped for the presence of Epstein-Barr virus (EBV) DNA by polymerase chain reaction. In situ hybridization revealed an exclusive localization of virus in the tumor cells and EBV markers were present in 30 HD cases (55%) and were mainly confined to the mixed cellularity (MC) and nodular sclerosis (NS) subtypes. 1-MH immunohistochemistry had similar results as in situ hybridization. EBV positivity with regards of HD subtypes were 64% (14/22) mixed cellularity (MC), 44% (12/27) nodular sclerosis (NS), 0% (0/1) lymphocyte depleted (LD) and 80% (4/5) lymphocyte predominance (LP). Epstein-Barr virus-specific DNA sequences were detected by PCR in DNA extracts from paraffin-embedded tissues of all LMP1 positive cases. Twenty-eight cases were type 1 EBV and 2 cases type 2 EBV. There was difference between EBV-positive and EBV-negative HD patients with regard to age. Analysis of age group 1-14 years, 15-49 years and over 49 years, revealed 73% (16/22), 35% (10/29), 100% (4/4) EBV positivity, respectively. These findings compared to the EBV association pattern with HD in developed and developing countries suggest an overall intermediate pattern of EBV association with HD and high incidence of EBV in children and elderly HD cases.

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Key Words: Epstein-Barr virus, Hodgkin's disease, latent membrane protein 1, in situ hybridization

INTRODUCTION

Epstein-Barr virus (EBV), the etiological agent of infectious mononucleosis, is also an important human oncogenic virus, being implicated in the pathogenesis of several neoplasms including Burkitt's lymphoma, nasopharyngeal carcinoma (NPC), immunodeficiency- associated lymphoma, peripheral T-cell lymphoma (PTLs), and Hodgkin's disease (HD) (1). Although EBV is ubiquitous in healthy population throughout the world, the incidence of different EBV associated tumors shows considerable geographical variations (2-5). HD is a lymphoid tumor that forms less than 1% of *de novo* neoplasms every year worldwide (6). Its diagnosis is based on the identification of characteristic multinucleated giant cells within an inflammatory milieu. These malignant cells, termed Hodgkin's Reed- Sternberg (HRS), represent the body of the tumor.

Recently, it has been shown that in most if not all HD cases, HRS cells belong to the same clonal population, which is derived from peripheral B and T cells in about 98% and 2% of cases, respectively

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(7-9). An association between EBV and HD had long been suspected from early epidemiological and serological studies (10-11). The observation that persons with a history of infectious mononucleosis have a twofold to threefold increased risk of HD (11), and the detection of elevated levels of antibodies to viral antigens in HD patients before or at the time of diagnosis (11-14) provided indirect evidence for a causal role for EBV in HD.

Demonstration of EBV genomic DNA in HD was first reported in 1987 (15). Subsequent in situ hybridization (ISH) studies localized the virus to the Reed-Sternberg cells and variants, which are the presumed neoplastic component of HD (15,16). Direct evidence for the association was supplied by the localization of EBV DNA to the malignant Hodgkin and Reed-Sternberg (HRS) cells. Perhaps the most widely used and most reliable method is in situ hybridization that targets the highly abundant Epstein-Barr virus encoded RNAs (EBERs) (11). In EBV-positive HD cases, the EBV is found in nearly all of the Reed-Sternberg cells, regardless of methodology.

However, the frequency with which EBV is demonstrated in HD tumors also shows geographical variability, with percentages between 40% and 50% for North American and European cases (17-20), 57% for HD in China (21) and nearly 100% of HD patients in developing countries as well as orientals (22,23,25). Furthermore, several investigators have demonstrated the clonality of EBV in HD tissue by hybridization with the viral terminal repeat sequences. These findings indicate clonal expansion of single EBV-infected cells and underlie a possible etiological role of EBV in a proportion of HD cases. In addition, immunohistochemical analysis has demonstrated that the HRS cells of EBV-positive cases express high levels of the EBV- encoded latent membrane protein-1 (LMP1) (27). The expression of LMP1 in several EBV associated tumors including immunoblastic lymphoma, Hodgkin's disease and nasopharengeal carcinoma is consistant with its ability to transform rodent fibroblasts, to induce the up-regulation of CD40, CD54 and Bcl-2 family members, and to inhibit epithelial differentiation.

