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EBER Oligonucleotide RNA in situ Hybridization in EBV Associated Neoplasms

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In virus associated diseases identification of viruses in cells can contribute to the understanding of the pathogenesis and may also help to establish the diagnosis. In the present communication, the effects of the microwave pretreatment (MWP) and that of the proteinase-K enzymatic predigestion (PKD) on EBER RNA oligonucleotide *in situ* hybridization (EBER-RNA-ISH) (EBER: *E*pstein-*B*arr-*E*ncoded-(*E*arly)-*R*NA) were studied. The efficacy of two EBV detecting methods, latent membrane protein-1 (LMP-1) immunohistochemistry and EBER-RNA-ISH were also compared. Our results show that microwave pretreatment enhances the intensity of the ISH signals and preserves significantly better the structure of the tissues compared with enzymatic predigestion. EBER-RNA-ISH, mainly in the nasopharyngeal carcinoma cases, showed a more frequent positivity than the immunohistochemical reaction for LMP-1, however in case of the Warthin's tumor only the LMP-1 protein was expressed. (Pathology Oncology Research Vol 4, No 3, 201–205 1998)

Key words: EBV, EBER, microwave, LMP-1, lymphoma, Warthin's tumor, nasopharyngeal carcinoma

Introduction

Epstein-Barr virus (EBV) is associated with a number of neoplastic and other diseases and may have some role in their pathogenesis. Beside *l*atent *m*embrane *p*rotein-1 (LMP-1) immunohistochemistry and DNA *in situ* hybridization (DNA-ISH), some years ago with the availability of oligonucleotide riboprobes a new RNA in situ hibridization method, namely the Epstein-Barr-Encoded-(Early)-RNA in situ hybridization (EBER-RNA-ISH) was developed. EBER-RNA-ISH can be used in formol-paraffin sections in part because EBV encoded EBER shows a high copy number in cells harboring the EBV genome.

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Abbreviations: EBV: Epstein-Barr virus; EBER: Epstein-Barr-Encoded (Early)-RNA; ISH: in situ hybridization; LMP-1: Latent membrane protein-1; IHC: immunohistochemistry; MWP: microwave pretreatment; PKD: proteinase-K predigestion; NHL: non-Hodgkin's lymphoma; NPC: nasopharyngeal carcinoma; LELC: lymphoepithelioma-like carcinoma; RS cells: Reed- Sternberg cells

Materials and Methods

Formalin fixed biopsy material of nasopharyngeal carcinoma (10 cases), Hodgkin's lymphoma (6 cases, including 4 cases of mixed cellularity, one each of nodular sclerosis and lymphocyte depletion type), one case of lymphoepithelioma-like carcinoma of the stomach (LELC), 7 cases of non-Hodgkin's lymphomas (including one case of Burkitt's lymphoma, and one sinonasal T-cell lymphoma), 2 cases of multiple/bilateral and 3 cases of solitary Warthin's tumor were examined. In nine cases of this series both type of pretreatments (PKD, MWP) were performed. All the 29 cases were studied by both the EBER-RNA-ISH and LMP-1 immunohistochemistry.

When comparing the two pretreatment methods two parameters were assessed: the signal intensity (weak, moderate and strong) and the preservation level of the cellular structures (poor, good). The probes were obtained from DAKO and Novocastra, the antibody to LMP-1 (a 60 kD protein encoded by the $BNLF_1$ gene of the viral genome) was from DAKO (clone CS 1-4). According to the specification, the in situ hybridization probe from DAKO is an oligonucleotide DNA mixture consisting of five FITC-conjugated 30-mers, which are complementary

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sequences to the two nuclear EBER-RNA molecules (EBER-1 and -2). The Novocastra probe is also a FITC labelled oligonucleotide coctail with the same specificity. The detection system was the Vectastain ABC-kit/3-amino-9-ethylcarbazole (Vector, SIGMA) in the case of the LMP-1 immunohistochemistry and the Rabbit F(ab) Anti-FITC/AP (Novocastra) in the case of EBER-RNA-ISH. Positive controls of nasopharyngeal carcinomas and Hodgkin's lymphomas have been used in all the reactions to rule out of false negativity. The reactions were performed according to the instructions of the manufacturer, with slight modifications: (1) to preserve the morphology, the slides were not dehydrated before applying the probe solution; (2) the incubation time with the probe was extended from 2 hours to overnight; (3) the amount of the probe solution used was reduced to 10–20 µl depending on the size of the sections; (4) to cover the sections, instead of the standard coverslips, plastic films (Oncor) were used which proved to be easily removable; (5) the pretreatment was modified: on parallel sections, 4x5 min. microwave pre-treatment (MWP) in citrate-buffer (pH 6, 0.01 M citrate-buffer, 700W, up to the boiling point) and standard proteinase-K predigestion (PKD) (15 µg/ml, 20 min) were performed; (6) the nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) substrate system (Novocastra) was changed to Naphthol AS-MX phosphate/Fast Red (Reanal) because it gave more contrast with haematoxylin counterstaining. The NBT/BCIP method with the resulting blue-black colour of NBT (without hematoxylin counterstaining) can be recommended only for the ambiguous cases (questionable or weak positivity).

