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PREVALENCE OF EPSTEIN BARR VIRUS IN BIOPSY SPECIMENS OF NASOPHARYNGEAL CARCINOMA FROM SERBIAN PATIENTS

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Abstract - The development of nasopharyngeal carcinoma (NPC) is the result of interaction between Epstein-Barr Virus (EBV) and many non-viral factors. The aims of this study were to determine the prevalence of EBV in NPC biopsies from Serbian patients and to investigate the correlation between EBV presence and demographic, anamnestic and clinical data. Ninety-three tissue blocks were included. For detection of EBV DNA, the C terminus of the LMP1 gene was amplified by nested-PCR. Twenty-eight biopsies were EBV-DNA-positive (30.1%), with a statistically significant difference in EBV DNA presence between geographical regions (p=0.02) and between the stages of tumor-node-metastasis (TNM) (p=0.02). A correlation was also found with the presence of EBV DNA and smoking (p=0.02). The correlation of EBV DNA presence, with or without smoking and the promising outcome of the disease was statistically significant (p=0.02; p=0.01). The EBV DNA findings from this study confirm the role of EBV in NPC carcinogenesis, and show the different distribution among TNM stages and correlation between the virus and outcome of disease.

Key words: Epstein-Barr Virus (EBV), nasopharyngeal carcinoma (NPC), smoking, TNM staging

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a human malignancy that is rare in most populations around the world. However, in South China and Southeast Asia, NPC is endemic, with incidence rates of 20-30 per 100 000 person-years (Jia and Qin, 2012). In Serbia, the undifferentiated carcinoma of nasopharyngeal type (UCNT, WHO type III) is dominant, as is case in high-risk areas, but with similar incidence to that found in Europe and the USA, 0.5-1 per 100 000 person-years (Jemal et al., 2011).

The development of NPC is the result of a complex interaction between early Epstein-Barr virus (EBV) infection, genetic factors and environmental carcinogens, which is shown in the clustering in families of diverse populations (Zeng, 2002). UCNT is universally associated with EBV, a human gammaherpesvirus that establishes a life-long, persistent infection in more than 90% of the population worldwide. This virus was classified as a group 1 carcinogenic agent by the IARC (International Agency for Research and Cancer). The discovery of EBV receptors on pharyngeal epithelial cells, as well as monoclonal viral episomes in UCNT tissues, has established the relation between EBV and NPC (Siegler et al., 2004; Raab-Traub and Flynn, 1986). The switch from the latent to the lytic phase of the infection is probably one of the crucial steps in NPC development. This switch could be identified by several specific serologic biomarkers widely used for the screening and early diagnosis

of UCNT in high-risk populations in South China. These biomarkers include antibodies against EBV lytic gene products, anti-VCA IgA and anti-Zta IgA (Chien et al., 2001). However, these markers can lack specificity, and usually remain elevated even after disease remission is achieved. Moreover, increasing plasma EBV DNA could be detected as a marker for the reactivation of viral replication, but in contrast to serology, it is also used for monitoring patients and predicting the outcome of treatment as it correlates well with tumor burden (Wang et al., 2010). The essential viral oncoprotein is latent membrane protein 1 (LMP1), an integral membrane protein that promotes the immortalization of B lymphocytes by inducing B-cell activation markers and expression of the antiapoptotic *A20* and *bcl-2* genes (Rowe et al., 1994; Kulwichit et al., 1998).

No specific natural inducer of EBV reactivation in NPC has been identified. However, epidemiological studies have determined several lifestyle risk factors for NPC, especially tobacco smoking, consumption of salt-preserved fish, consumption of preserved vegetables or inadequate intakes of fresh vegetables and fruits, and occupational exposure to chemicals (Chang and Adami, 2006). Although several chemicals (phorbol-12-myristate-13-acetate-TPA, sodium butyrate and trichostatin A) have been described as inducers of the EBV lytic cycle, smoking has been shown as the only lifestyle risk factor for NPC linked to EBV seropositivity (Xu et al., 2012). Moreover, chromosomal 3p loss of heterozygosity (LOH), specific human leukocyte antigen *HLA* polymorphisms and some other loci outside the (*HLA*) region also show association with NPC risk. They represent "pre-malignancy" genetic status of the epithelial cells where carcinogenesis develops (Jia and Quin, 2012).

