Detection and Genotyping of Epstein-Bar Virus Among Paraffin Embedded Tissues of Hodgkin and Non-Hodgkin's Lymphoma Patients in Ahvaz, Iran

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Received: 16 Jul. 2017; Accepted: 18 Dec. 2017

Abstract - Epstein-Barr virus (EBV) is a DNA virus which belongs to the Herpesviridae family, has been infected with 90% of the world’s population. EBV is transmitted through saliva and associated with different diseases such as Infectious Mononucleosis, Nasopharyngeal Carcinoma, Burkitt’s lymphoma, Hodgkin and Non-Hodgkin’s lymphoma. This study was designed to determine the frequency of EBV DNA and genotyping of this virus in histological tissues of Hodgkin and Non-Hodgkin’s lymphoma in Ahvaz, Iran. In this study, 12 samples of Hodgkin and 29 samples of Non-Hodgkin’s lymphoma were examined from Ahvaz Imam Khomeini Hospital. After deparaffinization and DNA extraction, Nested-PCR technique was carried out on Epstein-Barr nuclear antigen 1 (EBNA-1) and Epstein-Barr nuclear antigen 3C (EBNA-3C) regions in order to detect EBV genome and genotype in tumoral tissues. Among 12 cases of Hodgkin’s lymphoma, 3 (25%) cases and among 29 cases of Non-Hodgkin's lymphoma, 14 (48%) cases were positive for EBV. All 3 EBV positive cases of Hodgkin’s lymphoma and a number of 8 (57%) cases of Non-Hodgkin’s lymphoma belonged to the adolescence age group which proves an association between age and EBV positive in Hodgkin and Non-Hodgkin's lymphoma. This study revealed that EBV played a possible important role in Hodgkin and Non-Hodgkin’s lymphoma especially in adults. Also, the main genotype of virus in the current study was EBV-1.

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Keywords: EBNA-1; EBNA-3C; Epstein-Barr virus; Hodgkin lymphoma; Non-hodgkin lymphoma

Introduction

Epstein-Barr virus (EBV), or human herpesvirus 4, is a lymphotropic herpesvirus which belongs to the subfamily of γ-herpesvirus in Herpesviridae family (1). EBV is the causative agent of Infectious Mononucleosis (IM) as a primary infection, and it may be subclinical in early childhood (2,3). EBV is widespread in the human population. Its antibodies are found in most serums of children all over the world, and by adulthood, more than 90% of human populations are infected with EBV (1,4). After primary infection, the genome of virus is established latency in B-lymphocytes and then it can be expressed after a while as some malignant disorders like Hodgkin’s lymphoma (HL), Nasopharyngeal carcinoma, Gastric carcinoma, Burkitt’s lymphoma (BL), B and T cell non-Hodgkin’s lymphoma which were shown to be associated with EBV (4-6).

There are three different types of latency in the pattern of EBV gene expression. In fact, virus infection is described by a state of viral latency as an episome in association with host chromosome (3,7). During latency infection time, the virus can express some limited genes such as six EBV Nuclear Antigens (EBNA-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C and EBNA-LP), three Latent Membrane Proteins (LMP-1, LMP-2A, and LMP-2B) and two EBV encoded RNAs (EBER-1 and EBER-2) (8). Expression of all latent genes is observed in Lymphoproliferative Disease (LPD) in immunocompromised hosts and IM patients. This pattern refers to the third type of latency (latency III). In Hodgkin’s lymphoma and Nasopharyngeal Carcinoma
(NPC) which are seen in the second program (latency II), some restricted genes such as EBNA-1, LMP-1, LMP-2, and EBERs are expressed. In the first pattern of latency (latency I) EBNA-1 and two EBERs are expressed. This type of latency is associated with (BL) (3,7).