Results

In 9 cases of EBV-associated diseases the effects of the PKD and MWP on EBER-ISH were compared. The results are summerized in the *Table 1*. In the microwave treated cases the structure of the tissues and the microscopic details were well preserved, clear cellular outlines could be seen and strong signal intensity was registered. In contrast, only three of the nine PKD treated cases showed good cellular and nuclear details and were free of overdigestion. In five out of the nine cases the cells gave strong intranuclear signal, in four the signal intensity was moderate. The two EBV detecting methods has been compared on twenty nine different tumors *(Table 2)*. In six of them (all were nasopharyngeal carcinomas) both EBER probes (DAKO, Novocastra) were tested and no significant differences were found between them.

19 out of the 29 cases proved to be EBER positive, however only 12 were positive for LMP-1 (see *Table 2)*. The LMP-1 positive cases, except for the Warthin's tumors, were also EBER positive. The 10 nasopharyngeal carcinomas showed intensive intranuclear positivity of the tumor cells with the EBER-RNA-ISH *(Figure 1)*, while only two cases gave a weak or moderate cytoplasmic reaction with the LMP-1. The lymphoepithelioma like carcinoma of the stomach (LELC) was EBER positive and LMP-1 negative. 3 out of the 6 Hodgkins lymphomas – all of mixed cellu-

***Table 1.* Comparison of effects of MWP and PKD on EBER-RNA-ISH**

	PK-digestion		*MW pretreatment*	
diagnosis	*structure*	*signal intensity*	*structure*	*signal intensity*
NPC	–	+++	+	+++
NPC	–	+++	+	+++
NPC	+	++	+	+++
NPC	+	++	+	+++
NPC	–	++	+	+++
HL-MC	–	+++	+	+++
LELC	–	++	+	+++
NPC	+	+++	+	+++
NHL	–	+++	+	+++

PK: proteinase-K predigestion, MW: microwave pretreatment, -: poor, +: good, in case of signal intensity: +: weak, ++: moderate, +++: strong, NPC: nasopharyngeal carcinoma, HL-MC: mixed cellularity type of Hodgkin's disease, LELC: lymphoepithelioma-like carcinoma of the stomack, NHL: non-Hodgkin's lymphoma

***Table 2.* Effectiveness of the two virus detecting methods**

	EBER	*LMP*		*EBER*	*LMP*
NPC	+	–	NHL	+	–
NPC	+	–	NHL	+	+
NPC	+	–	HL-NS	–	–
NPC	+	–	HL-LD	–	–
HL-MC	+	+	NHL	+	+
NHL	+	+	NPC	+	–
NPC	+	–	HL-MC	+	–
NHL	–	–	NPC	+	–
FIL-MC	–	–	HL-MC	+	+
NPC	+	+*	NPC	+	–
NHL	–	–	NHL	+	–
LELC	+	–	NPC	+	+
WT	–	+	WT	–	+
WT	–	+	WT	–	+
WT		+			

NPC: nasopharyngeal carcinoma, NHL: non-Hodgkin's lymphoma, HL-MC, -NS, -LD: Hodgkin's lymphoma, -mixed cell, -nodular sclerosis and -lymphocyte depletion subtypes. LELC: lymphoepithelioma-like carcinoma of the stomach, WT: Warthin's tumor, +*: very weak reaction

