

Epstein-Barr Virus DNA frequency in paraffin embedded tissues of Non-Hodgkin lymphoma patients from Ahvaz, Iran

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Abstract

Background: Epstein-Barr Virus (EBV) is a DNA virus which belongs to the Herpesviridae family and in γ -herpesvirus subfamily, has been infected about 95% of the world's population. EBV is transmitted through saliva and associated with different disease such as Infectious Mononucleosis, Nasopharyngeal Carcinoma, Burkitt's lymphoma, Hodgkin and Non-Hodgkin's lymphoma.

The study proposed to determine the frequency of Epstein-Barr Virus expression in histological tissue of Non-Hodgkin's lymphoma in Ahvaz, Iran.

Materials and methods: In this study 29 samples of Non-Hodgkin's lymphoma were examined from Ahvaz Imam Khomeini Hospital, using Nested-PCR technique on EBNA-1 region in order to determine the presence of EBV genome in tumoral tissues.

Results: Among 29 cases of Non-Hodgkin's lymphoma, 14 (48%) cases were positive for EBV. Out of 14 samples of Non-Hodgkin's lymphoma, 4(28.57%) were female and 10 (71.42%) were male. A number of 8 (57%) cases belonged to the adults age group which proves association between age and EBV positive Non-Hodgkin's lymphoma according to Fisher's exact test ($P=0.03$). However, in the current study there was no significant difference between EBV positive cases and sex subject ($P=0.626$).

Conclusion: The result of the this study revealed that Epstein-Barr Virus played a possible important role in Non-Hodgkin's lymphoma especially on adults.

Keywords: Non-Hodgkin's lymphoma, Epstein-Barr Virus, Nested-PCR

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Introduction

Epstein - Barr virus (EBV), belongs to the Herpesviridae family and Gamma Herpesvirus subfamily. This virus has a linear double-stranded DNA genome of approximately 184 Kilobase pairs (Kbp) which is encased in an icosahedral protein nucleocapsid surrounded by a lipid envelope (1).

Infectious mononucleosis is the most common disease caused by Epstein-Barr Virus. Occurrence of some diseases like Hodgkin and Non-Hodgkin's lymphoma have been observed in people with the same infection background (2). Both Hodgkin and Non-Hodgkin's lymphomas are counted as malignant lymph nodes (1). Non-Hodgkin's lymphoma includes some malignancies such as Borkitt's lymphoma, NK-T lymphoma and lymphoproliferative disorder (LPD) which are progressive tumors. The annual incidence rate of this lymphoma is 15 per 100,000 population in the United States (3,4).

The virus can transmit mainly through saliva and less often through sexual and transplantation. Primary EBV infection occurs in childhood and it is frequently asymptomatic. The virus may have spread among members of the family by close contact; the virus can be latent in B lymphocyte and keep its DNA in B cell as an episom. Asymptomatic reactivation is common, that can lead to the transmission of infection to healthy individuals (5).

Usually when a virus is associated with a special tumor, it can reveal the specific characteristic of transformation of this virus. For example several latent proteins are encoded by EBV, which are responsible for immortalization of B cells; these proteins are Latent Membrane Protein 1,2 (LMP-1,LMP-2), six Epstein-Barr Nuclear Antigen (EBNAs) and two small EBV-Encoded RNAs (EBER) that are used in diagnosis (1,6) and likely have a crucial role in EBV associated malignancies (1).

EBNA1 is a DNA-binding protein that is required for the replication and

maintenance of the episomal EBV genome; EBNA1 also acts as a transcriptional transactivator and enhances gene expression (2). EBNA1 is the only nuclear antigen that continues to be transcribed when cells are activated to lytic infection and has an important role in cell growth and survival (7). EBNA1 is found in all malignancies associated with EBV (8).

Since Non-Hodgkin's lymphoma is one of the most common malignancies and regarding the EBV role in this disorder, the current study aimed to determine the frequency of Epstein-Barr Virus expression in histological tissue of Non-Hodgkin's lymphoma from Ahwaz, Iran.

Materials and methods

A cross-sectional retrospective study was carried out on 29 paraffin embedded tissues from Non-Hodgkin's lymphoma patients during over 2004-2011. These samples included 12 women (41%) and 17 men (58%) from 2 to 79 years old with the mean of 39.79 ± 27.48 which were classified into two age groups of children (under 15 years old) and adults (above 15 years old).

All samples were collected from the archives of Imam Khomeini Hospital in Ahvaz and diagnostic accuracy of Non-Hodgkin's lymphoma were confirmed by a pathologist. Around 8 paraffin embedded blocks were sectioned 10 μ m-thick and stored at 4°C till final stages of tests.