Immunohistochemically demonstration of LMP1 is an additional evidence suggesting that EBV is involved in the pathogenesis of a subset of cases of Hodgkin's disease. EBV strain can be classified into two main types, type 1 and type 2, based on polymorphism within the gene loci encoding EBNA 2, 3A, 3B and 3C proteins. Initial results suggested that type 2 strain were found predominantly in central Africa and New Guinea, whereas type 1 strains were more common in Western countries.

Reliable information regarding Iranian HD is not available. The current study was conducted to ascertain EBV types and to evaluate the association between EBV and HD in this region. For this study we collected HD patients samples from Tehran hospitals. Immunohistochemical staining for LMP1 and in situ hybridization for EBERs was used to define association of HD cases with EBV. We have also used sensitive polymerase chain reaction (PCR) methods for detection and typing of EBV in lymph nodes with HD involvement.

METERIALS AND METHODS

Patients and Histological Review

We studied archival paraffin wax-embedded tissue blocks from 98 suspected HD patients of Tehran hospitals. Hematoxylin and eosin-stained slides were prepared and used to review and subtype according to the Rye classification system by an expert pathologist (29). A histological diagnosis of HD was confirmed in 55 cases. The remaining cases were either not considered to have HD or there was insufficient material remaining in the tissue block to allow a precise histological diagnosis to be made.

Detection of Latent EBV Infection by EBERs ISH

In situ hybridization for the detection of EBERs was performed according to standard 15 methodology (30) and was used to detect the presence of latent EBV infection in all HD samples. A known EBER positive HD and cytospin slides preperation of lymphoid cell line (LCLs) containing EBV served as positive control

and EBV negative lymphid tissue served as negative control in each run. For RNA-RNA in situ hybridization, 4 μm paraffin sections were prepared, dewaxed, and dehydrated in ethanol, rinsed with PBS, digested with 0.1 mg/ml pronase in DEPC water at room temperature for 5 minutes, rinsed with PBS, dehydrated through graded ethanol solutions, and air dried. Digoxigenin-labelled RNA probe was generated from plasmids harboring EBER- specific inserts by in vitro transcription (39,40). The hybridization mix consisted of 50% formamide, 2× saline sodium citrate (SSC), 10% dextran sulphate, 10 mM DTT, and 250 μg/ml tRNA. The EBER1 and EBER2 digoxigenin-labelled probe were heated at 80 T for 30 seconds and added at a 1/100 dilution of the labelling reaction mix. Sense and anti sense probe were used separately with hybridization solution. A 25 μl aliquot of hybridization mix was added to each section. The sections were covered with parafilm and incubated at 50T overnight in a wet atmosphere. After RNA-RNA hybridization, the parafilm was removed and the sections washed in 2 × SSC for 5 minutes at 37°C, treated with 20 μg/ml of RNase A in 2× SSC for 30 minutes at 37°C, washed in 0.1× SSC for 5 minutes at room temperature, circled with DAKO wax pen and then transferred to phosphate buffered saline (PBS) for immunohistochemical detection of the bound probe with an anti-digoxigenin mouse monoclonal antibody (clone D1-22; Sigma, Poole, Dorset, UK), then added biotinylated goat anti-mouse/rabbit secondry antibody (DAKO Kit, Bottle C) and then used Strept AB Complex/HRP (DAKO Kit, Bottles MB) method, visualized by DAB-Urea/H_2O_2, rinsed off with PBS, counterstained with haematoxylin for 10-15 seconds, washed in water, dehydrated in ethanol, cleared in xylene and mounted in DPX.

