

Original Article

Study of Epstein-Barr virus expression in Burkitt's lymphoma by polymerase chain reaction and *in situ* hybridization: A study in Iran

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ABSTRACT

Background: The association of Epstein-Barr virus (EBV) with Burkitt's lymphoma (BL) is variable in different geographic regions. In developing countries, the association of EBV with BL is regarded to be of an endemic-type in equatorial Africa (> 95%) and sporadic-type in the developed countries (15-30%). The purpose of this study is to assess the frequency of EBV infection in BL, in Iran. The study also aims to compare Ribonucleic acid (RNA) *in situ* hybridization (RISH), the standard diagnostic method, with the polymerase chain reaction (PCR)-based method for diagnosing BL.

Materials and Methods: In this epidemiological study, the paraffinized specimens of 18 cases of BL were selected. Next, the ISH of EBV-encoded RNA (EBER-RISH) and PCR assays that were based on Epstein Barr Nuclear Antigen 2 (EBNA2) amplification were used. The EBV strain was determined by PCR. The data were analyzed using the SPSS10 software and by performing Pearson correlation coefficient formula at a significant level of 0.05.

Results: EBV RNA was detected in 50% of the BL specimens. Type 1 and 2 accounted for 70 and 30% of the cases, respectively. Regarding RISH as the standard method for EBV diagnosis, the PCR assays showed a sensitivity and specificity of 100 and 88.9%, respectively.

Conclusion: According to the obtained findings, the frequency of EBV in BL was 50% and PCR and RISH showed high concordance and sensitivity in EBV detection. Therefore, PCR can be used as a faster method for EBV detection in high-risk geographical regions.

Key Words: Burkitt's lymphoma, Epstein-Barr virus, *in situ* hybridization, polymerase chain reaction

Received: May 2013

Accepted: October 2013

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INTRODUCTION

About half of the childhood cancers in equatorial Africa are due to Burkitt's lymphoma (BL)

(i.e., endemic or African BL), while it occurs at a much lower rate in the rest of the world (i.e., sporadic or American BL).^[1]

The endemic type is associated with the Epstein-Barr virus (EBV) in almost all cases (DNA virus is present in more than 95% of the cases).^[2,3] The

association of BL with EBV is much less common in non-endemic areas. In the United States of America (USA), the association of BL with EBV is only 30%, while it is intermediate between endemic and sporadic types in developing countries,^[4] ranging from 55% in Taiwan,^[2] 80% in Pakistan,^[5] 27% in China,^[6] and 13% in Japan.^[7] The BL-type associated with the acquired immunodeficiency syndrome has also been introduced, in which the virus genome is detected in approximately 30 to 50% of the patients.^[8]

The EBV, a wide-spread human herpes virus, has a high affinity to B-cells.^[9] The first exposure to the virus depends on the socioeconomic conditions and usually occurs in the first decade of life. In fact, more than 90% of the adults are infected with the virus. Initial infection with the virus leads to a persistent, latent, and asymptomatic infection, which remains in a limited amount in the memory B-cells.^[10,11]

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In recent years, the serological and molecular assessments have evidenced that EBV is associated with a number of malignancies.^[12] Thus, a deep understanding of this relationship can be useful for clinical and therapeutic interventions.^[13,14]

To identify EBV infection, the following methods are used: *In situ* hybridization (ISH), southern blotting, and polymerase chain reaction (PCR).^[15] As a high amount of EBV-transcripts are expressed in the latently infected cells, the RNA-ISH (RISH) method is used as the standard method for diagnosing infected cells, to detect EBV-encoded RNA (EBER).^[16,17] The PCR-based methods are also used to determine the strain of virus (1 or 2).^[18]

In this research, we studied the prevalence of EBV in BL, in Iran, and also compared the sensitivity and specificity of PCR, as an easier and faster method for EBV detection, with RISH as the standard method.

