

Original Article

The Incidence of EBV in Diffuse Large B-Cell Lymphoma: A Comparative Study of Immunohistochemical and PCR Techniques

Farid Kosari¹, Naghmeh Amin Taheri², Alireza Sadeghipour³,
Kamran Alimoghaddam⁴, Ardeshir Ghavamzadeh⁴

1. Dept. of Pathology, Tehran University of Medical Sciences, Tehran, Iran

2. Tehran Branch, Islamic Azad University, Tehran, Iran

3. Dept. of Pathology, Iran University of Medical Sciences, Tehran, Iran

4. Hematology & Oncology Research Center, Shariati Hospital, Tehran University of Medical Sciences, Tehran, Iran

ABSTRACT

Background and Objectives: Epstein Barr Virus (EBV) is one of the members of herpesviridae family and a sub-category of Gamma herpes virinae. EBV, which normally has CR2 or CD21 receptors on B-lymphocytes, has mutagenic features for them. The virus plays an important role in causing some malignant cancers. About 30% of the cases with non-Hodgkin's lymphoma are diffuse large B-cell lymphoma (DLBCL). In the present study, the incidence rate of EBV in DLBCL was evaluated.

Materials and Methods: Immunohistochemistry (IHC) and PCR methods were used for studying the relationship between EBV and DLBCL. Paraffin blocks of 116 patients from Sina & Shariati hospitals, Tehran, Iran, with DLBCL diagnoses in 2005-2009 were collected. EBV-LMP in IHC and PCR virus genome in PCR were examined.

Results: Findings of the PCR method showed that 28 cases of the total 116 patients with DLBCL were EBV positive (the frequency of EBV positivity was correspondingly 40% and 60% in females and males) and this shows a 25.8% EBV frequency in DLBCL. IHC findings showed that six cases were EBV positive. The compatibility of positive IHC and PCR responses was two cases and there are four conflicting cases.

Conclusion: It seems that PCR is a more appropriate method for diagnosing EBV and IHC cannot solely prove the presence of EBV in DCBCL patients.

Key words: Epstein Barr Virus Infections, Diffuse Large-Cell Lymphoma, B Cell Lymphoma, Immunohistochemistry

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Address communications to: Dr Farid Kosari, Department of Pathology, Tehran University of Medical Sciences, Tehran, Iran

Email: faridkosari@yahoo.com

Introduction

Diffuse large B-cell lymphoma (DLBCL) includes about 30% of non-Hodgkin's lymphoma, which are commonly seen in older ages; however, it can emerge at any age (1) and is more prevalent in men (1, 2). On the other hand, Epstein Barr Virus (EBV) is one of the members of herpesviridae family and a sub-category of Gamma herpes virinae (3). Most people of the world are infected by the virus (3) (95% of American people between 30-40 yr.). This infection usually emerges in the first decade of life and infects the cells without any specific signs (3, 4). EBV naturally has a receptor on B lymphocytes called CR21 or CR2 (3, 4), which at the same time has mutagenic characteristics for B lymphocytes; in other words, it is the cause of the ploy-colonal stimulation of cells. Following the contamination of epithelial cells, the active replication of the virus leads to the lysis and destruction of the cell (3, 4). Moreover, EBV causes the infected B cells to replicate and this leads to a genetic mutation in new B cells and eventually transformation to lymphomas (3-5).

The most common methods of detection of the virus in the different lymphomas include IHC, RISH and PCR (6). In the present study, the incidence rate of EBV in DLBCL was evaluated

MATERIAL & METHODS

IHC procedures:

Paraffin blocks of patients with the pathologic diagnosis of DLBCL confirmed by IHC were collected. Initially, the paraffin blocks were cut by microtome and slides of the required blocks were prepared. The slides were kept in 37°C for 24 hours. In the hydration stage, they were passed through xylol, three times (each time lasted 10 minutes). The slides were respectively passed through absolute, 70%, and 96% alcohol (5 minutes each) and then they were rinsed by water. The 1/10 density H₂O₂ was mixed with methanol and the slides were put in H₂O₂ solution for 20 minutes. After being rinsed, the slides were put in Trice EDTA solution with pH=9 and then in the autoclave. They were taken out after 30 minutes and were washed in PBS solution

after cooling. The EBV-LMP antibody thinned by 1/100 ratio was put on a slide to cover the tissue. After 45 minutes, the slides were washed by PBS solution. Envision solution and a chromogen were added after washing in order to make the antibodies visible under the microscope (the background color would be brown under the microscope). After washing the slides with water, they were kept in hematoxylin for 15 seconds and were then rinsed. Finally, the slides were passed through 70%, 96% and absolute alcohol as well as xylol before being observed under the microscope (in each run, the EBV control sample is used as a proof for positive staining).