LMP1 Immunohistochemistry

lmmunohistochemistry for LMP1 was performed on all cases to confirm the presence of EBV infection in HRS cells (31). Sections (4 μm thick) were cut from each formalin fixed, paraffin wax embedded tissue samples, adhered to vectabond ticated slides (Vector Laboratories, Peterborough, UK), and incubated overnight at 37°C. After dewaxing and clearing, endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol. Sections were microwave-pretreated and the sections were transferred to PBS at pH 7.6. Sections were preincubated in normal rabbit serum (Dako, Cambridge, UK) for 10 minutes and then incubated at room temperature with a prediluted (1/50) mouse polyclonal antibody directed against LMP1 (CS1-4; Cancer Research Center, Birmingham University, UK), for one hour. Then added biotinylated goat anti-mouse/rabbit secondry antibody (DAKO Kit, Bottle C) for 30 minutes and the strept AB Complex/HRP method, visualized by DAB-Urea/H_20_2, rinsed off with PBS, counterstained with haematoxylin for 10-15 seconds, washed in water, dehydrated in ethanol, cleared in xylene and mounted in DPX. One section known to give strong staining was included in each subsequent run as a positive control. Negative controls consisted of the substitution of the LMP1 primary antibody with appropriate non-immune serum of the same IgG subclass.

EBV typing

DNA extraction

DNA was extracted from paraffin blocks as described previously (5). Briefly, tissue sections were deparaffinized and digested at 55°C for 48 h with 0.28 mg/ml proteinase K (Roche, Cat. No: 1964372) in 250 ml digestion buffer (50 mM Tris-HCI, 1 mM EDTA and 0.5% Tween 20, pH 8.5). Proteinase K was inactivated at 95°C for 20 min. The supernatant was used as the template for PCR amplification (5).

DNA PCR Amplification

DNA for PCR was prepared as reported previously and the PCR was carried out using 22 automated thermal cycler. Cases were EBV subtyped by analysis of the EBNA2 and EBNA3C coding regions. EBV cell lines B95.8 and AG876 carrying type 1 and type 2, respectively, were used as controls (32).

DNA aliquots (100 ng to l μg) were amplified in 100 μl reactions containing 1 μmol/L of each primer. 200 μmol/1 dATP, dCTP, dGTP, dTTP, 10 mmol/L Tris HCl (PH 8.8) 1.5mmol/L $MgC1_2$, 50 mmol/L KCl, 0.1% Triton X-100, and overlaid with mineral oil. The samples were denatured for 5

minutes at 94°C. Two units of Taq DNA polymerase (Roche, Cat.No:1146173) were added and PCR amplification performed in a Perkin Elmer-Cetus (Warrington, UK) 480 Thermal Cycler. The primer combination used, cycle parameters for PCR amplification, and product sizes of the different regions of EBV are described in table 1. The PCR products were visualised by electrophoresis in a 2% agarose gel (Sigma, Cat.No:A7431) containing 0.5 μg/mL ethidium bromide and photographed. DNA isolated from the EBV-negative T-cell line HS32, water, and lysis buffer were included as negative controls in each assay (3,5,32).

RESULTS

Each case reported in this study was analyzed for EBERs by in situ hybridization and LMP1 by immunohistochemistry. Expression of LMP1 was identified in the cytoplasm of the HRS cells (Fig. 1). Expression of EBERs was identified in the nuclei of these cells (Fig. 2). All in situ hybridization-positive cases showed abundant EBERs in virtually all HRS cells. These EBERs positive cases also expressed LMP1 with the membranous and cytoplasmic pattern of staining. Although one of LMP1 positive case was EBERs negative, the number of EBERs positive cells were higher than the number of LMP1 positive cells in parallel sections. EBV signals that were occasionally detected in small lymphocytes, were excluded as a positive reaction of the disease if EBV signal was not present in HRS cells.

The overall EBV positivity rate was 55% (30 out of 55), including 29 for EBERs and 30 for LMP1. It is noteworthy that EBV was present in all patients older than 49 years (4 out of 4), in contrast with those younger than 49. The details of histological subtypes, EBERs and LMPI results are shown in table 2. Twenty-seven were classified as nodular sclerosis, twenty-two as mixed cellularity, one as lymphocyte depleted, and five as lymphocyte predominant subtype. The EBERs and LMN positivity rate for these histological subtypes were 44% (12 out of 27), 64%(14 out of 22), 0% (none out of one), and 80%(4 out of 5), respectively (Table 2).

