Molecular diagnosis of Epstein-Barr virus in paraffin-embedded tissues of tumors with abundant lymphoid infiltration*

M. Korabečná[1], M. Ludvíková[2], A. Skálová[2]

[1]*Department of Biology, Faculty of Medicine, Charles University, 301 66, Pilsen, Czech Republic, e-mail: marie.korabecna@lfp.cuni.cz;*
[2]*Department of Pathological Anatomy, Faculty of Medicine, Charles University, 305 99 Pilsen, Czech Republic*

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To evaluate the significance of Epstein-Barr virus (EBV) infection in tumorogenesis, we examined ten archival samples of acinic cell carcinomas of salivary glands with lymphoid-rich stroma, four archival samples of lymphoepithelioma-like carcinomas (LELC) of the urinary bladder, ten samples of oncocytic papillary carcinoma of the thyroid (Warthin-like tumors) and one sample of lymphoepithelioma-like carcinoma of the cervix, together 25 paraffin-embedded tumor tissues. Polymerase chain reaction (PCR) and *in situ* hybridization (ISH) assays were used. The EBV genome was detected by PCR using primers targeting the IR region. ISH was performed using EBER oligonucleotide probes. Each examination was repeated two times. Positive PCR result was obtained in 12% of samples only. However, this result was not confirmed by the subsequent second PCR examination. ISH revealed negative signals in all samples. Our results demonstrate the importance of the diagnostic strategy based on combination at least two independent methods. PCR due to its sensitivity may produce false positive results depending on the degree of infiltration the tumor sample by EBV carrying lymphocytes.

Key words: Epstein-Barr virus, EBV associated tumors, PCR, ISH.

Epstein-Barr virus (EBV) is associated with a variety of human diseases, including infectious mononucleosis. EBV is constantly present in Burkitt's lymphoma and nasopharyngeal carcinoma. In addition, EBV has been also associated with Hodgkin's disease, immunodeficiency-related lymphoproliferative disorders, and some T-cell lymphomas [31]. In contrast, the association between EBV and lymphoepithelioma-like carcinomas (LELCs) appears to be related to the location of the tumor and the race of the patient. LELCs of the lung, salivary gland, thymus, and stomach have been consistently associated with EBV infection [6, 7, 12, 20, 23, 26, 29, 37, 41], whereas LELCs in other organs such as uterine cervix, vagina, skin, urinary bladder, and breast that have been tested for EBV infection have usually rendered negative results [4, 10, 11, 13, 18, 19, 22, 25, 28, 34, 36, 40]. While restricted to Asian patients in lung and salivary glands, EBV appears to be universal in gastric and thymic tumors [8, 20]. We focused on the molecular diagnosis in tumors with abundant lymphoid infiltration. The detection of the EBV positive B-lymphocytes in tumors may signalize the presence of viral reservoirs for the infection of tumor cell originated from epithelia. Bayliss and Wolf [5] have shown that virally infected B-lymphocytes can fuse with epithelial cells and thus provide a mechanism by which the virus is transferred from one cell type to another. Griffin and Xue [17] cited an unpublished observation of this type from their laboratory. The infected B-lymphocytes were able to fuse in culture with immortalized primate epithelial cells and to produce giant syncytia, multinucleated cells. Such observations raise the question why only some types of LELC are EBV positive when the abundant lymphoid infiltration is characteristic feature of this type of tumors. It has been shown, that virus HIV can ex-

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ploit M cells to penetrate epithelium. The M cells are highly specialized epithelial cells within the lymphoid follicle-associated epithelium of the gastrointestinal and respiratory tracts, which perform non-degradative vesicular transport of foreign particles and antigens to the lymphoid tissue in order to activate immune response. EBV can probably use the same way during infection [14], and therefore the presence of M cells in the epithelium may be an important factor for the development of EBV infection in tumor cells of LELCs originated from gastrointestinal and respiratory tracts. It has been described [16, 32] that EBV contains more than one set of oncogenes. These sets of oncogenes are specific in expression and responsive to the individual host cell in question. The genes, which take part in B cell transformation, seem to be well defined. The set of genes that are responsible for transformation of epithelial cell is not full characterized. The virus can contain a number of genes associated with cell-growth regulation that are silent in one cell type, but active in another [17]. The activity of these genes may be dependent also on the state of cell differentiation that can differ in the diverse types of LELC.