PCR: In this method, DNA virus was looked for on paraffin blocks. The required primer was provided by TIB molBio synthesis Labor Company and their nucleotide base sequence was as follows:

- 1- 5'-AGT CTG GGA AGA CAA CCA CA
- 2- 5'-CCC GCC TAC ACA CCA A

PCR Procedures

Before the cut, the microtome unit and the tools should be disinfected by HCL.

1. 8-10 μ thick paraffin blocks were cut by the microtome

2. Deparaffinizing:

in order to deparaffinize the samples, xylol was poured on them by sampler and were then vortexed (three times and the surface solution was outpoured). During the third time, the sample was centrifuged by 1200 cycles for 5 minutes and again the surface solution was outpoured. Next, absolute and then 70% alcohol were added to the sample (like the previous phase, the sample was vortexed and the surface solution was outpoured) and it was rinsed by distilled water.

3. DNA extraction:

The extraction kit No.667327 L of Roche Company including 3 buffers was used for extracting DNA:

A mixture of 1000 microliter of buffer 1 (RBCs lyses) and 300 microliter of buffer 2 (WBCs lyses) along with proteinase K were kept at 56°C for 2 days.

Buffer 3 (for protein sedimentation) was added to the samples kept at 56°C. This time, after

centrifuging, the surface solution was poured into a new tube and 500 microliter of absolute alcohol was added to the samples, mixed, and centrifuged. The surface solution was outpoured, 70% alcohol was added and centrifuged; the solution was outpoured. Finally, the microtube was put under 37°C so that the sample dried; then, distilled water was added.

4. PCR thermal cycling program:

- a. Predenaturation: 94°C for 5 minutes: 1 cycle
- b. Denaturation: 94°C for 30 seconds: 40 cycle
- c. Annealing: 60°C for 30 seconds: 40 cycle
- d. Elongation: 72°C for 30 seconds: 40 cycle
- e. Post-elongation: 72°C for 5 minutes: 1 cycle

5. Electrophoresis in 2% Agarose gel; Voltage: 1v

Results

Among the 116 cases (Fig. 1) in the study, there were 73 males (63%) and 43 females (37%). Using PCR method, EBV in 28 cases with DLBCL became positive (Fig. 2); this shows the frequency of 25.8% EBV in DLBCL (17 male & 11 females EBV⁺ cases). The frequency of EBV positivity was 40% and 60% in women and men, respectively.

The EBV positive cases of the present study were classified into four age groups:

- Less than 20 yrs.: 6 cases
 - 20-40 yrs.: 8 cases
 - 40-60 yrs.: 7 cases
 - Older than 60 yrs.: 7 cases of EBV⁺
- In IHC, EBV became positive in six cases (Fig. 3), and the compatibility of PCR and IHC positive responses was two and the conflicts were four cases.