The aims of the present study were: (i) to determine the prevalence of EBV in biopsy specimens of nasopharyngeal carcinoma from Serbian patients, and (ii) to investigate the correlation between EBV presence and demographic and anamnestic data, TNM (tumor-node-metastasis) staging and therapy effect in UCNT patients.

MATERIALS AND METHODS

Patients and samples

This study included 93 patients with histologically confirmed undifferentiated carcinoma of nasopharyngeal type (UCNT) after endoscopic biopsy. After diagnosis, they were treated with radiotherapy (RT) as the mainstay treatment; some were also treated with chemotherapy (CT), chemo-radiotherapy (CRT), surgically or only symptomatically.

Archived tissue blocks from the 93 patients, fixed in formalin and embedded in paraffin, were retrieved from the Clinic of Otorhinolaryngology and Maxillofacial Surgery, Clinical Center of Serbia. According to accessible data, all patients were classified by 5 different criteria: sex (male, female); region of living (Vojvodina, Beograd, Šumadija and western Serbia, south and east Serbia, Kosovo and Metohija); tobacco smoking (smokers, non-smokers), TNM staging by the American Joint on Cancer (AJCC) (Tx, I, II, III, IV); and the last known outcome of disease (Th-therapy in progress, CR-complete remission, PR-partial regression, S-stabilization, P-progression and/or metastasis). Age ranged from 18 to 76 years (mean 52.9±1.4 years).

Deparaffinization and DNA Isolation

Three 10-mm-thick tissue sections from each block were placed in a sterile, plastic 1.5-ml PCR tube, deparaffinized with xylene, rehydrated in alcohol, and then air-dried. The tissue was then resuspended and lysed overnight in 180 µl digestion buffer (QIAGEN, Hilden, Germany) and 20 µl proteinase K (QIAGEN, Hilden, Germany) at 56^0C. Viral DNA was isolated using a QIamp Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

PCR for Detection of LMP1

Amplification of the C terminus of the LMP1 gene was performed by nested PCR using primers flanking the characteristic 30-bp deletion region: OF 8081: 5'- GCTAAGGCATTCCCAGTAAA-3' and OR 8744:

