

**SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)**

**Prevalence of Epstein-Barr virus in oral squamous cell carcinoma and premalignant lesions, and the genetic and epigenetic aberrations of p14<sup>ARF</sup> and p16<sup>INK4A</sup> tumour suppressor genes in head and neck cancers**

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PREVALENCE OF EPSTEIN-BARR VIRUS IN ORAL SQUAMOUS CELL CACINOMA  
AND PREMALIGNANT LESIONS, AND THE GENETIC AND EPIGENETIC  
ABERRATIONS OF P14ARF AND P16INK4A TUMOUR SUPPRESSOR GENES IN  
HEAD AND NECK CANCERS

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## INTRODUCTION

The oral and laryngeal cancers represent a serious public health problem in Hungary; in Europe, Hungary tops the morbidity list regarding both oral and laryngeal cancer. In addition, Hungary is ranked first in Europe regarding mortality of oral tumours in case of both sexes and laryngeal cancer mortality Hungary is second among women and fourth among men. Despite the developed diagnostic and therapeutic opportunities as well as modern surgical techniques, the prognosis of oral cancer is unfavourable. Its major known risk factors are alcohol and tobacco consumption; however, some studies have reported oral cancer in patients without exposure to these risk factors. This fact suggests the role of additional factors involved in carcinogenesis.

The Epstein-Barr virus (EBV) infection is associated with lymphoid- (Burkitt's lymphoma) and epithelial tumours (nasopharyngeal carcinoma; oral squamous cell carcinoma, OSCC), however, the direct causal relationship between the virus and the development of tumours is controversial. The frequency of EBV varies according to the geographic regions. In our present study, we determined the prevalence of EBV in patients with OSCC and premalignant lesions (oral leukoplakia, OL; oral lichen planus, OLP) in an Eastern Hungarian population. To determine the possible etiologic role of EBV we also collected a sample of the apparently healthy mucosa of the patients and the obtained data were compared with a healthy control group.

Besides the external factors, changes of the cellular regulatory process may play a role in the development of head and neck cancer. The p14<sup>ARF</sup> and p16<sup>INK4A</sup> tumour suppressor genes localized on short arm of chromosome 9p21 are key factors in preventing carcinogenesis. Inactivation of these genes was studied in a number of malignant lesions. In the second part of our study, our aim was to examine the frequency of genetic changes and promoter methylation of p14<sup>ARF</sup> and p16<sup>INK4A</sup> tumour suppressor genes in patients with head and neck cancer in an Eastern Hungarian population.

## REVIEW OF THE LITERATURE

### *Head and neck cancers*

Cancers of the head and neck are the sixth most common malignancy in the world with approximately 500 000 new cases and 350 000 deaths annually. Oral cancers are the largest group of head and neck cancers and the laryngeal cancer is the second most common cancer of head and neck region. According to epidemiological studies, in Europe, Hungary tops both the morbidity and mortality lists regarding head and neck cancer. According studies, mortality due to head and neck cancers increased nearly five-fold between 1975 and 2007 in Hungary. Despite the fact that the diagnostic and therapeutic opportunities were improved over the past decade, prognosis of oral cancer remains unfavourable; in Hungary, the five-year survival rate of oral cancer is less than 50%. The aetiology of oral malignant tumours is complex; the known risk factors are tobacco and alcohol consumption both in oral- and in laryngeal squamous cell cancer. However, other factors may play a role in the development of cancers, such as dietary habits, betel chewing, genetic predisposition, poor dental status or fungal infection (*Candida*). Oncogenic viruses, such as the presence of human papillomavirus (HPV) or Epstein-Barr virus (EBV) may also contribute to the tumour formation. The etiologic role of these viruses in tumourgenesis is still controversial. In addition to these external factors, changes in the retinoblastoma (RB) and p53 pathway may play a role in the carcinogenesis.