Polymerase Chain Reaction (PCR) method is used for detection and strain distinction (9). Based on this method, there are two different types of EBV, A and B or 1 and 2, sequencing of EBNA-3A, EBNA-3B, and EBNA-3C, are known to be targeted for genotyping of EBV. The alleles of two types are different in predicted primary sequence by 16%, 20%, and 28%, respectively. Type 1 of EBV is predominant in the developed countries, while type 2 of EBV is common in Africa and New Guinea (3,10). EBNA-3C plays an important role in the transformation of B lymphocyte to lymphoblastoid cell lines (LCLs) (3,11).

EBNA-1 has a critical role in maintaining EBV in the infected cells, and it is essential for replication of the genome in the latently infected cells. It has also activator role for transcription (8,12). It has been considered to have a role in cell growth and survival (3).

Materials and Methods

In this cross-sectional study, 29 non-Hodgkin’s lymphoma and 12 Hodgkin’s disease paraffin-embedded tissue blocks were collected during March 2005 to March 2012, from the Imam Khomeini Hospital of Ahwaz. Their diagnoses and histological classifications were confirmed in a pathological part of the hospital, and their immunohistochemical assay was approved at the Pars laboratory in Ahvaz.

Removal of paraffin
Deparaffinization was done using of xylene and ethanol. 10 µm slices were put on the 1.5 ml autoclaved microcentrifuge tubes. Each tube of samples exposed to 1 ml xylene for 15 minutes for twice and then centrifuged at 14000 rpm for 1 minute after each stage and the supernatant aspirated. Then added 1ml absolute ethanol for 10 minutes, centrifuged at 14000 rpm for 1 minute and discard its supernatant. Also added 70% ethanol in the same condition, discarded supernatant and took the pellet to use as a tissue for DNA extraction.

DNA extraction
High Pure PCR Template Preparation kit (Roche, Germany, Code No: 11796828001) was used for the extraction of DNA, according to the manufacturers’ instruction.

Detection of EBV by PCR for EBNA-1
PCR for the region of EBNA-1 gene was carried out using extracted DNA from tissue in a 25µl mixture, containing 2.5 µl PCR Buffer (Roche), 0.5 µl deoxynucleotide triphosphate (Roche), 0.2 µl Taq Polymerase (Roche), 0.2 µl of each primer was used. The thermal conditions used in two steps PCR described in table 1.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>PCR thermal cycling conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>First cycle</td>
<td>5'-GTAGAAGGCCATTTTTCAC-3'</td>
<td>94°C for 5 min: 1 cycle</td>
</tr>
<tr>
<td></td>
<td>5'-CTCCATCGTCAAAGCTGCA-3'</td>
<td>94°C for 45 s, 54.5°C for 45 s, 72°C for 35 cycles</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72°C for 10 min; final extension</td>
</tr>
<tr>
<td>Nested cycle</td>
<td>5'-AGATGACCCAGGAGAAGCCCAAGC-3'</td>
<td>94°C for 5 min: 1 cycle</td>
</tr>
<tr>
<td></td>
<td>5'-CAAAGGGGAGACGACTCAATGGTG-3'</td>
<td>94°C for 45 s, 58.5°C for 45 s, 72°C for 35 cycles</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72°C for 10 min; final extension</td>
</tr>
</tbody>
</table>

Nested PCR

The PCR conditions were same as described aforementioned with the set of Nested primers and different annealing temperature which are reported in Table 1.

The positive control was extracted DNA from an EBV cell line, B95.8, and the negative control was a mixture of samples without DNA.

The products of PCR were separated on a 2% agarose gel and developed by Safe Stain under voltage at 100 V. The results were observed under ultraviolet transilluminator. The sizes of bands were compared with 100 bp Ladder (Fermentas). Cell line B95.8 was used as a positive control to detect EBV DNA (13). Amplification with EBNA-1 primers resulted in production of 300 base-pair fragments (10).
EBV genotyping in hodgkin and non-hodgkin patients

EBV typing by PCR for EBNA-3C

In order to determine the type of EBV, PCR was performed by amplifying a strain-specific sequence in the EBV 3C Nuclear Antigen (EBNA-3C). As described aforementioned, all PCR reactions were performed in a total volume of 25 µl which contained 2.5 µl PCR Buffer (Roche), 0.5 µl deoxynucleotide triphosphate (Roche), 0.2 µl Taq Polymerase (Roche), 0.2 µl of each primer sequence and PCR condition which are reported in Table 2.