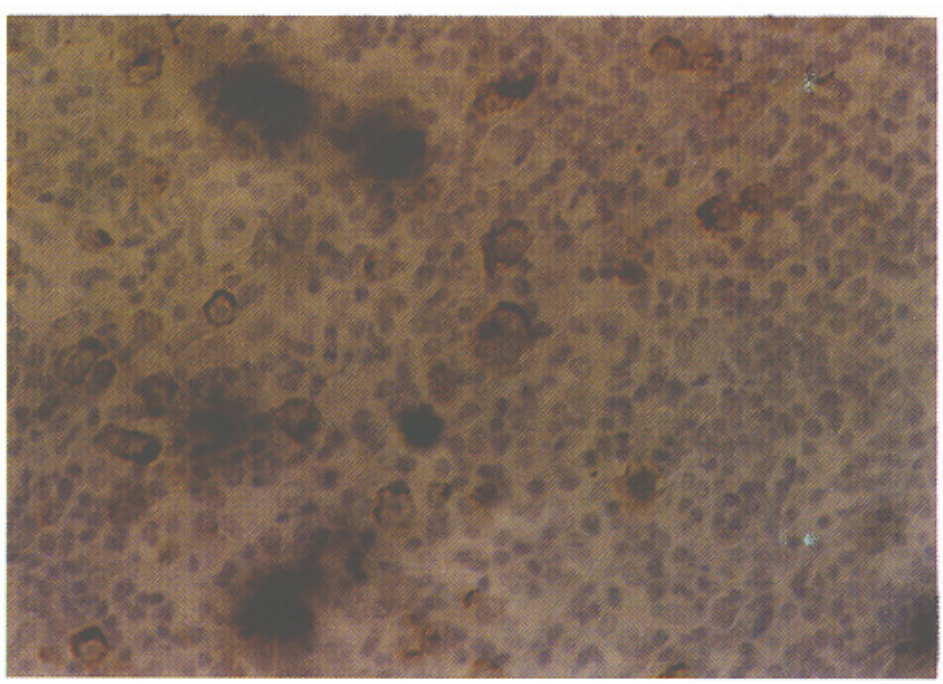

Fig. 1. LMP1 immunohistochemical analysis in Reed- Sternberg and Hodgkin malignant cells of paraffin- embedded section from lymph node with HD