### *Oral premalignant lesions*

Premalignant lesions are defined as pathologically altered tissue conditions, in which malignant lesion is more likely to develop than in its apparently, healthy tissue. In addition, precancerous conditions are also known. Precancerous conditions are defined as a generalized condition of the individual (anaemia, diabetes mellitus, hepatitis C virus infection, bone marrow transplantation, stress) which is associated with a significantly increased risk of cancer. The oral leukoplakia (OL) is a premalignant lesion, but oral lichen planus (OLP) is classified currently as precancerous condition.

In the development of OL, many chemical and mechanical factors may participate, such as smoking, alcohol consumption, spicy foods, poor dental condition or denture related to irritation. In addition, *Candida* infection can be associated to some types of OL and may

contribute to the malignant transformation. Furthermore, the role of various viral factors is possible in the pathogenesis, such as HPV or EBV. The role of EBV was studied mainly in HIV (human immunodeficiency virus) infected patients with oral hairy leukoplakia. According to our knowledge, data on EBV prevalence in other forms of OL are not available in the literature.

The precise aetiology of OLP is unknown, but in the recent years, the role of autoimmune response and psychological factors has been postulated. Current data suggest that OLP is a T-cell-mediated autoimmune disease in which autocytotoxic CD8+ T cells trigger the apoptosis of oral epithelial cells. There are some factors, which predispose to malignant transformation, such as diabetes mellitus, hepatitis C or Candida infection, tobacco and alcohol consumption, poor oral hygiene and status. In addition, the HPV and EBV may contribute to the malignant transformation, but the viral origin of the disease has not been demonstrated yet.

### ***Epstein-Barr virus***

Epstein- Barr virus (EBV; human herpesvirus 4) belongs to the *Herpesviridae* family, Gammaherpesvirinae subfamily and Lymphocryptovirus genus, has a 175 kbp long, double-stranded DNA genome that encode nearly 100 genes. EBV genome is surrounded by an icosahedral protein capsid. A protein tegument lies between the capsid and the envelope. Mature virions with capsid are approximately 120 to 180 nm in diameter. EBV is a common, ubiquitous virus; nearly 90% of the population become infected during their life. Seroepidemiologic studies suggest that primary infection occurs in childhood in developing countries due to poor hygiene. EBV is transmitted from host to host via oral contact with saliva or via sexual intercourse. Initial infection is thought to occur mainly in the oral and nasopharyngeal mucosa. In vivo, EBV can infect B-lymphocytes as well as oropharyngeal and cervical epithelial cells. After the infection of epithelial cells, B-cell infection occurs in the surrounding lymphoid tissues. Like other herpesviruses, EBV life cycle consists of a lytic and a latent phase. The lytic phase is characterized by active virus production, which causes lysis of the host cell. After the primer infection, latent infection develops in a small number of resting B-lymphocytes (1-50 cells/10<sup>6</sup> B-lymphocytes). EBV may reactivate and return to the lytic cycle to infect new B cells. However, these subsequent reactivations do not produce symptoms in healthy individuals.

Little information is available about the early intracellular processes after primary infection, i.e. about the processes during the incubation period lasting 4-6 week before appearance of symptoms. First the immediate early genes are expressed, which encode transcriptional activators and involve in the regulation of early gene expression and the promoter activation of the host cell. Early genes encode replication proteins (e.g. viral DNA polymerase) and regulate the viral DNA replication. Late genes encode structural antigens (viral capsid antigen, VCA).

Latency is the state of persistent viral infection without active virus production. In contrast to the lytic replication, viral DNA replication during the latent phase occurs via the host DNA polymerase. There is a limited expression of EBV nuclear antigen (EBNA), latent membrane protein (LMP) gene product, and EBV encoded RNAs (EBER). These include six EBNAs (EBNA-1, -2, -3A, -3B, -3C, -LP), three LMPs (LMP-1, -2A, -2B), two EBERs (EBER1, EBER2) and transcripts of BamHI region. Characterization of different promoter usage and gene expression patterns in different cell lines revealed that there are three different latency programs. Only EBNA-1 and the EBERs are expressed in type I latency, which is seen in Burkitt's lymphoma. EBNA-1, LMP-1 and -2 and the EBERs are expressed in type II latency, which is observed in nasopharyngeal carcinoma, Hodgkin disease and peripheral T-cell lymphoma. Type III latency program, in which all of the latency gene products are expressed, is often detected during acute infectious mononucleosis or in some lymphoproliferative diseases.