The experiments in this study were initiated through the following four stages:

1-Deparaffinization: Deparaffinization was done by xylene and ethanol (Germany, Merk). Initially, all the specimens were placed in microtubes then xylene was added and kept at 45°C for 15 min followed by centrifuge at 14000rpm. This stage was repeated again. The supernatant was discarded and 1ml absolute ethanol was added to precipitate and stored at the room temperature for 10 min and centrifuged again at 14000rpm for 1 minute. The

supernatant was discarded. This process was repeated by adding 70% ethanol, followed the same condition. Finally supernatant was discarded and all microtubes were placed at 65°C for 5 min to vaporize the ethanol residue and the pellet was used in DNA extraction (9).

2-DNA extraction: High pure PCR template preparation kit (Roche, Germany, code No: 11796828001) was used for the extraction of DNA, according to the manufacturer's instruction. For better results, the extracted DNA was stored at -70°C until PCR amplification.

3-Nested-PCR amplification: Nested-PCR test was performed in two cycles of primary PCR and Nested by thermocycler (Techne TC-5000, UK). The first round of PCR was performed in 25µl mixture, containing 7µl of extracted DNA, 2.5µl PCR buffer 10X (Roche), 0.5 µl deoxynucleotide triphosphate 10nM (Roche), 1U Taq Polymerase (Roche), 20µM of each primer sequence. The second round was carried out with 4µl of the first round product, under the same condition described previously with the set of Nested primers and different annealing temperature which are reported in Table 1.

4-Gel electrophoresis: the second round PCR product was separated on a 2% agarose gel and developed by Safe Stain

under voltage at 100V. The result was seen under ultra violet in transilluminator. The sizes of bands are compared with 100bp Ladder (Fermentas) which was placed on the well as an indicator. Cell line B95.8 was used as a positive control to detect EBV (11).

Statistical analysis

The obtained results were analyzed by the version 17 of SPSS software and the role of age and sex on positive cases were surveyed by the Fisher's exact and CHI square test.

Results

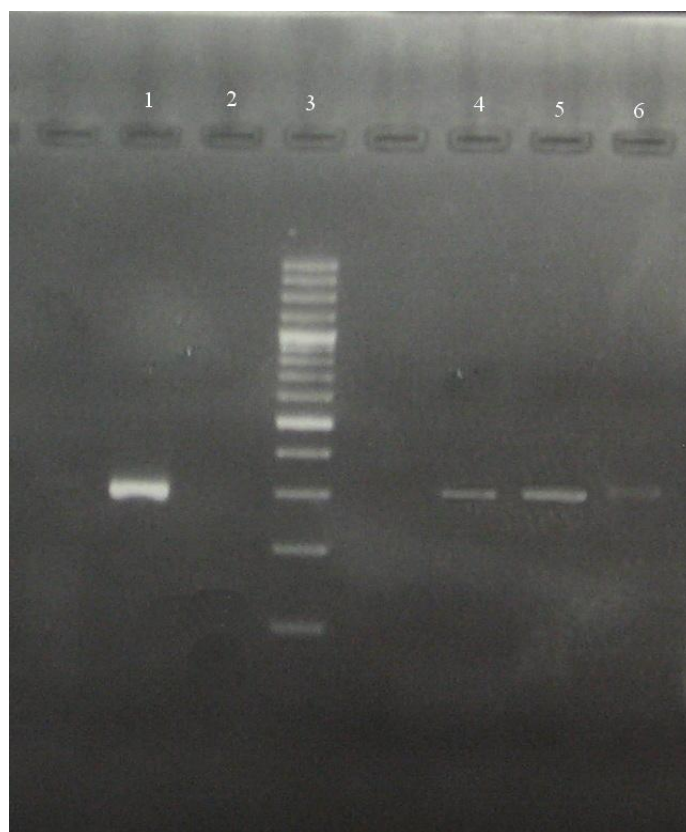
Among 29 cases of Non-Hodgkin's lymphoma, 14 (48%) cases were positive for EBV. Picture of the results of PCR is shown in Fig.1. These EBV positive cases include 4 (28.57%) female and 10 (71%) male. From these positive cases the most frequent, 8 (57%) cases, belonged to the adults age group. The results of Fisher's exact test have shown that the frequency of Epstein-Barr Virus in adults in comparison with children in Non-Hodgkin's lymphoma patients were significantly higher (P=0.03). Assayed histological cases with positive EBV are shown in two age groups and two different genders (Table 2).