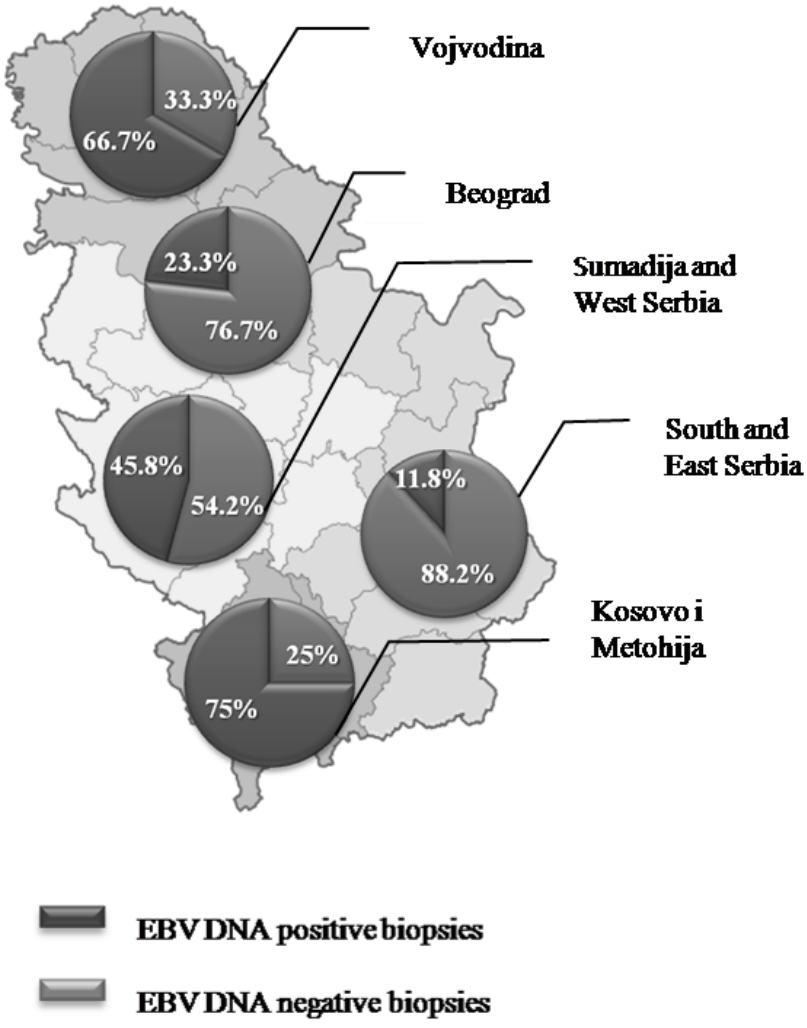

Fig. 1. Regional distribution of EBV positive and EBV negative biopsies from NPC patients

5'-GATGAACACCACCACGATG-3' as outer primers, and IF 8213: 5'-CGGAACCAGAAGAACCCA-3' and IR 8719: 5'-TCCCGCACCCTCAACAAG-3' as inner primers, as previously described by Li et al. (2009). The PCR reactions were carried out in 40 cycles at 95^{0}C for 1 min, at 47^{0}C for 1 min, and at 72^{0}C for 1 min. PCR products were analyzed using gel electrophoresis with ethidium-bromide staining.

Statistical analyses

For statistical analyses, the chi-squared and Spearman rank correlation tests were used. Analysis was performed by SPSS v.15 for Windows (SPSS Inc., Chicago, IL) software. $P<0.05$ was considered significant.

RESULTS

Twenty-eight of the ninety-three tested tissue samples were positive for EBV DNA (30.1%). Patients' data from 5 different categories were obtained, and the results are summarized in Table 1.

To investigate a potential correlation between carcinoma tissue finding of EBV DNA and patient data

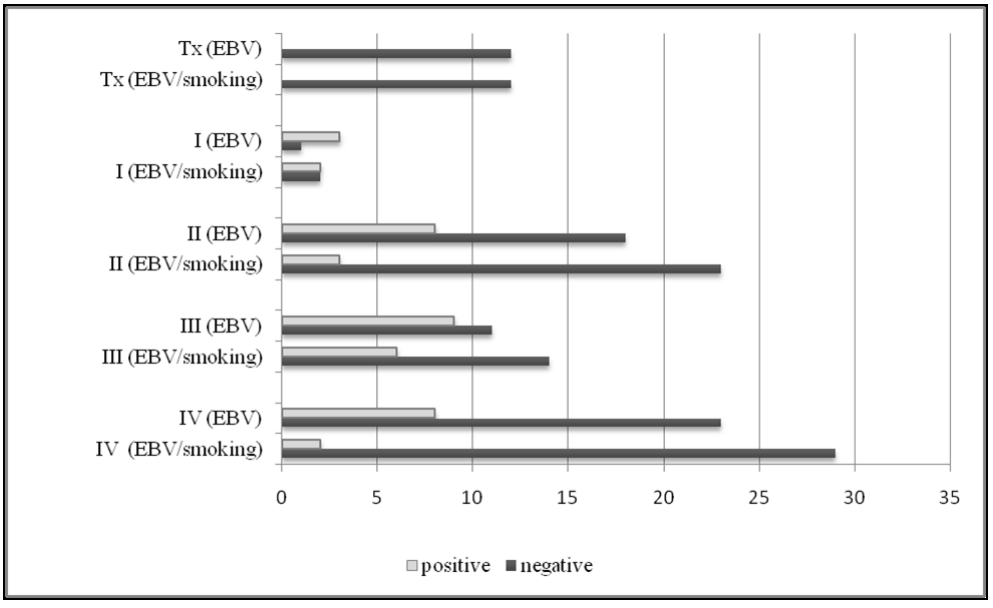

Fig. 2. Distribution of EBV positive and negative biopsies with and without association with smoking according to TNM stage
EBV – number of positive and negative patients according to EBV DNA presence
EBV/smoking –– number of positive and negative patients according to association of EBV DNA presence and tobacco smoking