### ***EBV-associated diseases***

Primary EBV infection occurring early childhood is usually asymptomatic or, in case of young adults, infectious mononucleosis (MI) can develop. Mononucleosis is an acute disease; most common symptoms are fever, sore throat and swollen lymph nodes. Chronic active EBV infection (CAEBV) is a rare, prolonged illness, which affects various organs (pneumonia, hepatitis, haematological changes). EBV infection is associated with the development of different lymphoid tumours, such as Burkitt's lymphoma, Hodgkin's disease and lymphoproliferative diseases of immunocompromised individuals (X-linked lymphoproliferative disorders, transplantation- and AIDS-related lymphoproliferative disorders). In addition, EBV may be responsible for the development of epithelial cancers; EBV may have a role in nasopharyngeal carcinoma, gastric cancer, salivary gland or breast carcinoma as well as in the development of OSCC belonging to head and neck cancer and in

some oral premalignant lesion (OL, OLP). The presence of EBV in oral samples varies widely in different studies therefore the etiological role of EBV is doubtful in OSCC.

### ***Cell cycle and regulation***

The different phases of cell cycle are regulated by complex regulatory systems and check points. Different proto-oncogenes, cyclin-dependent kinases (CDK), transcription factors, DNA repair genes as well as tumour suppressor genes participate in the regulation of cell cycle. Tumour suppressor genes are agonists of cell replication. Their role is to check the cell cycle and to arrest replication and induce apoptosis in case of gene damage. Loss of their function, which requires inactivation of both alleles, leads to uncontrolled cell cycle regulation, continuous cell division and malignant transformation. Development of malignant disease is a multi-step process, in which aberrations of tumour suppressor genes and a number of genetic changes in cell cycle regulatory genes may participate. The most important ways affected in the development of malignant tumours are the retinoblastoma (RB / p16INK4a / cyclin) and the p53 (p53 / p14ARF / MDM2) pathways.

### ***The p14<sup>ARF</sup> and p16<sup>INK4A</sup> tumour suppressor genes***

The INK4A/ARF gene on chromosome 9p21 encodes two structurally distinct tumour suppressor proteins, the p14<sup>ARF</sup> and p16<sup>INK4A</sup>. The protein p16<sup>INK4A</sup> is encoded by exon 1 $\alpha$ , exon 2 and exon 3, while p14<sup>ARF</sup> is encoded by exon 1 $\beta$  located approximately 20 kb upstream of p16 exon 1 $\alpha$ , and exon 2 and 3. The two proteins have different promoter and use the second exon with alternative reading frames, thus the two proteins have no physical homology. Both proteins function as tumour suppressors and arrest G1-S transition of the cell cycle, but through different pathways. The p16<sup>INK4A</sup> is a cyclin-dependent kinase inhibitor and inhibits cyclin D-CDK4/6 complex, thus prevents hyperphosphorylation of pRb. The p14<sup>ARF</sup> protein can inhibit the cell cycle through the p53 pathway. The p14<sup>ARF</sup> interacts physically with MDM-2 and stabilizes the p53 tumour suppressor protein in the nucleus by blocking its cytoplasmic transport and MDM-2-mediated degradation. In this manner, both p14<sup>ARF</sup> and p16<sup>INK4A</sup> plays an indirect role in inhibition of G1 to S transition in the cell cycle. Genetic and epigenetic alterations of tumour suppressor genes, including p14<sup>ARF</sup> and p16<sup>INK4A</sup>, were found to contribute to carcinogenesis in various types of cancer, such as lung carcinoma, colorectal tumour, melanoma and breast cancer as well as bladder carcinoma. Aberrations of p14<sup>ARF</sup> and p16<sup>INK4A</sup> genes and gene products are one of the most studied alterations regarding head and

neck cancers. Inactivating mechanism of these genes include deletion, polymorphism, mutation, loss of heterozygosity or promoter methylation. Considering, that head and neck cancers are a heterogeneous group regarding their aetiology, histology and prognosis; it is possible that the frequency of the different alterations in the tumour suppressor genes is also variable by tumour type. Studying the epigenetic profile of head and neck cancer may provide possibilities to detect new biomarkers, which may help for early diagnosis and therapy of disease.