MATERIALS AND METHODS

Case selection

In this epidemiological study, 18 cases of BL were studied. These cases had been diagnosed at Al-Zahra Hospital and the Dental School of Medical Sciences (Isfahan, Iran) from 2008 to 2013. The specimens were formalin-fixed, paraffin-embedded tissues. The cases were intra-abdominal biopsies (six cases), head and neck region biopsies, including jaws, brain, nose, scalp, submandibular region, and cervical lymph nodes (ten cases), and thorax and pelvis (one case each). Other clinical information including age and sex was also available.

To begin with, the histopathological diagnoses were reviewed by two independent pathologists, according to the Revised European–American Lymphoma Classification (REAL).^[19]

Immunohistochemical staining, which was used as a criterion for entrance, was carried out in all the cases by the standard avidin-biotin-peroxidase technique. The antibody panel included CD20, CD10, and CD3 (DAKO, Carpinteria, CA).^[20]

Finally, EBV detection was performed by the two methods, namely, RISH and PCR.

EBER-1 RNA *in situ* hybridization

To detect EBER-1, an EBV transcript expressed actively in latently infected cells, based on Zytovision

(Bremerhaven, Germany) protocol, RISH with a biotinylated probe was done.^[21,22]

Briefly, 5 µm tissue sections were prepared on poly-D-lysine-treated glass slides and incubated at 70°C for a day. Then, the sections were deparaffinized and 3% solution of H₂O₂ was used to block the endogenous peroxidase. Enzymatic digestion of the sections was done with protease, followed by dehydration in ethanol and drying at room temperature. The slides were incubated in pre-hybridization solution for 20 minutes at 95°C. After washing with phosphate buffer saline (PBS), an EBER-1 biotinylated probe was added and incubated overnight (Invitrogen, Germany). The slides were washed again with PBS and placed in the washing solution (PBS) for 10 minutes at 25°C. Next, the slides were placed in AG solution, after that in the DAB solution, and finally counterstained with hematoxylin and mounted with Entellan.

The cells that presented with dark brown to black nuclear staining were considered as infected. Positive control was a case previously diagnosed as an EBV-positive BL.

Polymerase chain reaction amplification

Sections of 5 µm were used to extract high molecular weight DNA from tissue samples by using the 5-Prime kit (5 prime, Hamburg, Germany). During sectioning the samples, care was taken to avoid cross-contamination between them. A spectrophotometer was used to check the amount and purity of the DNA.^[23]

EBNA2 primers have been described previously.^[24] In this study, two primers were used for EBV detection and typing [Table 1].

Briefly, the reaction mixture consisted of 10 mM TRIS-HCl (PH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM of the dNTP mix, gelatin 0.001%, 0.3 µM of each primer, 1 U of Taq DNA polymerase, and 1 µg of the DNA template or 5 µl of 1:10 diluted lysate.

The reaction tubes were placed in a thermo cycler (Applied Biosystem, Germany) and 35 cycles of amplification (30 seconds at 96°C, 30 seconds at 60°C, and one minute at 72°C) were conducted.

Table 1: Primers used for EBNA2 pcr typing

Primers	Sequence (5'-3')	Use in the PCR reaction
EBNA-2G	GCCTCGGTTGTGACAGAG	Type-1
EBNA-2B	TTGAAGAGTATGTCTTAAGG	Type-2

After the last cycle, the process was followed by an extension step for 10 minutes. The amplified DNA was analyzed by an Applied Biosystem.

After the data were collected, the Pearson correlation coefficient was used in the SPSS10 software (SPSS Inc., Chicago, IL, USA) to analyze the data. The significance level was determined to be 0.05.

RESULTS

Patient characteristics

The clinical data of the BL cases are concisely presented. There were 13 males and five females, aged between two and 81 years, with a mean of 21 years.

Regarding the site of tumor involvement, the head and neck region was the most frequent (ten cases) followed by intra-abdominal organs (six cases), and two cases of thorax and pelvis.

Immunohistochemical findings

B-cell origin was documented by expressing CD10 and CD20 in all the cases of BL.