Material and methods

We examined 25 paraffin-embedded tumor tissues: ten archival samples of acinic cell carcinomas with lymphoid rich stroma of salivary glands, four archival samples of lymphoepithelioma-like carcinomas (LELC) of the urinary bladder, ten samples of oncocytic papillary carcinoma of the thyroid (Warthin-like tumors) and one sample of lymphoepithelioma-like carcinoma of the cervix.

For EBV detection, both methods polymerase chain reaction (PCR) and *in situ* hybridization (ISH) were used.

DNA samples were extracted from 4 μm-thick paraffin sections. The sections were treated with lysis buffer (50 mM Tris/HCl, pH 7.6) containing 200 μg/ml Proteinase K (Boehringer Mannheim,) at 56 ˚C over night. After incubation, the samples were boiled for 10 min. For purification of DNA, the CHROMA SPIN 400 Columns (Clontech) were successfully used according to manufacturer recommendations.

The quality of the DNA extracted was confirmed with Gene Print STR system LPL (Promega) which amplifies polymorphic tetranucleotide repeats in the human lipoprotein lipase gene.

For detection of EBV DNA, the primers (E1 and E2) which targeting a 129 base pair segment of the BamH1W internal repeat (IR1) region described by Akao et al [1] were employed. A 25 μl volume of polymerase chain reaction mixture was prepared and the procedure performed with 40 cycles of a 1-minute denaturation step at 94 ˚C, a 2-minute annealing step at 55 ˚C, and an extension step at 72 ˚C. A Hodgkin disease case, a nasopharyngeal carcinoma case, and an EBV positive tonsil were used as positive controls, while water was substituted for DNA as a negative control for each examination. Polymerase chain reaction was carried out in duplicate. The amplified DNA was electrophoresed on 10% polyacrylamide gels in 1 x TBE.

In situ hybridization (ISH) for EBV-encoded RNA (EBER) was performed using an EBV-ISH detection kit (Novocastra) in accordance with the manufacturer instructions. Briefly, after deparaffinization, sections were digested with 15 μg/ml proteinase K (Novocastra) for 2 h at 37 ˚C. The mixture of fluorescein-conjugated EBER oligonucleotide probes was then applied and hybridization was allowed to proceed for 2 h at 37 ˚C. To detect signals, alkaline phosphatase-conjugated rabbit anti-fluorescein isothyocyanate (anti-FITC) antibody was employed. The reaction was developed with nitroblue tetrazolium/5-bromchloro-indole phosphate. As positive controls, the samples of Hodgkin lymphoma and nasopharyngeal carcinoma were used.

Results

Amplified PCR product, demonstrating the expected 129

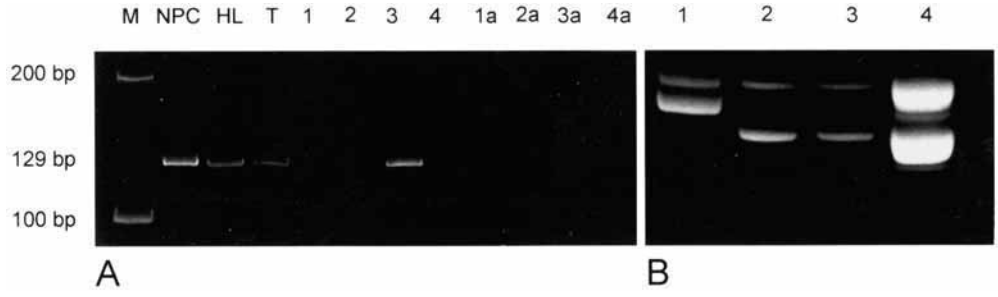

Figure 1. A) PCR detection of EBV genomes in four samples of lymphoepithelioma-like carcinomas of urinary bladder. Lane M, DNA size marker; positive controls: lane NPC, nasopharyngeal carcinoma; lane HL, Hodgkin lymphoma; lane T, the tonsil from a patient with infectious mononucleosis; lanes 1, 2, 3, 4, the first PCR examination of tumor samples; 1a, 2a, 3a, 4a, the second PCR examination of tumor samples. B) Amplification with Gene Print STR system LPL as the test for the quality of isolated DNA samples. Lanes 1, 2, 3, 4, samples of DNA isolated from paraffin-embedded sections of the examined LELCs.