### *Aims*

Data provided in the literature suggest that the prevalence and the aetiologic role of EBV in OSCC may vary according to geographical regions. In some population (e.g. Japanese, Chinese, South Africans), EBV may play a role in OSCC, in contrast, in other populations (North American, North European) the role is less probable. To our knowledge, prevalence of EBV in Hungarian population with OSCC has not been studied yet. As alterations in cellular processes are also involved in the development of tumours, the aberrations of tumour suppressor genes were also studied in specimens obtained from head and neck cancers.

- We collected data on the prevalence of EBV DNA in patients with OSCC, OLP and OL in an Eastern Hungarian population. To study the possible etiologic role of EBV, we took a sample from the apparently healthy mucosa of the patients and the obtained data were compared with a healthy control group.
- We examined the frequency of genetic and epigenetic (promoter methylation) alterations of p14<sup>ARF</sup> and p16<sup>INK4A</sup> tumour suppressor genes in patients with head and neck cancer with known virological (HPV and EBV) status in an Eastern Hungarian population.



## PATIENTS AND METHODS

### *Study groups*

All patients enrolled in the study attended the Department of Dentoalveolar and Maxillofacial Surgery or the Department of Periodontology, Faculty of Dentistry, as well as the Clinic of Otorhinolaryngology and Head and Neck Surgery, at the University of Debrecen, Hungary, during 2003-2007. Sixty-five patients with OSCC (51 men, 14 women; mean age: 54.4 yrs; age-range 25-80 yrs), 116 patients with OLP (29 men, 87 women; mean age: 55.0 yrs; age-range 23-79 yrs) and 44 patients with OL (14 men, 30 women; mean age: 56.3 yrs; age-range 29-91 yrs) were enrolled. Fresh tissue samples were obtained from the central part of the tumours during operation. The apparently healthy mucosa of the patient was also sampled by means of cytobrush. The age-matched control group consisted of 69 individuals without a history of oral disease or malignancy, with a healthy oral mucosa (16 men, 52 women; mean age: 52.5 yrs; age-range: 22-77 yrs). These individuals attended the Faculty of Dentistry for regular oral screening. Exfoliated buccal epithelial cells were collected from the controls using cytobrush. To assess complication-free survival (without recurrence, growth of new tumour or metastasis), patients were followed-up after surgical intervention, the mean follow-up time was 31 months (range: 1.6-60 months). Data were also collected on exposure to known risk factors (smoking, alcohol consumption) and pathological characteristics of tumours.

In another project, we determined the frequency of genetic and epigenetic alterations of p14<sup>ARF</sup> and p16<sup>INK4A</sup> tumour suppressor genes in 65 patients with head and neck cancer. Thirty-seven patients with OSCC (28 men, nine women; mean age: 54.5 yrs, age-range: 39-80 yrs) and 28 patients with laryngeal squamous cell cancer (LSCC) (27 men, one woman; mean age: 56.8 yrs; age-range: 43-71 yrs) were enrolled in head and neck cancer group. Data were compared with 68 healthy, age-matched control individuals.

### *Immunohistochemistry*

Latent membrane protein (LMP-1) of Epstein-Barr virus was detected by using immunohistochemistry performed with the Dako-Cytomation LSAB+ System AP Kit (Dako Denmark, Glostrup, Denmark), using monoclonal mouse antibodies against LMP-1, according

to the manufacturer's recommendations. A paraffin-embedded lymph node preparation from a patient with EBV-positive Hodgkin's disease was used as a positive control, while lymph node sections from EBV-negative Hodgkin's disease served as negative controls.