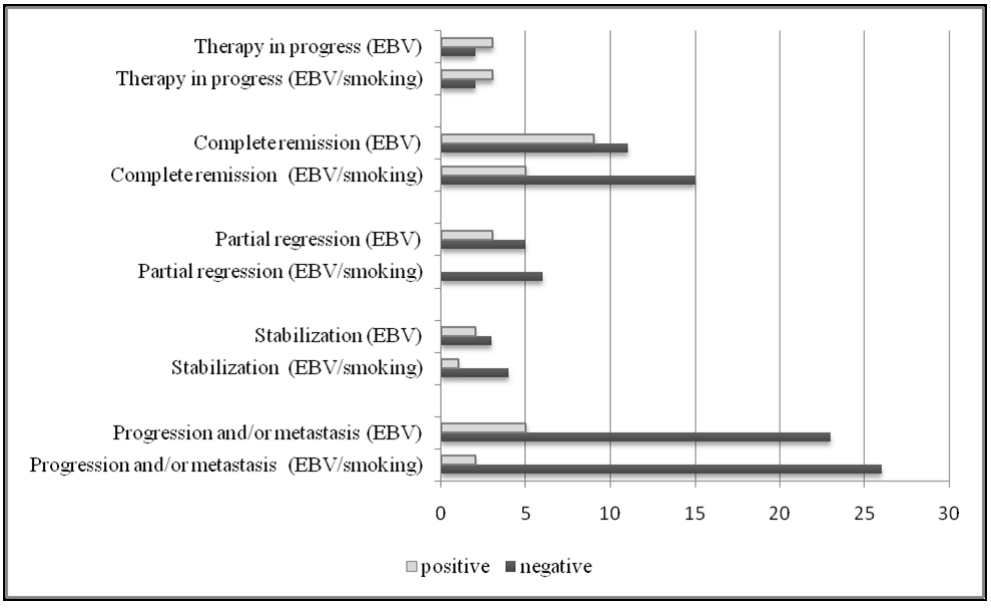

Fig. 3. Distribution of EBV positive and negative biopsies with and without association with smoking according to last known outcome of disease
EBV – number of positive and negative patients according to EBV DNA presence
EBV/smoking –– number of positive and negative patients according to association of EBV DNA presence and tobacco smoking

Table 1. Distribution of patients according to five different criteria

Category	Number and valid percent of patients					Missing data
Sex	Male 67 (72%)			Female 26 (28%)		0
Region of living	Vojvodina 3 (3.3%)	Belgrade 43 (47.3%)	Šumadija and west Serbia 24 (26.4%)	South and east Serbia 17 (18.7%)	Kosovo and Metohija 4 (4.4%)	2
Tobacco smoking	Smokers 43 (64.2%)			Non-smokers 24 (35.8%)		26
TNM staging [a]	Tx 12 (12.9%)	I 4 (4.3%)	II 26 (28%)	III 20 (21.5%)	IV 31 (33.3%)	0
The last known outcome of disease [b]	Th 5 (7.8%)	CR 20 (31.3%)	PR 6 (9.4%)	S 5 (7.8%)	P 28 (43.8%)	29

[a] TNM staging by The American Joint on Cancer (AJCC): tumor-node-metastasis
[b] Therapy in progress (Th), Complete remission (CR), Partial regression (PR), Stabilization (S), Progression and/or metastasis (P)

from 5 categories, statistical analysis was performed. A significant difference in the EBV DNA presence between regions of living (χ^2=12.07, p=0.02) (Fig. 1) was observed. It has also been shown that there was a significant difference in EBV DNA-positive biopsies and TNM staging (χ^2=11.39, p=0.02), even if the positive finding of EBV DNA was associated with tobacco smoking (χ^2=12.13, p=0.02) (Fig. 2). The highest frequencies were found at early TNM stages, especially TNM I.