RISH and PCR assays

All samples were evaluated by RISH and PCR. The findings are summarized in Table 2. EBV RNA was detected in nine out of 18 BL cases by RISH [Figure 1] and 10 out of 18 cases by PCR. The discordant result was one PCR positive/ISH negative. As ISH is considered the standard test for EBV detection, 100% sensitivity and 88.9% specificity

were obtained for the PCR assay. Type 1 EBV was identified in six out of nine cases (70%), while type 2 was found in three samples (30%).

DISCUSSION

The incidence rate of BL shows considerable geographic variations. In equatorial Africa, the annual incidence is between five and ten per 100,000 children younger than 15 years old, whereas, in USA it occurs at a rate of about two per million persons.^[4,25] Patients with acquired immunodeficiency syndrome (AIDS) are at higher risk of developing BL. The exact reasons for these differences are unclear, although environmental (e.g., malarial infection) and genetic factors, as also immunosuppression may play a role.^[6]

In Iran, BL is the second most common high grade B-cell lymphoma.^[26] In a study conducted in Fars province (in Southern part of Iran), 10.8% of the lymphoid malignancies in children under 15 years were BL.^[27] In another study in Mashhad (in Northeast of Iran), BL accounted for 0.5% of all lymphoid malignancies in adults.^[28]

Jaw lesions are the most characteristic manifestations of the disease and are commonly seen in 50 to 70% of the patients with endemic BL, while in the sporadic type, the most common site of involvement is the abdomen.^[5] In this study, the major site of involvement has been the head and neck region (55.5% of the cases) followed by intra-abdominal organs (11.1% of the cases).

Table 2: Detection of ebv by RISH and PCR

Case number	EBER	Real time PCR	EBV Sub type
1	+	1.1×10 ³	1
2	—	—	—
3	+	4.2×10 ³	1
4	—	—	—
5	+	1.4×10 ³	1
6	+	3.6×10 ³	1
7	—	—	—
8	+	1.9×10 ³	2
9	+	3.8×10 ³	1
10	+	1.3×10 ³	2
11	+	5.2×10 ³	2
12	+	1.6×10 ³	1
13	—	—	—
14	—	—	—
15	—	0.3×10 ³	—
16	—	—	—
17	—	—	—
18	—	—	—

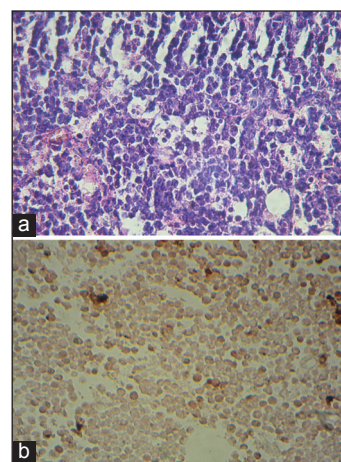


Figure 1: Burkitt's lymphoma. (a) Histological section (H and E, ×400) showing diffuse infiltration of medium-sized, non-cleaved lymphoid cells, with interspersed macrophages presenting a 'starry-sky' appearance. (b) Dark brown RNA *in situ* hybridization signals by EBV-encoded RNA (400×)

Among other differences between endemic and sporadic types, the mean age at the time of diagnosis is noticeable. In the endemic type in Africa, it is 6.09, while for the sporadic type it is 19.2 years.^[1] In this study, the patients' age range was 2-81 years, with a mean of 21 years.