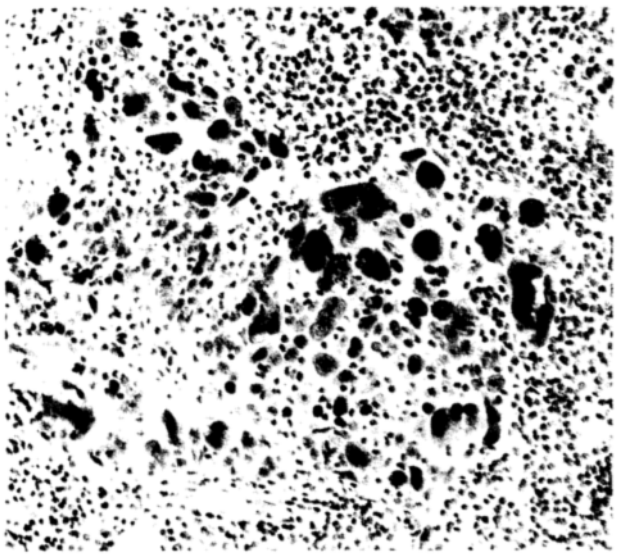

Figure 1. Strong intranuclear in situ hybridization reaction of anaplastic cells in nasopharyngeal carcinoma. (EBER-RNA-ISH, AP/NBT/BCIP, x150)

larity type – were positive for EBER in the Reed-Sternberg cells (RS) *(Figure 2)*. However, only two of them were positive with the LMP-1 antibody. All of the LMP-1 positive cases were also positive for EBER-RNA-ISH, but there was a mixed cellularity type of Hodgkins disease in which only the in situ hybridization demonstrated the viral genome. Among the seven NHL-s, five showed EBER positivity *(Figure 3)*, three were also LMP-1 positive, and all of the LMP-1 positive cases were EBER positive. In all Warthins tumors moderate to strong, mainly perinuclear and granular cytoplasmic positivity could be found in the basal layer of the oncocytes, however, no signal could be seen in the reactive lymphoid stroma. The negative controls (the same reaction without primary antibody) remained negative ruling out of false positivity. There was no reaction with the EBER-RNA-ISH in any of the examined cases, however the positive control cases showed strong intranuclear signal.

Discussion

Formalin fixation quite frequently either destroys or masks antigen epitopes rendering them inaccessible to immunohistochemical detection. Restoration of immunoreactivity (unmasking) can be achieved by pretreatment of sections.[2,17,21] Similar problems may arise in the course of DNA or RNA *in situ* hybridization, and therefore the most fequently used unmasking procedure, the enzymatic predigestion is recommended as a pretreatment. One of the setbacks of enzymatic predigestion is that it much depends on the tissue type and the duration of the fixation. PKD needs calibration of the enzyme concentrations and time of the digestion.[1,22] In case of ISH, until now MWP has been used only for light chain mRNA-ISH or the Philadelphia FISH.[14,21,22]

The oligonucleotide RNA hybridization has some advantages in contrast to the DNA-DNA hybridization: it does not need denaturation, and because the target is also a single stranded molecule, the reaction takes place at significantly lower temperatures (37°C up to 55°C) prevent-

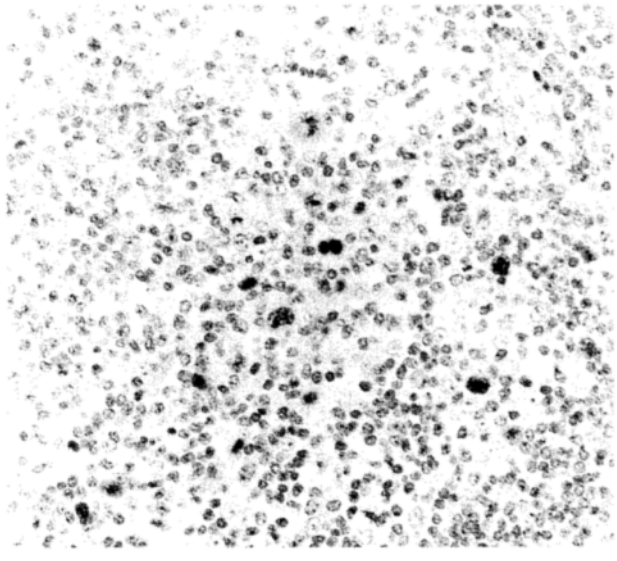

Figure 2. Hodgkin's lymphoma, mixed cell type. Note the modearate to strong intanuclear signals in the nuclei of Reed-Sternberg cells. (EBER-RNA-ISH, AP/ Naphtol-AS-MX-phosphate /fast red, x250)

