Iranian Journal of Virology 2017;11(4): 1-7

Original Article

Evaluation of Epstein - Barr virus Frequency in Paraffin-Embedded Tissues of Hodgkin and Non-Hodgkin Lymphoma Patients in Kerman Province

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Abstract

Background and Aims: EBV infection usually occurs in humans and is very common. Sero-epidemiological surveys show that over 95% of adults worldwide are faced with EBV virus. The Epstein-Barr virus causes infectious mononucleosis and its association with a number of human cancers, including Hodgkin's lymphoma, non-Hodgkin lymphoma (especially lymphoma BL), nasopharyngeal carcinoma, gastric carcinoma and breast cancer has been established.

Materials and Methods: This study was conducted cross-sectional. This study was designed to determine the presence of the Epstein-Barr virus genome in tissue samples of patients with Hodgkin lymphoma and non-Hodgkins. In this study, 40 samples of patients with non-Hodgkin's lymphoma and Hodgkin's disease, while had been kept in archives of the Shahid Bahonar Hospital, Afzalipour Hospital and Payambar-e-Azam Hospital of Kerman were examined by real-time PCR technique. The data collected by SPSS software and chi-square test were analyzed.

Results: The prevalence of EBV in patients in this study was 5/27% of the 40 sample, of which 20 cases were of Hodgkin lymphoma and 20 cases were of non-Hodgkin lymphoma. No significant correlation was found between the prevalence of EBV in Hodgkin and non-Hodgkin lymphoma. There was also no correlation between men and women of different ages for the presence of EBV DNA in patients samples ($P>0.05$).

Conclusions: EBV DNA associated with the tumor could be detected by molecular methods which are useful for the diagnosis of EBV-associated diseases, also biological factors such as age, sex, health status, social and economic factors in the pathogenesis of EBV and its relationship to lymphoma was not observed.

Keywords: Hodgkin's lymphoma, Non-Hodgkin`s lymphoma, Epstein-Barr Virus, Real - Time-PCR.

Introduction

Epstein - Barr virus (EBV) is a member in the family Herpesviridae, subfamily Gamma-Herpesvirus (1). EBV has a linear, double-stranded DNA genome of about 184 Kilobase pairs (Kbp), encased in an icosahedral protein nucleocapsid surrounded by a lipid envelope (2). EBVs have been classified as Type I or Type II based on DNA sequence divergence in the EBNA-2 and -3 regions (3) type 1 is prevalent in China and type 2 is common in Africa and New Guinea (4-7).

Infectious mononucleosis, that often called "mono", corresponds to a group of symptoms often created by the Epstein-Barr virus (EBV). It typically occurs in teenagers, but it can afflict any age group (8). EBV can spread through direct contact with saliva or expose to cough or sneeze, by kissing, or by sharing food or drinks with someone who has mono (9). The incubation period of the disease is 4 to 6 weeks. The signs and symptoms of mono typically, last for one to two months. Symptoms may include fever, sore throat, swollen lymph glands in the neck and armpits, headache, fatigue, muscle weakness, swollen tonsils, night sweats. Occasionally, spleen or liver may also swell. Also, EBV can infect B-lymphocytes and epithelial cells. EBV exhibits two phases in its infective cycle, latency, and lytic replication (4). In EBV-associated tumors, the virus establishes a latent infection, which is characterized by the limited expression of a subset of viral latent genes (10).

The incidence of diseases such as Hodgkin and Non-Hodgkin`s lymphoma have been seen in people with the same infection experience (11). EBV is an omnipresent human herpes virus with oncogenic action. The EBV genome was detected in malignancies of both lymphoid and epithelial cell origin, such as nasopharyngeal carcinoma (NPC) (12). EBV has been associated with various human malignancies, including Burkitt's lymphoma, Hodgkin 's disease, nasopharyngeal carcinoma (NPC), some T cell lymphomas, post-transplant lymphoproliferative disease, and newly, some cancers of the stomach and smooth muscle (13, 14).