Table 2. Primers of the EBNA-3C used for PCR and thermal cycling conditions

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>PCR thermal cycling conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>First cycle</td>
<td>5'-GAGAAGGGGAGCGTGTTGTGTT-3' 5'-GGCTCGTTTTTGACGTCGGC-3'</td>
<td>94°C for 5 min: 1 cycle 94°C for 45 s, 53°C for 45 s, 72°C for 45 s: 35 cycles 72°C for 10 min; final extension</td>
</tr>
<tr>
<td>Nested cycle</td>
<td>5'-TCATAGAGGTGATTGATGTT-3' 5'-ATGTTCCGATGTGGCTTAT-3'</td>
<td>94°C for 45 s, 47°C for 45 s, 72°C for 45 s: 35 cycles 72°C for 10 min; final extension</td>
</tr>
</tbody>
</table>

Nested PCR

The PCR conditions were the same as described previously with the set of Nested primer and different annealing temperature which are reported in table 2.

Gel electrophoresis and PCR product purification

8 µl of each product was analyzed using the electrophoresis. The results of amplification reactions were expected 246 and 153 base-pair for EBV type 2 and EBV type 1 respectively. In the following round, the amplified fragment by nested PCR was 75 base-pair for EBV-1 and 168 base-pair for EBV-2.

Sequencing

The second cycle of PCR products were sent out to Korean Bioneer Company to sequence DNA. After use of Bioinformatics program in www.ncbi.gov, EBV genotype was surveyed, and the mentioned results were confirmed.

Statistical analysis

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

Out of 41 paraffin-embedded tissue blocks, 12 (29.26%) samples were Hodgkin’s lymphoma, and 29 (70.73%) of them were non-Hodgkin’s lymphoma.

Nested-PCR results were demonstrated three of 12 (25%) Hodgkin’s lymphoma, and fourteen of 29 (48%) non-Hodgkin’s lymphoma were positive for EBNA-1, and they were associated with Epstein-Barr Virus (Figure 1).

Single and nested-PCR were performed in all positive cases for amplifying the EBNA-3C gene. The frequency of type 1 and 2 of EBV infection was determined that all of 3 EBV positive Hodgkin’s lymphomas were EBV type 1. Also among 14 EBV positive of non-Hodgkin’s lymphomas, all cases were EBV type 1 (Figure 2).

Out of 3/12(25%) cases of Hodgkin’s lymphoma which were associated with EBV, 2/12 (16.66%) patients were male, and 1/12 (8.3%) was female. There is no significant difference between EBV positive cases and sex (P=0.626). All 3 cases belonged to Mixed Cellularity subtype of Hodgkin’s lymphoma, and all of them belonged to the age of 15-34 years (Table 3).

Among 29 cases of Non-Hodgkin’s lymphoma, 14 (48%) cases were positive for EBV. Out of 14 cases of non-Hodgkin’s lymphoma, 10 (71.4%) were male, and 4
(28.5%) were female. The frequency of Epstein-Barr virus was observed 42% in children and 57% in adults. The most prevalent belonged to the adults who prove association between age and EBV positive Non-Hodgkin’s lymphoma according to Fisher’s exact test (P=0.03).

![Image](image_url)

**Figure 2.** Analysis of EBV using EBNA-3C (positive band, left: 153bp, right: 75bp). Lane 1: Positive control (B95.8 cell line), Lanes 1 - 3: EBV positive Lane 4: Negative Control, Lane 5: 50-bp size marker, Lanes 6-8: EBV positive

**Table 3. Age classification in Hodgkin and Non-Hodgkin’s lymphoma**

<table>
<thead>
<tr>
<th>Age group</th>
<th>Hodgkin</th>
<th>Non-Hodgkin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Children (under 14-year-old)</td>
<td>0</td>
<td>6 (42.8%)</td>
</tr>
<tr>
<td>Adults (above 14-year-old)</td>
<td>3 (100%)</td>
<td>8 (57.1%)</td>
</tr>
</tbody>
</table>