Table1: Primers used for PCR and thermal cycling conditions

Primer	Sequence	PCR thermal cycling conditions
First cycle	5`-GTAGAAGGCCATTTTCCAC-3` 5`-CTCCATCGTCAAAGCTGCA-3`	94°C for 5 min: 1 cycle 94°C for 45 s, 54.5°C for 45 s, 72°C for 45 s: 35 cycles 72°C for 10 min; final extension
Nested cycle	5`-AGATGACCCAGGAGAAGGCCCAAGC-3` 5`-CAAAGGGGAGACGACTCAATGGTGT-3`	94°C for 5 min: 1 cycle 94°C for 45 s, 58.5°C for 45 s, 72°C for 45 s: 35 cycles 72°C for 10 min; final extension

Table 2: Characteristics of patients with Non-Hodgkin`s lymphoma

Age group	Gender		Total
	Female	Male	
Children (under 15 years old)	0	6 (42.8%)	42.8%
Adults (above 15 years old)	4 (28.5%)	4 (28.5%)	57.1%
Total	4 (28.5%)	10 (71.4%)	

**Fig: 1 Analysis of EBV using EBNA-1 (positive band 308bp)**

Lane 1: Positive control (B95.8 cell line), Lane 2: Negative control, Lane 3: 100-bp size marker, Lanes 4-6: EBV associated Non-Hodgkin`s lymphoma

Discussion

Non-Hodgkin`s lymphoma which is known as a fifth most commonly occurring cancer in both men and women, includes a group of hematologic tumors that are originated from T and B cells in the lymphatic system and have different genetic, phenotypic, morphologic and clinical features. Beside, viral factors that might be associated Non-Hodgkin`s lymphoma, there are several risk factors such as acquired immunodeficiency state, congenital agents, autoimmune disorders and environmental factors. Incidence of this disease is more common among men than women and this

increases while growing old, the highest prevalence of this disease has been observed in developed countries such as United States and the least frequent incidence rate is in East Asia (2 per 100,000 person) (12-15).

Using EBNA-1 as a target gene and PCR test, Kasprzak *et al.* reported that EBV DNA was detected in 12 (46%) cases from 26 paraffin embedded tissue of Non-Hodgkin`s lymphoma(16).

In the present study it was demonstrated EBV DNA in 48% of Non-Hodgkin`s lymphoma that was consistent with the

result of study in Poland. Moreover, the result of other studies reported the association of Non-Hodgkin's lymphoma with EBV infection 71.5% in Thailand (17), 40% in Argentina (18) and 70% in Egypt (19).

PourAkbari *et al.* using primers designed from EBNA-2 gene, reported that EBV DNA was detected in 10.5% cases of 19 histological samples by Nested-PCR (20).

Kosari *et al.* applied EBER as a target gene as well as Chromogenic In Situ Hybridization test and detected EBV DNA in 8 (16%) cases from 50 histological cases of Non-Hodgkin's lymphoma (21).

Several studies have demonstrated different results of the association between Non-Hodgkin's lymphoma and EBV infection which may be attributed to the sensitivity of the applied methods in studies. Other factors such as used samples (blood, fresh tissues, paraffin embedded tissues), experimented region of genome may also be effective on the results of study (19). Nested-PCR is one of the most strong and best techniques used for detection of special segments of DNA in tissues. Only limited amount of the template are needed for this method (22).

In the current study all samples were classified into two age groups: children and adults. The frequency of Epstein-Barr Virus was observed 42% in children and 57% in adults. The most prevalent belonged to the adults which proves association between age and EBV positive Non-Hodgkin's lymphoma according to Fisher's exact test ($P=0.03$). Kosari *et al.* have reported that the incidence of B cell lymphoma (the most common type of Non-Hodgkin's lymphoma) was higher in adults too (21). Tumwine *et al.* reported that the most frequent (66.7%) of Non-Hodgkin's lymphoma with positive EBV DNA among the adults and there was an association

between age and presence of EBV DNA (23).

In the current study EBV DNA was detected more in men than women, 71% and 28.57% respectively and there is no significant difference between EBV positive cases and sex ($P=0.626$). there are no any significant difference between the presence of EBV DNA in Non-Hodgkin's lymphoma and gender in the previous studies (20,21,23).

Since the current study was on retrospective basis, so the limited patients profile was available however it seems a cohort study with more demography of the patients requires for further investigation. In conclusion according to the result of the present study, some different agents were responsible for Non-Hodgkin's lymphoma that role of Epstein-Barr Virus may seems very important. The highest rate of infection was observed in adults. Whereas reactivation of EBV is a causative of Non-Hodgkin's lymphoma in a patient who has been infected by EBV previously. The risk of this disease is higher in adults.

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