### ***PCR and SSCP***

After DNA isolation, we performed PCR amplification of the  $\beta$ -gobin gene using pCO3 and pCO4 primers to confirm the quality of the DNA. For EBV detection, we used a nested PCR, amplifying a 97 bp region of the internal repeat of the *Bam*H1-W fragment of the EBV genome. Exon deletions of p14<sup>ARF</sup> and p16<sup>INK4A</sup> tumour suppressor genes were analysed by means of PCR assays and primers described earlier (Baur et al, 1999; Chen et al, 2000; Nagy et al, 2003) Changes (single nucleotide polymorphisms, point mutation) of tumour suppressor genes were detected by SSCP analyses.

### ***Sequencing***

Fragments with electrophoretic mobility different from the wild type were analysed by direct sequencing to confirm and characterize the nature of the alteration. After amplification of the exons, PCR product was purified and sequenced using the BigDye Terminator Kit (Applied Biosystems, Foster City, CA, USA) in an ABI 3100-Avant Genetic Analyser. Resulting sequences were compared to the GenBank reference sequence (Accession number: NG007485).

### ***Methylation-specific PCR (MSP)***

Methylated and unmethylated promoter was distinguished by sodium-bisulphite modification. Modified DNA was purified on Wizard Clean-Up System (Promega, Madison, WI, USA) according to the protocol provided by the manufacturer. Promoter hypermethylation of the p14<sup>ARF</sup> and p16<sup>INK4A</sup> genes was determined by methylation-specific PCR.

### ***Statistical analysis***

Statistical comparison of EBV prevalence data and frequency of genetic differences or epigenetic alterations was performed using chi-square and Fisher's exact tests. Logistic regression was used to analyze the association between EBV carriage and patient characteristics (gender, age) as well as the clinical appearance of OLP. Tumour-free survival

of OSCC patients was analyzed using Kaplan-Meier test. All tests were carried out with a confidence interval (CI) of 95% using SPSS 15.0 for Windows software.

## RESULTS

### *EBV prevalence data*

The prevalence of EBV DNA was 19.1% (13/68) in the control group. In patients with OSCC, OL and OLP, the carriage rates in the lesion were 73.8% (48/65), 29.5% (13/44) and 46.6% (54/116), respectively. In the apparently healthy mucosa of the patient, EBV positivity was 66.2% (43/65), 22.7% (10/44) and 31.9% (37/116) for OSCC, OL and OLP patients, respectively.

We studied the EBV carriage rate of apparently healthy mucosa of the patients depending on EBV positivity of the lesions. When patients were divided according to the presence or absence of EBV DNA in their lesions, prevalence rates in the apparently healthy mucosa of patients with EBV-positive vs. EBV-negative lesion were 34/48 (70.8%) vs. 9/17 (52.9%, 5/13 (38.5%) vs. 5/31 (16.1%), and 29/54 (53.7%) vs. 8/62 (12.9%) for OSCC, OL and OLP patients, respectively.

When patients with OLP were divided into EA-OLP (erosive and atrophic) group and non-EA-OLP (plaque-like and reticular) group according to the clinical appearance of the OLP, the two subgroups showed comparable EBV prevalences, both in the lesion and on the healthy mucosa. The EBV positivity of non-EA-OLP and EA-OLP lesions was 45.6% (26/57) and 47.5% (28/59). The prevalence of EBV DNA was 33.3% (19/57) and 30.5% (18/59) in the healthy mucosa of non-EA-OLP and EA-OLP, respectively.

### *Statistical analysis of EBV prevalence data*

The prevalence of EBV in the lesion, as well as on the apparently healthy mucosa of patients with OSCC, was significantly higher than prevalence in the controls or in the other two groups of patients. The lesion of patients with OLP carried EBV DNA more frequently than controls and OL. Regarding only patients with EBV-negative lesions, only OSCC patients showed significantly different EBV carriage rate in their healthy mucosa compared to the control group and two premalignant lesion groups. However, in OSCC patients carrying EBV DNA in their lesions, EBV carriage rate of the apparently healthy mucosa was significantly higher than the prevalence of EBV in controls and in patients with OL. In OLP

patients with EBV positive lesions, EBV prevalence was also significantly higher in the apparently healthy mucosa than in control individuals.