One of the most obvious differences between endemic and sporadic types is their different associations with EBV in various geographical regions.^[29] In tropical Africa, EBV is present in almost all the cases, whereas, in the sporadic form, in developed countries, it turns up in 15 to 30% of the samples. Also, in developing countries, the association is intermediate between endemic and sporadic types.^[9]

Although the precise role of EBV in the BL pathogenesis is unclear even after several decades, it seems EBV has an initiator role and malaria has a promoter role in tumor development in Africa.^[1]

Today, in EBV-positive lymphomas, EBV is considered as a therapeutic target in the new therapeutic interventions. The focus on this issue has resulted in the improvement of EBV detection methods.^[9]

According to the studies conducted in Asian countries, the prevalence of EBV in BL was reported to be 80% in Pakistan,^[5] 55% in Taiwan,^[2] 27% in China,^[6] and 13% in Japan.^[7]

The standard diagnostic method to assay EBV-infection in tumor cells is ISH, which detects EBER. PCR-based methods are applied to determine the virus subtype. Although due to its simplicity, PCR is regarded as the first-line method for virus detection, its high sensitivity may cause false-positive results. This high sensitivity can be accounted for by the fact that memory cells and non-tumor bystander lymphocytes may also be traced.^[10,11,17]

In this regard, Klumb *et al.* observed a high concordance (more than 90%) between EBV detected by ISH and by PCR in the BL samples. They also introduced PCR as a suitable method for diagnosing EBV infection in the pathological samples of non-Hodgkin B-cell lymphoma.^[4] Hassan *et al.* also found high sensitivity and concordance (more than 96%) between ISH and PCR procedures in tracing EBV in BL samples. They further suggested that EBV detection could be done with PCR as a more rapid and simpler method, followed by RISH for confirmation.^[9]

In the present study, by regarding ISH as the standard test for EBV diagnosis, an incidence rate of 50% (nine of 18 samples) was found, which is higher than that in the developed countries and is intermediate between the endemic and sporadic types.

The comparison of EBV detection through PCR and RISH procedures indicated high sensitivity (100%) and specificity (88.9%) for PCR assessment. Furthermore, a correlation coefficient of 88.9% ($PV < 001$) was achieved between PCR and RISH.

The results of this study and previous studies can validate that the PCR assay detects EBV in BL samples. The PCR method can have an important role in diagnosing EBV in lymphoma samples, because in those care centers that serve large populations, ISH is conducted as the second or third method. Note that ISH is used only after histopathological and immunohistochemical diagnosis. The financial cost of ISH in low-income communities is also a limiting factor. Therefore, PCR can be performed as a first-line procedure, followed by ISH for confirmation.^[4,9,17]

Previous studies have identified the subtype 2 virus as the predominant type in Africa and subtype 1 as the predominant one in other regions.^[30-32] However, in Egypt, Anwar *et al.* has observed that subtype 1 virus is predominant on the contrary to the endemic type. This difference could be due to such environmental factors as malaria and AIDS, which are predisposing risk factors in BL, but observed less commonly in Egypt than in other parts of Africa.^[3]

In this study, determining the virus subtype by PCR, revealed sub type 1 in 70% and subtype 2 in 30% of the samples. This is consistent with the previous studies. Accordingly, we can conclude that BL in Iran has a lower association with environmental risk factors such as malaria and AIDS than Africa.

CONCLUSION

The current study shows that both the PCR and ISH methods have high concordance and sensitivity in EBV detection. Therefore, it is suggested that PCR can be used for EBV diagnosis, followed by RISH confirmation.

Identifying the subtype 1 virus as the dominant type in 70% of the samples, being similar to the sporadic type in Asian countries, probably confirms that the environmental predisposing risk factors such as malaria and AIDS have a less significant role in BL pathogenesis in Iran.

ACKNOWLEDGMENTS

This article was based on a thesis submitted to the School of Dentistry, Isfahan University of Medical Sciences, in partial fulfillment of the requirements for an MSc degree in Oral and Maxillofacial Pathology. This study was supported by the Isfahan University of Medical Sciences Grant # 391154. The authors also thank Miss Mahmoodi for preparing the manuscript.

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How to cite this article: Deyhimi P, Kalantari M. Study of Epstein-Barr virus expression in *Burkitt's lymphoma* by polymerase chain reaction and *in situ* hybridization: A study in Iran. *Dent Res J* 2014;11:380-5.

Source of Support: Nil. **Conflict of Interest:** None declared.