Primary EBV infection, which is frequently asymptomatic, occurs in childhood (15). With the close contact, the virus can be spread among the family members and can establish latency in B lymphocyte where its DNA will be in the form of the episome. Asymptomatic reactivation of the virus is common, which can be lead to the transmission of infection to healthy individuals (16). The relation of a virus with a special tumor can typically reveal the exact feature of the transformation of this virus. For example, a number of latent proteins encoded by EBV, are responsible for the 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), which are used in the diagnosis (2, 17) and likely play a crucial role in EBV- associated malignancies (2). EBNA1 is a DNA- a binding protein that is necessary for the replication and maintenance of the episomal EBV genome; EBNA1 also acts as a transcriptional trans activator and increases gene expression (11).

When cells are activated to lytic infection, EBNA1 is the only nuclear antigen that can be transcribed and plays a key role in cell growth and survival (18). EBNA1 as the only protein expressed in all EBV-associated tumors play a critical role in the maintenance, replication, and transcription of the EBV genome in latently infected cells (4, 19). Hodgkin lymphoma or Hodgkin's disease is a type of lymphocytes cancer and characterized by multinucleated Reed–Sternberg cells (RS cells) (16). HL account for 1 per 100000 cases (both genders) worldwide (17). Low occurrence of Hodgkin lymphoma has been reported in Iran (17). Non-Hodgkin lymphoma is the cancer type of B, T, or natural killer (NK) cells (16).

The etiology of lymphoma has not yet been fully understood and may be influenced by genetic susceptibility, immune status, ethnicity, viruses, environmental factors, cultural factors

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and geographic factors (18). Low prevalence of Non-Hodgkin lymphoma has been proclaimed in Iran (19).

Methods

Patients and specimens. The samples used in this study included 20 paraffin-embedded tissues blocks from Hodgkin disease and 20 paraffin-embedded tissues blocks from Non-Hodgkin,s lymphoma patients. These blocks were archived from the department of pathology of both hospitals, Shahid Bahonar, Afzalipour and Payambar-e-Azam in Kerman during 2006-2014.

Diagnostic accuracy of Hodgkin and Non-Hodgkin,s lymphoma confirmed pathologically. Initially, all paraffin-embedded blocks were sectioned 10 µm-thick and stored at 4°c until following stages of experiments.

Deparaffinization and DNA extraction. using "high pure DNA paraffin kit "(Interlab service, Italy) Initially all paraffin-embedded sections(10µm) became deparaffinized and next total DNA extracted from these samples according to the manufacturer's instruction and then the extracted DNA was stored in micro-tube until Real-Time PCR amplification.

Real-Time PCR. In the first experiment, using Real-Time PCR was performed using Hydrolysis probes where the

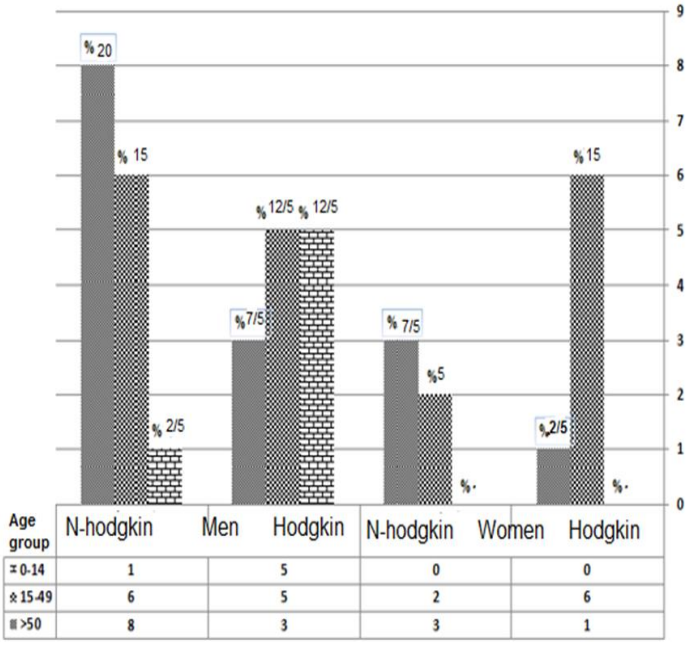
VBC Biotech F and R VBC Biotech primers that are common among all types of Epstein-Barr virus were used to identify positive examples. At this trial, the "Amplicon ш Master mix" manufactured by Inter lab service was used.