**Discussion**

EBV is an important pathogen which causes of variety of malignancies, such as Hodgkin’s lymphoma, Non-Hodgkin’s lymphoma, post-transplant lymphoproliferative disorder and nasopharyngeal carcinoma (14,15). Since most of the children are infected by EBV during childhood, more than 90% of populations are lifelong carriers of this virus and incidence latent infection up to two decades after the diagnosis of Infectious Mononucleosis (16,17).

Recent studies have shown the association of molecular epidemiology between EBV and Hodgkin’s lymphoma, Burkitt’s lymphoma, and the other types of Non-Hodgkin’s lymphomas. This evidence were confirmed by some techniques like polymerase chain reaction in latent infections of tumors (18,19).

Many of the mentioned tumors are only sometimes associated with EBV, with the determinants of association varying from cofactors such as malaria (Burkitt’s lymphoma) or other factors (Hodgkin’s lymphoma). In fact, epidemiological factors such as age, sex, race, geographical region and social level play an important role in pathogenicity (1). Whereas EBV positive Hodgkin’s lymphoma is less common in developed countries, the percentage of prevalence is variable from 25% to 50% in European cases (20), 31% in America (21), and much higher rates in underdeveloped countries like Peru and Kenya (11).

It has been demonstrated by American researchers on patients with Hodgkin’s lymphoma, that Asians are in low encounter with this disease (22,23). In point of fact, the most frequent incidence rate of Hodgkin and Non-Hodgkin’s lymphomas are in Africa because of some mosquito-transmitted disease such as malaria (11).

In the current study, we detected 3 (25%) cases from 12 Hodgkin’s lymphoma, and 14 (48%) cases from 29 Non-Hodgkin’s lymphoma were positive for EBV DNA.

According to MacMahon hypothesis, Hodgkin’s lymphoma was classified on the basis of age. This classification is involved in three different age groups: children, Adult and elderly persons that were distinguished 0-14, 15-34 and 50 year and above, respectively (20).

Hodgkin’s lymphoma is a bimodal distribution in association with age which the first peak is in childhood.
period (0-14 year) and the second peak is in above 50; although these peaks vary according to the socioeconomic condition. The incidence of Hodgkin’s lymphoma is in adolescence (15-35 year) in developed countries and is in childhood (0-14 year) in undeveloped countries (24).

Hodgkin’s lymphoma, used to be called Hodgkin’s disease, is classified into 2 main groups by World Health Organization (WHO) according to how the cells look under a microscope: Classical Hodgkin’s lymphoma and Nodular lymphocyte predominant Hodgkin’s lymphoma. There are 4 types of classical Hodgkin’s lymphoma on the basis of abnormal cells called Reed-Sternberg cell which is essential to the diagnosis of Hodgkin lymphoma may be seen under the microscope. The 4 types of Classical Hodgkin’s lymphoma are 1. Nodular Sclerosis (NS), 2. Mixed Cellularity (MC), 3. Lymphocyte Rich (LR), and 4. Lymphocyte Depleted (LD) (25,26). Using EBER as a target gene and Chromogenic In Situ Hybridization test and immunohistochemistry to identify latent membrane protein-1 (LMP1) expression, Najafipour et al., detected EBV markers were presented in 30 cases (55%) among 55 Hodgkin’s lymphoma (27). Using primers designed from EBNA-1 gene, Kim et al., reported that EBV DNA was detected in 85% cases of 20 histological samples by PCR (10). Expression of Epstein-Barr virus in Hodgkin lymphoma in a population of United Arab Emirates nationals revealed that 17 of 45 (38%) cases of Hodgkin’s lymphoma were EBV positive and MC subtype predominately seen in this cases (28). Moreover, the result of other studies reported the association of Hodgkin’s lymphoma with EBV infection 44.8% in Brazil (29), 34% in the Netherlands (30), 85% in China (31). Although the occurrence of EBV in Hodgkin’s lymphoma varies from country to country, but it has been reported to be higher in developing countries than in developed countries (32,33).