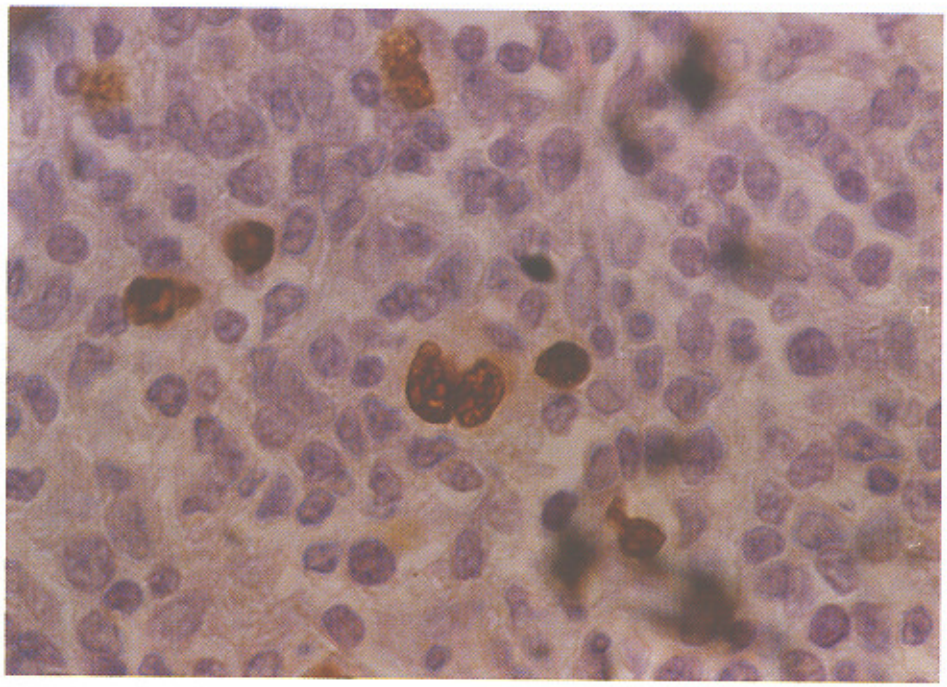

Fig. 2. EBERs in situ hybridization analysis in Reed- Sternberg and Hodgkin malignant cells of paraffin- embedded section from lymph node with HD

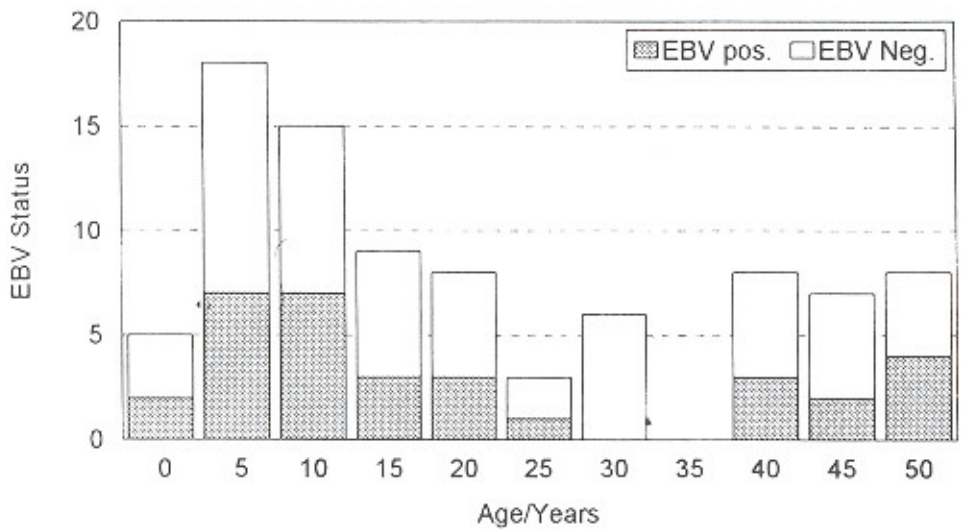

Fig. 3. Bar graph showing distribution of patients by age and percentage of Epstein- Barr virus positivity (shaded areas)

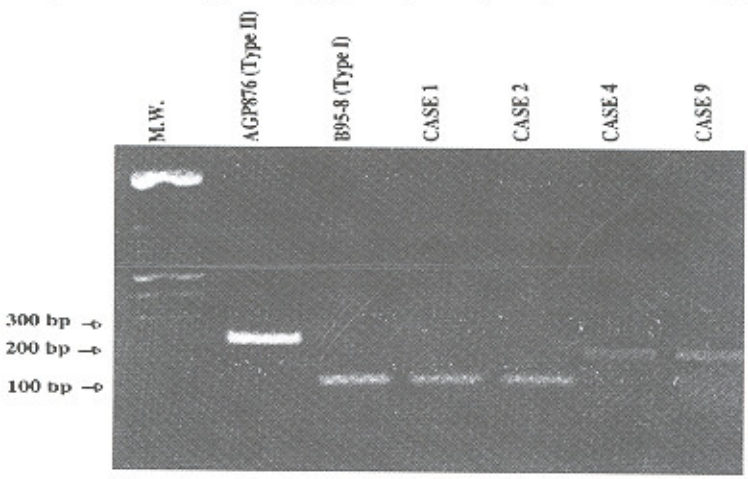

Fig. 4. Type- Specific PCR amplification of EBV EBNA3C gene. Cases 1, 2 contain type 1 and cases 4, 6 contain type 2 M. W.; size markers, B95-8 cell line carrying EBV type 1, AG876 cell line carrying EBV type 2