The correlation of EBV DNA presence and a promising outcome of the disease (patients receiving therapy and patients with complete remission) was shown to be statistically significant (ρ=0.29, p=0.02). However, the correlation between a promising outcome of disease was highly significant when EBV DNA presence was associated with tobacco smoking (ρ=0.32, p=0.01) (Fig. 3).

The differences in the EBV DNA presence between sex, age or tobacco smoking habit did not reach statistical significance (p>0.05). Statistical analysis of the difference between demographic, anamnestic and clinical data revealed a significant difference only between sex and TNM staging (χ^2=14.62, p=0.01).

DISCUSSION

UCNT is type of NPC that is, in almost all cases, EBV-positive, irrespective of geographical origin (Ozyar et al., 2004). It has been suggested that activated EBV might have a different impact on NPC tumorigenesis during different stages. At the early stage, the reactivation of EBV in B cells might facilitate nasopharyngeal epithelial cell infection with EBV by cell-to-cell contact. Changing cytokine were observed to effectively enhance EBV infection in the nasopharyngeal epithelial cells (Tsang et al., 2010).

Although EBV DNA was detected in only 30.1% of samples, this is in accordance with the hypothesis concerning the early oncogenic involvement of EBV (Perez-Ordonez, 2007). In support of this hypothesis is the observation that more than 75% of biopsies from the early TNM stage were EBV-positive. This was found for archived biopsies that were not processed immediately after incision, which is known to potentially decrease the probability of viral DNA isolation.

Although human genetic variation has an influence on the development of NPC, differences in lifestyle and dietary factors, in concert with environmental factors, are likely to increase the risk of developing this cancer and lead to the unique distribution of NPC. The regional diversity of EBV-positive UCNT patients in Serbia could be caused by different combinations of all these factors added to individual genetic predisposition, especially smoking habits, EBV infection and occupational exposures to chemicals. Therefore, in view of the absence of data about the patients' occupations, it might be assumed that the lowest frequency of EBV-positive biopsies from patients living in south and east Serbia are the result of the domination of chemicals and toxins from the many industrial zones in this region. The association of NPC and formaldehyde, wood dust, solvents such as phenoxy acid and chlorophenol were reported previously (Thompson and Grafström, 2009; Hildesheim et al., 2001; Hardell et al., 1982). Together with EBV reactivation, these carcinogens may lead to a dramatic change in genome stability (Fang et al., 2012).

According to the results from this study, the presence of EBV DNA is correlated with a promising outcome of disease. Interestingly, it was found that EBV DNA presence associated with tobacco smoking also correlates with a promising outcome of disease. This is an important contribution to the hypothesis that the presence of the EBV genome and EBV antigens might become a specific factor of vulnerability for the malignant cells and immunotherapeutic target for novel therapeutic approaches (Lin et al., 2002).

Tobacco smoking produces several reactive forms of agents resulting in the formation of DNA adducts which cause DNA damage. However, in NPC patients, especially UCNT, smoking may play an alternative role via induction of EBV reactivation, as demonstrated earlier (Xu et al., 2012). Among Serbian patients, smokers' frequency was approximately 65%, which correlates with previously reported data (Anghel et al., 2012). Moreover, smoking contributed to EBV presence in biopsy cells and was more frequent in a promising outcome of disease, but also in the early TNM stages. Thus, the association of EBV presence and tobacco smoking in clinical entities of Serbian patients correlates with the "smoking driven model" of inducing EBV reactivation (Jia and Quin, 2012; Xu et al., 2012).

Consistent with the reported data was the frequency of UCNT found in males (2.6-fold higher than in females) (Brennan, 2006, Anghel et al., 2012). The difference between sexes may reflect the effects of habit and lifestyle diversity or potential biological particularities. The peak of age for acquiring this tumor was between 50 and 59, which is in accordance with data from high-risk areas where UCNT is the dominant type of NPC, as observed in neighboring Romania (Chang and Adami, 2006; Anghel et al., 2012).

Although the etiological mechanism of the latent-to-lytic phase transformation of EBV is unclear, the EBV DNA findings from this study confirm the role of EBV in NPC carcinogenesis. Also shown are the association of EBV positivity with tobacco smoking, the different distribution among TNM stages and the correlation between the virus and outcome of disease.

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