Najafipour and et al., demonstrated that EBV was found in age group 1-14 and above 50 and were mainly confined to the mixed cellularity (MC) and nodular sclerosis (NS) subtypes (27); meanwhile in the current study all of these 3 EBV positive cases belong to the age of 15-34 years, and there are no EBV positive cases among two other age groups; also all 3 cases were in the Mixed Cellularity type. In fact, our result reveals changing and transforming in socioeconomic conditions in Iran as a developing country.

In our study EBV frequency using EBNA-1 as a target gene in PCR technique revealed that only 3 (25%) Hodgkin’s lymphoma patients among 12 cases were EBV positive; 2 (66.6%) patients were male, and 1 (33.3%) was female.

Non-Hodgkin’s lymphoma which is known as a fifth most commonly occurring cancer in both men and women 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 the United States, and the least frequent incidence rate is in East Asia (2 per 100,000 people) (34-37).

In the current study, Non-Hodgkin’s lymphoma patients were classified into two different age groups, children, and adults. In this study among 29 cases of Non-Hodgkin’s lymphoma, 14 (48%) cases were positive for EBV. The frequency of EBV was observed 42% in children and 57% in adults. The most prevalent belonged to the adults who prove association between age and EBV positive Non-Hodgkin’s lymphoma according to Fisher’s exact test (P=0.03).

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 (38). Using primers designed from EBNA-2 gene, PourAkbari et al., reported that EBV DNA was detected in 10.5% cases of 19 histological samples by Nested-PCR (39). Using EBER as a target gene and Chromogenic In Situ Hybridization test, Kosari et al., detected EBV DNA in 8 (16%) cases from 50 histological cases of Non-Hodgkin’s lymphoma and there was an association between age and presence of EBV DNA (40). Moreover, the result of other studies reported the association of Non-Hodgkin’s lymphoma with EBV infection 71.5% in Thailand (41), 40% in Argentina (42) and 70% in Egypt (43).

In the current study, there was no significant difference between EBV positive cases and sex (P>0.05). There were no any significant differences between the presence of EBV DNA in Non-Hodgkin’s lymphoma and sex in the previous studies (40,41).

Different prevalence of EBV in patients with Non-Hodgkin’s lymphoma maybe because of the sensitivity of the applied methods in studies. Some factors such as used samples (blood, fresh tissues, paraffin-embedded tissues), experimented region of the genome may be effective on the results of the study (43). 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 is needed for this method (44).

EBNA-1 is the only protein that is persistently expressed in all malignancies which were associated with EBV. EBNA-1 is expressed latency phase, it may maintain EBV in infected cells and promote replication of episome (8,45,46). In the current study, researchers
have used EBNA-1 protein to detect and found the frequency of EBV in Hodgkin’s and Non-Hodgkin’s lymphoma. They have applied EBNA-3C to identify the EBV genotype in all samples.

Two different types of EBV, EBV-1, and EBV-2 were indicated by amplification of DNA from EBNA-3A, 3B, and 3C. The predominant type in Asia, Europe, and the United States is EBV-1 whereas EBV-2 is distinguished in Africa, New Guinea and in individuals who are HIV seropositive even in regions in addition of Africa (10,47).

In the present study, EBV genotyping study using sequencing of positive samples revealed that all EBV positive in both Hodgkin’s and Non-Hodgkin’s lymphoma were belonged to the type 1 and were EBV-1.

Acknowledgments

Sincere gratitude is extended to indefinite efforts of the honorable supervisor of the department of pathology of the Imam Khomeini Hospital of Ahvaz. This project with registration No 91103 was financially supported by Health Research Institute Infectious and Tropical Diseases Research Centre, Jundishapur University of Medical Sciences, Ahvaz, Iran.

References


EBV genotyping in Hodgkin and non-Hodgkin patients