Table 1. Relation of EBV positivity between PCR and ISH in examined tumor samples

	PCR		ISH	
	+	–	+	–
Acinic cell carcinoma of salivary glands with lymphoid rich stroma	1	9	0	10
LELC of the urinary bladder	1	3	0	4
Papillary carcinoma of the thyroid	1	9	0	10
LELC of the cervix uteri	0	1	0	1
Total	3 (12%)	22 (88%)	0	25(100%)

LELC – lymphoepithelioma-like carcinoma, PCR – polymerase chain reaction, ISH – *in situ* hybridization

base pair size was found only in 12% of examined samples. However, this finding was not verified in the second PCR examination of these samples (Fig. 1). Negative controls were included in each experiment to rule out the possibility of contamination or carry over. All the negative controls were negative.

The ISH results revealed only negative signals in all the samples. We failed to detect EBER RNA in the tumor infiltrating lymphocytes. We demonstrate that positive results on PCR can be obtained in samples in which tumor cells are EBV negative (Tab. 1). If approximately 1 in 10^6 B lymphocytes in the circulation carry the EBV [9], then there is high probability that the positive results on PCR in such cases are caused by the presence of these EBV positive lymphocytes in the sample and by the extreme sensitivity of this detection method. The same situation has been described in cervical neoplasia where detection of EBV is closely related to the degree of infiltrating lymphoid cells. SHOJI et al [35] in their statistical study, detected positive lymphocytes using ISH in approximately 15% of pre-invasive (CIN III) and invasive lesions of the uterine cervix. However, no positive reactions were observed in normal cervical samples. The authors concluded that the degree of lymphoid cell infiltration was positively related to the histological grading. They found a significant correlation between the presence of EBV genomic sequences detected by PCR and the extent of lymphocyte infiltration. According to this report, our results may be explained by the presence of abundant inflammatory infiltrates in samples.

Discussion

KIM et al [21] examined 87 cases of salivary gland diseases and showed that EBV may be implicated in some of these lesions in which the lymphocytes are abundant. EBV has been detected using PCR, *in situ* PCR and imunohistochemistry. Two samples of acinic cell carcinoma were involved in this study. Results of all detection methods were negative in these cases. We found PCR positivity in 1 (10%) of 10 examined samples of acinic cell carcinoma, ISH shows only negative tumor cells in all our samples. KIM et al [21] identified EBV specific fragment after PCR in 21.2% samples of chronic sialadenitis; whereas using *in situ* PCR the signals were found only in nuclei of infiltrating lymphocytes. ATULA et al [3] detected EBV in salivary gland tumors using PCR only; therefore their results can not cast light on the significance of EBV infection in the etiology of these tumors.

The presence of EBV in oncocytic papillary carcinomas (OPC) with lymphoid stroma (Warthin-like tumors) of the thyroid has not been studied [33, 38, 39]. The absence of EBV encoded transcripts (EBER RNAs) indicates that, with the present knowledge on EBV, an active role in OPC of the thyroid for this virus is unlikely [24].

GAZZANIGA et al [15] studied 35 biopsies from urinary bladder carcinomas using only PCR and detected EBV genomes in 34% of samples. As only one detection method has been employed in this study, it is difficult to interpret the results. The finding of EBV infections in examined bladder tumors may represent only the presence of EBV carrying lymphocytes in tumors. For the exact evaluation of the role of the EBV in the urinary bladder carcinogenesis, cytological localization of viral genomes by *in situ* hybridization would be helpful. Our results document the importance of a combined diagnostic strategy for this purpose.

Lymphoepithelioma-like carcinoma of the urinary bladder is an extremely rare entity. ANGULO et al [2] reviewed the 14 cases reported as such till the date. Only some of the reported LELC of urinary bladder have been studied for the presence of Epstein-Barr virus. DINNEY et al [13] and GULLEY et al [18] described only EBV negative cases. According to the results of our combined diagnostic strategy, we evaluated the tumor cells in our samples as negative ones.

LELC of the cervix uteri belongs to lymphoepithelial carcinomas that fail to show an EBV association [20, 27, 28]. In our sample, we obtained negative results with both applied techniques.

Our results indicate that EBV-infected non-neoplastic cells such as lymphocytes can be a cause of false positivity, if a study is conducted with PCR alone. Some studies of this type [3, 15] may be found in literature. It is difficult to interpret their results and to use them for comparison with other reports. As viral loads in healthy volunteers ranged from less than one in $1x10^6$ to one in $6.25x10^4$ [30], the second detection method allowing the direct cellular localization of viral transcripts (ISH) or proteins (immunohistochemistry) should be used for the correct evaluation of the significance of EBV infection in the tumorogenesis.

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