M. W.; size markers, B95-8 cell line carrying EBV type 1, AG876 cell line carrying EBV type 2

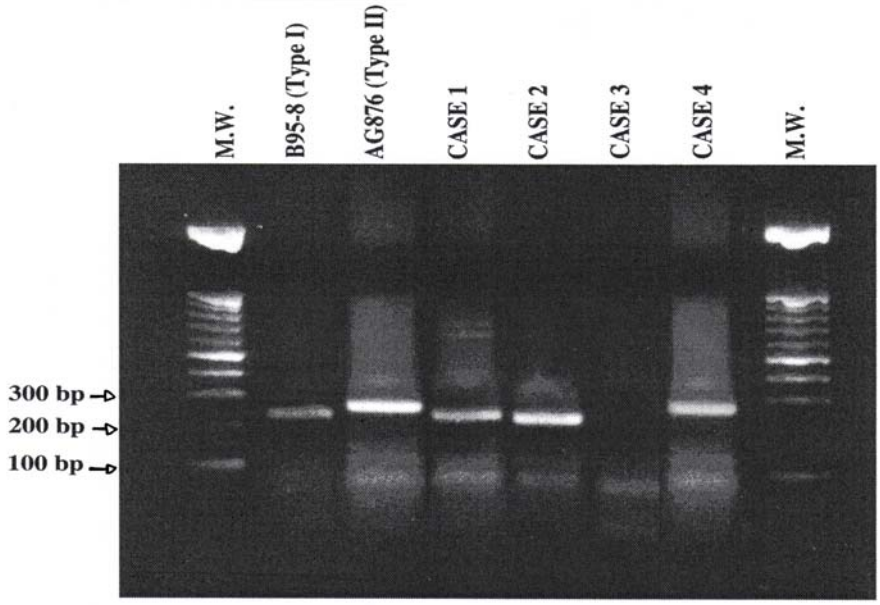

Fig. 5. Type- Specific PCR amplification of EBV EBNA 2 gene. Cases 1, 2 contain type 1 and 4 contain type 2

The subtype data and EBV positivity were analyzed statistically by fishers exact test ($P<0.26$) and any significant relationship between subtype and EBV positivity was not seen in this study. The EBV positivity for different age groups were 16 out of 22 (73%) for age younger than 15 years, 10 out of 29 (35%) for age 15 to 49 years, and 4 out of 4 (100%) for age 50 or higher. The age groups data and EBV positivity, statistically were analyzed by fisher's exact test ($P<0.001$) and confirmed the previously reported observation that the association of EBV with Hodgkin's disease was stronger at the extremes of life (Fig. 3). From 38 males and 17 females HD cases 22 and 9 were EBV positive, respectively E13V type was determined over two of the latent EBV genes, EBNA2 and EBNA3C, using well characterized primers and amplification conditions from Sample et al reference. Twenty-eight (93%) out of 30 EBV positive cases harbored type 1 EBV and two (7%) contained type 2. For control, we used BDL3 (Akata virus type l), B95-8 LCL (type 1), LCL-C2BL and AG876 (type 2) (Fig. 4 & 5).

Table 1. Type specific PCRs primer combination and condition (32)

PCR	Primer	Primer sequence 5'-3'	B95-8 Coordinates	PCR Conditions	PCR Product Size (bp)
EBNA 2	2A-3'	TTGTGACAGAGGTGACAAAA	49058-039		
	2B-3'	TTGAAGAGTATGTCCTAAGG			
		AGGGATGCCTGGACACAAGA	48810-829	94°C, 30sec	Type 1= 249
				52°C, 60sec × 35	
				72°C, 60sec	Type 2= 300
EBNA 3C	5'	AGAAGGGGAGCGTGTGTTGT	99939-958		
	3'	GGCTCGTTTTTGACGTCGGC	100091-072	94°C, 30 sec	Type 1= 153
				52°C, 60 sec × 35	
				72°C, 60 sec	Type 2= 246

Abbreviation: 5', 5' PCR primer; 3', 3' PCR primer; Sample et al reference (32).