Materials. Amplicon ш Master mix: a polymerase enzyme Taq, dNTP, Forward Primer, Reverse Primer, Distilled water, primer-probe was used

Primer used:

Table 1. primers used in the experiment.

Genes	Primer Sequence	TM
VBCF	5'-TAGAGGACCTGGAAATGG-3	59/4
VBCR	5′- TCTTTGAGGTCCACTGCC -3′	58/8
VBCP	FAM-AGGGAGACACATCTGGACCAGAAG-BHQ 1	68



Age group	N-hodgkin	Men	Hodgkin	N-hodgkin	Women	Hodgkin
¤ 0-14	1	5		0		0
¤ 15-49	6	5		2		6
ш >50	8	3		3		1

Fig. 1. EBV positive cases in Hodgkin`s and Non- Hodgkin's lymphoma

Preparations of Master Mix. Primers were reconstituted according to the manufacturer instruction.At this state 20 µl sterile water was added to the forward primer and 20µl sterile water was added to reverse primer. Primer probe was dissolved in 10 µl sterile water according to the brochure.

To build 400 µl master mix per each reaction, 50µl forward primer, 50µl reverse primer, and 100 µl primer-probe were mixed with 200 µl of distilled water.That finally 400 µl master mix was prepared. Five µl master mix for each sample was required proportionally to the number of samples.

Then, 10 ml from each sample and 5 µl from Master Mix were placed in 0.2 ml microtubes and were put in the device. test time was 128 minutes.

Statistical analysis. The variable such as mean, standard deviation, were analyzed using SPSS version 21, age and sex on positive cases were analyzed by chi-square exact tests.

Results

From 20 cases of Hodgkin's lymphoma, 8 cases and from 20 cases of Non-Hodgkin's, 3 cases were positive for EBV. These EBV positive cases in Hodgkin's lymphoma

included 3 (37,50%) female and 5 (62,50%) male. These EBV positive cases in Non-Hodgkin's lymphoma included 1(37,50%) female and 2 (66,66%) male. From these positive cases the most frequent, 8 (57%) cases, belonged to the adult's age group (figure 1). The results of Fisher`s exact test have showen that the frequency of Epstein-Barr Virus in adults in comparison with children in Non-Hodgkin`s lymphoma patients was significantly higher (P=0.03). The results of histological cases with positive EBV are shown in two age groups and two different genders.

Discussion

Epstein-Barr virus (EBV) causes infectious mononucleosis and its relationship with some human cancers, including Hodgkin's lymphoma, non-Hodgkin's lymphomas (especially Burkitt's lymphoma), nasopharyngeal carcinoma, gastric carcinoma and breast cancer has been observed (20, 21).

Although the global prevalence of the virus is estimated to be at 95% of adults have been reported, but the tumors associated with this virus have been reported in different regions (22). Hodgkin's lymphoma and non-Hodgkin's and Burkitt's lymphoma by the presence of EBV are of the lymphoproliferative disorders (23). Recent studies on the molecular epidemiology evidence linking Epstein-Barr virus disease Hodgkin's lymphoma, Burkitt's lymphoma and other types of non-Hodgkin lymphoma have been reported (24).

The evidence and findings of latent infection by EBV in these tumors have been confirmed by using various techniques (including Real-time PCR) (25).