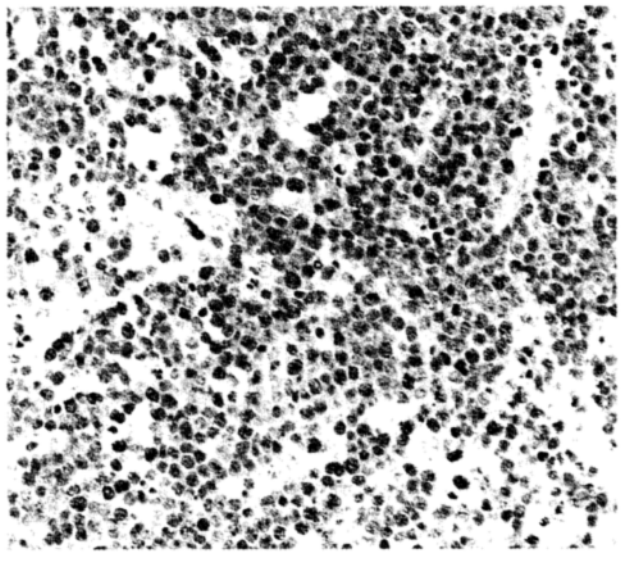

Figure 3. Burkitt's lymphoma with the "starry sky" pattern. Moderate to strong positivity of the immature cells. (EBER-RNA-ISH, AP/ Naphtol-AS-MX-phosphate /fast red, x250)

ing the structural damage.[5] Hybridization with double stranded probes on double stranded targets needs significantly higher denaturation temperatures (95°C) and sections may be damaged by overdigestion. In these cases other pretreatment than enzymatic predigestion can be recommended. Two further advantages of the oligonucleotide RNA hybridization are the usually higher target copy number (sometimes 10^6–10^7 copies)[11,24] and the high penetrability of the oligonucleotides.

Table 1 shows, that MWP results in better cellular and nuclear details. The signal intensity in the individual cases was moderately stronger or as strong as with PKD. Thus, based on these results, MWP is highly recommended for ISH procedures.

Except for the Warthins tumor cases, the EBER-RNA-ISH proved to be a more reliable method for detecting the virus. The close relationship between NPC and EBV is well known.[8,11,24] In all the cases of our NPC series, the EBER-RNA-ISH gave strong positive reactions, therefore it can be a really good choice for the detection of the virus, however LMP-1 immunohistochemistry in these cases may play only a secondary role.[4] One of the causes of these findings may be the differences in the latency types. The LMP-1 is expressed only in the type II and III latency, but not in the type I, however in latent phase, virtually all the EBV infected cells express the EBER sequences.[13]

In the recent years, undifferentiated carcinomas, similar to nasopharyngeal tumors (lymphoepithelioma-like carcinomas, LELC-s), were described at various sites, such as the salivary glands,[12,19] lung,[3,23] stomach,[6,10,15] small and large bowels,[26] thymus,[25] vulvar region and bladder.[4,7] Thus, LELC-s developed in the foregut derived organs, are frequently assciated with EBV.[4,23] The LELC case in our series occured in the stomach and proved to be EBER positive, just as the majority of the formerly reported cases.[15]

Only a slight superiority of the EBER-RNA-ISH to the LMP-1 immunohistochemistry could be demonstrated in Hodgkin's lymphoma. Comparing our Hodgkin's lymphoma cases to other European series, the rate of the EBER positivity is similar.[9,20] Examining the NHLs, the EBER in situ hybridization showed more frequent positivity (5/7) on the same cases than LMP-1 immunohistochemistry (3/7), therefore the *in situ* hybridization seems to be more reliable method for detecting the virus.

In contrast to the discussed cases above, all the presented Warthin's tumors expressed the LMP-1 protein, but failed to be positive for the EBER sequences. Expression of EBV in these tumors has been demonstrated in previous studies by means of PCR reaction for the viral BZLF gene and DNA-ISH respectively.[16,18] These studies proved convincingly the presence of the virus in the tumor cells, but either morphological data were not presented, and/or the number of examined markers were limited. In the present study two methods were used for detecting of EBV. The absence of EBER RNA molecules in an EBV-associated tumor is unusual, so further examinations have to be performed to elucidate the type of infection.

As a final conclusion, the EBER-RNA-ISH with MWP was found to be a quick, non isotopic method, which allows the detection of the EBV sequences at nucleic acid level and which can be easily fitted into the every day surgical pathological routine.

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