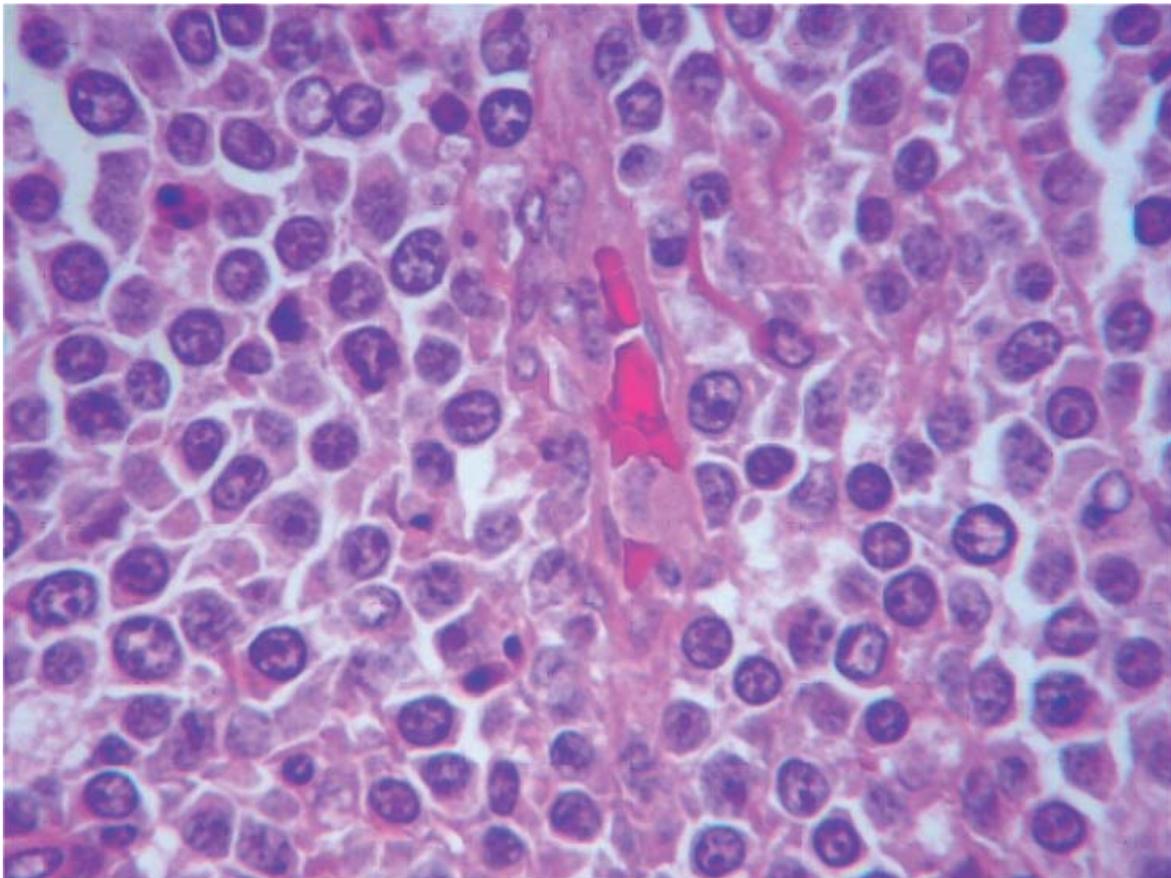


Fig. 1: Histopathologic view of Diffuse Large B-cell Lymphoma (H&E × 400)

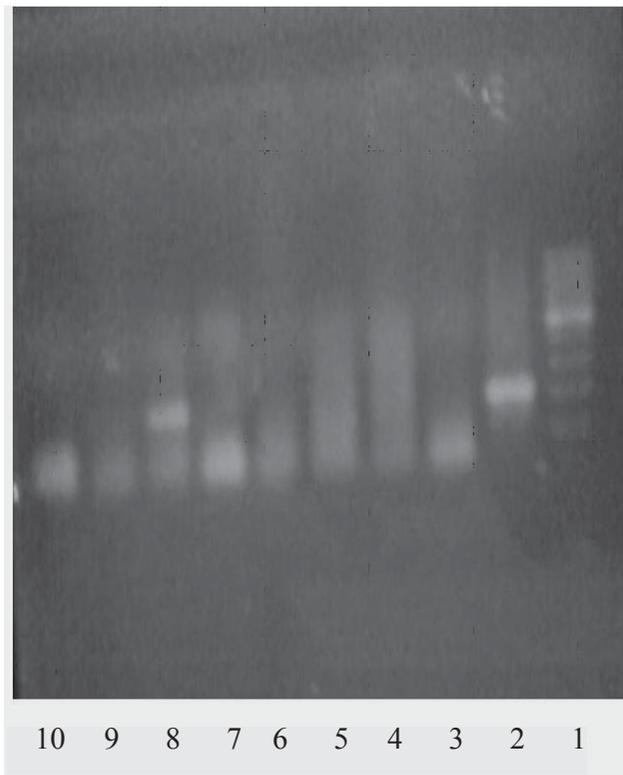


Fig. 2: Agarose gel electrophoresis of PCR products

- 1- Molecular weight marker 210bp (ladder)
- 2- Positive control
- 3- Negative control
- 4- Negative sample (DNA from paraffin block)
- 5- Negative sample (DNA from paraffin block)
- 6- Negative sample (DNA from paraffin block)
- 7- Negative sample (DNA from paraffin block)
- 8- Positive sample (DNA from paraffin block)
- 9- Negative sample (DNA from paraffin block)
- 10- Negative sample (DNA from paraffin block)

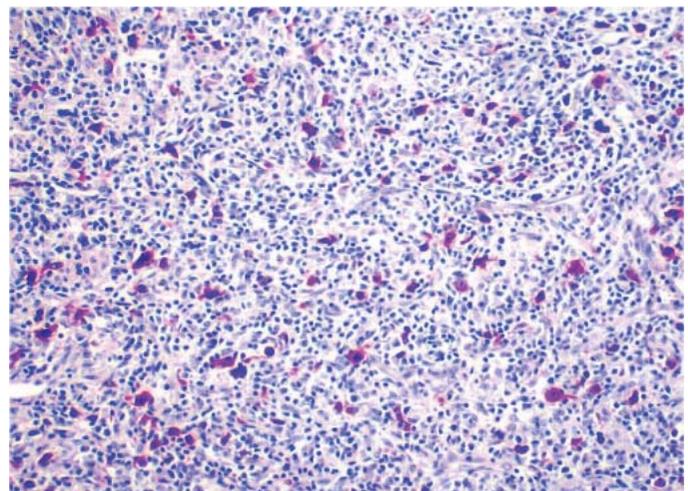


Fig. 3: Positive EBV-LMP staining in Immunohistochemical method, $\times 400$

Discussion

In this study, 28 cases of the total 116 patients with DLBCL were EBV positive based on PCR method (40% and 60% in females and males, respectively) and this shows a 25.8% EBV frequency in DLBCL.