Epidemiological factors such as age, sex, race, geographic region, social class and the important role of an infectious agent (11) and in this regard is highly variable in different populations and different contexts.

The correlation of Hodgkin's lymphoma with Epstein-Barr virus in the developed Western countries from 25% to 50% (21), 31/3 percent in America (26), 28% in Denmark (27), in China 57% (28) and in Turkey 55 the percentage obtained (25), and the values are higher in underdeveloped countries such as Peru and Kenya have been observed (29).

The presence of EBV in Hodgkin's disease reported in most developing countries compared to Western countries, although the amounts vary from one country to another (30).

The hypothesis MacMahon, Hodgkin's lymphoma was based on age categories. These categories in three age groups of children, youth and the elderly have been conducted and prevalence, descriptive epidemiology, and assessment of risk factors included (22). Curve incidence of Hodgkin's disease is related to the age of two peaks, a peak in childhood and another in adulthood (over 51 years).

Although these peaks are associated with different geographical regions, the prevalence of childhood disease in affluent communities at the age of 15 to 35 years in affluent societies occurs in 5 to 10 years (31, 32).

Hodgkin's lymphoma has four sub-groups, including mixed cell (MC), nodular sclerosis (NS), with a predominance of lymphocytes (LP) and decreased lymphocytes (LD) (33).

Childhood Hodgkin's lymphoma frequently typed MC, in young adults frequently typed NS and in older adults mostly Type MC. Type MC compared with NS has more connection with EBV (33).

In developing countries, primary infection usually occurs in early childhood and the relationship between Epstein-Barr virus and Hodgkin's lymphoma subtype in children's and MC and LD are common (8, 34). In contrast, youth Hodgkin's lymphoma is more common in developed countries and is the dominant subtype NS. The risk of Hodgkin's lymphoma in young adults with a higher level of Socio-Economic childhood is linked to the delay in dealing with an infectious agent, particularly EBV poses (35).

In this study from of 20 patients with Hodgkin's lymphoma, 8 (40%) positive EBV cases were found that 6 cases (75%) were in the age group of 15 to 48 years old and from of foregoing 8 positive cases, 6 positive cases were belonging to the nodular sclerosis (NS)

and two cases of mixed cell (MC) of the disease.

And in this study due to the larger number of samples in the age group of 15 to 49 years (young adults), more samples were tested in the type NS. With some studies in developing countries does not match. Perhaps reflecting gains in economic growth and led to the development.

In this study from of 8 positive EBV cases in Hodgkin lymphoma, 3 (37.50 %) cases in women and 6 (62.50) cases in men have been observed. Also from of 3 positive cases in non-Hodgkin lymphoma, 1 (33.33 %) cases in women and 2 (66.66 %) cases in men has been observed. From statistical conception, any correlation with rate of the prevalence of EBV between women and men suffering to Hodgkin's lymphoma and non-Hodgkin lymphoma and also between the age of women and men has not been observed ($p > 0.05$). that have no matching with some other studies in developing countries, maybe causes by the method of performance test or a low number of cases.

These results demonstrate that epigenetic form Enlarge EBV DNA was associated with tumor mass and its identification with molecular techniques to diagnose diseases associated with EBV, is possible, as well as biological factors such as age, sex, health, social and economic factors in the pathogenesis of EBV and its relationship and create a variety of lymphoma are.

Conclusion

Epithelial form of EBV DNA associated with the tumor was detected by molecular methods for the diagnosis of EBV-associated diseases, also biological factors such as age, sex, health status, social and economic factors in the pathogenesis of EBV and its relationship to creating a variety of lymphoma is possible.

Acknowledgment

Sincere gratitude is extended to indefinite efforts of the department of pathology of both hospitals shahid bahonar and Afzalipour and Payambar-e-Azam in Kerman. This project with registration No 9352 was financially supported by Infectious and Tropical Disease Researc.

Conflict of interest

The authors declare they have no conflict of interest.

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