### ***Immunohistochemistry***

The lymph node of the Hodgkin's disease patients (positive control) were consistently strongly positive for EBV LMP-1, but none of the OSCC patients with EBV positive tumour (n=48) tested, or the lymph nodes from EBV-negative Hodgkin's disease patients, were found to be LMP-1 positive.

### ***Analysis of the association of EBV with clinicopathological data***

Epstein-Barr virus positive and EBV negative OSCC patients did not statistically differ in patient characteristics (age, gender) and exposure to risk factors (smoking and alcohol consumption) or clinical data (localization, TNM stage, histological grade) of tumour. The presence of EBV in the lesion or in the apparently healthy mucosa was not found to influence survival, and did not increase the risk of poor outcome. In OSCC patients, higher T stage of the tumour significantly reduces the tumour free survival of the patients (p=0.011). Additionally, the higher T stage (p=0.010) and unfavourable tumour localisation (p=0.013) decrease the tumour free survival among OSCC patients with EBV positive lesion.

In case of patients with OL, younger age (under 55 yrs) was a risk factor associated with EBV carriage in the lesion (p=0.047). In patients with OLP, EBV infection appeared more frequently in men than in women (p=0.02), but the age was not significantly associated with EBV carriage. Furthermore, EBV virus carriage did not influence the risk of unfavourable clinical appearance (EA-OLP).

### ***Analysis of genetic and epigenetic changes of tumour suppressor genes***

We detected one or more exon deletion in four control individuals, one showed p16 exon 1 $\alpha$  deletion, another one exhibited lack of the p16 exon 2 amplicon. Two individuals had deletion in two exons, one in p16 exon 1 $\alpha$  and 2, another one in p14 exon 1 $\beta$  and p16 exon 3. Out of the OSCC patients (n=37), only one patient showed lack of p16 exon 1 $\alpha$ , amplification of all other exons was successful in all other patients. In patients with LSCC, deletion of at least one of the three exons (exon 1 $\alpha$ , 2 and 3) of p16<sup>INK4A</sup> was observed in 21 cases (75.0%), while 10 cases (35.7%) showed p14 exon 1 $\beta$  deletion. Ten of 28 LSCC samples showed deletion in p14<sup>ARF</sup> exon 1 $\beta$ , 19 in exon 1 $\alpha$ , nine in exon 2 and only two samples in exon 3 of p16<sup>INK4A</sup>. Regarding inactivation by exon deletion, p14 was inactivated

in three controls, none of the OSCC and 14 of the LSCC patients. Tumour suppressor gene p16 was inactivated in four controls, one OSCC and 21 LSCC patients. Both genes were found in inactive state in three controls, none of the OSCC and thirteen of the LSCC patients. This corresponds to a significantly different distribution of deletions in LSCC as compared to the controls or to OSCC patients ( $p < 0.001$  in both comparisons).

The SSCP alterations confirmed the presence of two mutations, a homozygous T24610A nucleotide change in the non-coding region of p16 exon 1 $\alpha$  and a heterozygous C24702A change in the coding region of p16 exon 1 $\alpha$ , leading to an Ala13Asp amino acid change. We identified three polymorphisms, G28575A heterozygous polymorphism affecting exon 2 was detected in three patients and one control. This polymorphism corresponds to alanine/threonine variants at codon 140. The G28608A polymorphism was detected in the non-coding region of exon 2 in four patients and always heterozygously. The third polymorphism G31292C was found in the non-coding region of exon 3, it was found in homozygous and heterozygous forms in six and seven patients, respectively. This polymorphism corresponds to the C540G polymorphism at the mRNA level.