In a study, the concordance of DLBCL and EBV was mostly reported in the elderly (7); however, no meaningful difference was observed in the EBV frequency among DLBCL patients. Moreover, in another study of Park carried out on 380 DLBCL

patients, the frequency of EBV was reported as 9% (7). EBV and DLBCL are related and EBV frequency is cited at 12% (3).

In previous studies, EBV frequency (using In situ hybridization method) in DLBCL patients was reported at about 8-35%, which is statistically comparative (8, 9). In a study performed on patients with NHL in 2006 (10), PCR sensitivity and specificity for diagnosis were reported as 96% and 100%, respectively and the prevalence of EBV NHL patients was 12% (10). Considering the point that in PCR and IHC methods, only 2 cases are

compatible, it seems that PCR is a more appropriate method of diagnosis for EBV in DLBCL patients and IHC cannot solely suffice for providing the presence of EBV in patients with DLBCL. Therefore, it is suggested that PCR is used for diagnosing EBV (11); however, further studies on the patients might be required, as well. In a study conducted on Brazilian children with NHL in 2006, RISH and PCR methods were compared and it was found that PCR sensitivity and specificity were 96 and 100%, correspondingly and the method was reported as the first and the fastest method of diagnosing EBV accompanied by NHL. In the same study, EBV prevalence in NHL and DLBCL were expressed as 68% and 17%, respectively.

Conclusion

Considering the fact that few studies have been conducted on the topic across the country, it is proposed that PCR is also used for diagnosing EBV in tissues without DLBCL so that the role of EBV in the incidence of DLBCL can be demonstrated more clearly.

Acknowledgment

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References

1. Kimura H, Hoshino Y, Kanegane H, Tsuge I, Okamura T, Kawa K, *et al.* Clinical and virologic characteristics of chronic active Epstein-Barr virus infection. *Blood* 2001;98(2):280-6.
2. Risdall RJ, McKenna RW, Nesbit ME, Krivit W, Balfour HH Jr., Simmons RL, *et al.* Virus-associated hemophagocytic syndrome: a benign histiocytic proliferation distinct from malignant histiocytosis. *Cancer* 1979;44(3):993-1002.
3. Oyama T, Yamamoto K, Asano N, Oshiro A, Suzuki R, Kagami Y, *et al.* Age-related EBV-associated B-cell lymphoproliferative disorders constitute a distinct clinicopathologic group: a study of 96 patients. *Clin Cancer Res* 2007;13(17):5124-32.
4. Feng WH, Kenney SC. Valproic acid enhances the efficacy of chemotherapy in EBV-positive tumors by increasing lytic viral gene expression. *Cancer Res* 2006;66(17):8762-9.
5. Delecluse HJ, Kremmer E, Rouault JP, Cour C, Bornkamm G, Berger F. The expression of Epstein-Barr virus latent proteins is related to the pathological features of post-transplant lymphoproliferative disorders. *Am J Pathol* 1995;146(5):1113-20.
6. Buisson M, Morand P, Genoulaz O, Bourgeat MJ, Micoud M, Seigneurin JM. Changes in the dominant Epstein-Barr virus type during human immunodeficiency virus infection. *J Gen Virol* 1994;75(Pt 2):431-7.
7. Park S, Lee J, Ko YH, Han A, Jun HJ, Lee SC, *et al.* The impact of Epstein-Barr virus status on clinical outcome in diffuse large B-cell lymphoma. *Blood* 2007;110(3):972-8.
8. Armitage JO, Weisenburger DD. New approach to classifying non-Hodgkin's lymphomas: clinical features of the major histologic subtypes. Non-Hodgkin's Lymphoma Classification Project. *J Clin Oncol* 1998;16(8):2780-95.
9. Hoffman N, Rickinson A, Kieff E. *Field's Virology*, 3rd ed. Philadelphia: Lippincott-Raven Publishers; 1996.
10. Perrine SP, Hermine O, Small T, Suarez F, O'Reilly R, Boulad F, *et al.* A phase 1/2 trial of arginine butyrate and ganciclovir in patients with Epstein-Barr virus-associated lymphoid malignancies. *Blood* 2007;109(6):2571-8.
11. Hoffman N, Sugden B. *Immune Deficiency and Cancer*. New York: Plenum Press; 1984.