Table 2. Histopathologic subtypes, LMP1 and EBERs status in HD in patients from Tehran hospitals

		EBV expression		
HD subtype	**No**	**EBERs +**	**LMP1 +**	**EBERs+ or LMP1+**
LP	5 (9%)	4	4	4 (80%)
NS	27 (49%)	11	12	12 (44%)
MC	22(40%)	14	14	14 (64%)
LD	1 (2%)	0	0	0 (0%)
Total	55 (100%)	29	30	30 (55%)

DISCUSSION

HD is seen worldwide and also total incidence rates appear not to vary substantially with geography. There are interesting differences between countries in age incidence and subtype distribution. In Europe and United States, where HD incidence is low in childhood, the disease shows a marked young adult peak dominated by NS disease, then appear plateau through later life with equal contribution from both NS and MC subtypes. In less developed countries, childhood HD is more common, and the disease shows a steady age-related increase with no young adult peak and overall contribution of MC is higher. In Western world about half the cases of HD arising in children or in old age are EBV-positive, whereas the figure falls below 30% for the young adult peak of disease: overall, some 35% of HD patients carry the viral genome. In developing countries more than 60% to 70% of all HD cases are EBV genome-positive, some of this increase being explained by the absence of young adult peak. It is worth recalling that EBV infection occurs very early in life in these areas, and several reports now indicate that every case of childhood HD from the above population is EBV genome-positive (28,33). The increased incidence of EBV positive Hodgkin's disease in under-developed countries could result from the existence of an underlying immunosuppression similar to that observed for African Burkitt's lymphoma in a malaria infected population (22).

We have analyzed 55 cases of HD for the presence of EBV and have correlated the results with the age, sex and subtype of the cases. The EBER-ISH results due to large of abundance EBERs in HRS cells is gold standard of EBV detection in HD samples. On the other side, RNA of some samples may be degraded and this should be detected by LMPI-MC. We detected EBERs in 29/55 and LMP1 in 30/55. This EBV association pattern is more than in Western countries and less than in developing countries (33). Previous study about eight Iranian children samples reported 80% EBV association (25). Analysis of our results for EBV detection according to the age of cases showed that EBV was more frequently found in age groups 1-14 (73%) years and above 49 years (100%), and that this trend was statistically significant (P-value has been shown in results section). The majority of other studies have reported the same age pattern in developing countries (33). Analysis of our data according to subtype despite a higher frequency of EBV-positive cases among the mixed cellularity subtype compared to other subtypes, has not shown statistical relationship ($P<0.26$), contrary to some other studies in developing countries (28,25,34).

There is not any study about EBV type and presence in Iranian population and EBV genomic detection has not been done till now. The age and subtype distribution of our EBV-positive cases support a role for this virus as a transmissible agent associated with HD at least in pediatrics and elderly in Iranian patients. The results are in keeping with the hypothesis put forward by MacMahon (1996), which suggests that HD is a grouping of at least three entities, which probably have distinct etiologies and can be distinguished on the basis of age (35).

The whole picture might be unified by an extension of Sixbey's hypothesis as generated to explain the Burkitt's lymphoma data (36). EBV might be an important cofactor for Hodgkin's disease in general, but the viral episome might be lost from malignant cells, where tumor progression had rendered the viral episome unnecessary for survival, and in patients with relatively intact immune systems, where the presence of the viral

episome and expression of associated viral antigens rendered the tumor susceptible to immune surveillance (37). In this study, presence of EBV in considerable number of infiltrated cells of HD tissues were seen in EBV positive cases. EBV typing results has revealed dominance of EBV type 1, and is similar with results of some other studies.

Sex and ethnicity are also factors that are related to EBV positivity in Hodgkin's disease. Various studies have shown that EBV positive rates are higher in male patients than in female patients (38). Because the detection of EBV in HRS cells was mainly based on the detection of the latently expressed gene LMP1 or on the detection of EBER transcripts, it remained an open question whether, in HRS cells classified as EBV-negative, the EBV genome might have escaped standard screening methods, due to the absence of latent gene expression.

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