### ***Study of promoter methylation of p14ARF and p16INK4A tumour suppressor genes***

Examination of the p14<sup>ARF</sup> promoter methylation patterns was successful for all 68 controls, for 30 of 37 OSCC and for all 28 LSCC samples. In case of the p16 promoter success rates were all controls, 29 of 37 for OSCC and 21 of 28 for LSCC patients. Neither p14 nor p16 promoter was found to be completely methylated in samples obtained from healthy individuals. However, two and three individuals showed partial methylation of p14 and p16 promoters, respectively; i.e. the p14 and the p16 promoters were unmethylated in 97.1% (66/68) and 95.6% (65/68) of the controls, respectively.

In OSCC tumour samples, p14 promoter was unmethylated (fully functional) in 86.7% (26/30) of the patients. Complete and partial methylation was found in one and three patients, respectively. The p16 promoter was unmethylated in 69% (20/29) of patients, while complete and partial methylation was detected in three and six patients. Unmethylated promoters were significantly less frequent in case of p16 promoter ( $p = 0.001$ ) as compared to the control group. In case of the p14, unmethylated promoters were less frequent in OSCC group as well, as compared to the control, but this was not statistically significant ( $p = 0.069$ ).

In case of LSCC, the p14 promoter was unmethylated in 85.7% (24/28) of patients. Completely and partial methylation of p14 was detected in one and three tumour samples,

respectively. The p16 promoter was unmethylated in 76.2% (16/21) of the patients, five patients showed partial methylation of the promoter, complete promoter methylation was not found. Regarding the methylation status of the p16, data differ significantly from healthy controls ( $p=0.016$ ), but in case of the p14 promoter methylation we did not find significant difference ( $p=0.058$ ). Between the methylation status of the two patient groups there was no statistically significant difference in any comparisons.

Combining the two patient groups to a group of head and neck cancer patients, unmethylated promoter was significantly less frequent in case of both p14 and p16 ( $p=0.043$  and  $p=0.001$ , respectively) compared to the control group.

### ***Effect of genetic and epigenetic changes of the tumour suppressor genes to the tumour free survival***

Mean tumour-free survival time was 870 (93-1807) days and 951 (167-2988) days for OSCC and LSCC patients, respectively. Exon deletions in case of LSCC and p16 promoter methylation in case of OSCC were associated with poorer tumour free survival, but neither differences were statistically significant ( $p=0.054$  and  $0.108$  in LSCC and OSCC, respectively).

## **DISCUSSION**

Out of the many potential aetiological factors influencing the development of OSCC, smoking and alcohol consumption are recognized as the two most important, however, the tumour may develop in patients who do not smoke or drink alcohol. This fact suggests the existence of additional important factors, which also increase the risk of tumourigenesis. These may include betel chewing, dietary habits, poor oral hygiene, chronic periodontal diseases, fungal infections and infections with oncogenic viruses (HPV, EBV). Besides these exogenous factors, endogenous predisposition may also be important. The two tumour suppressor pathways most commonly implicated in malignant tumours are the retinoblastoma and the p53 pathways. In head and neck malignancies, the most frequently examined tumour suppressor genes and proteins are the p14<sup>ARF</sup> and p16<sup>INK4A</sup> involved in these pathways.

Though the role of EBV is established in some malignant tumours, the role as an aetiological agent in OSCC or in premalignant lesions of the oral cavity is debatable. Authors reporting low EBV prevalence conclude that EBV has no or only a minor role in the

development of oral cancer; a group from Northern Europe did not find EBV in the examined epithelial head and neck tumours, a Japanese group found only seven EBV positive among 46 OSCC tissue samples.

Our workgroup found a high prevalence of EBV DNA (73.8%; 48/65) in the tumour tissue samples from OSCC, which was significantly higher than that found in the controls or in samples from precancerous oral lesions. This prevalence is comparable to the findings of those studies which presume an etiological role of EBV in development of OSCC. Japanese studies demonstrated a >70% prevalence in OSCC; the samples originated in a region where the EBV-associated nasopharyngeal cancer is prevalent. Others, depending on the sensitivity of the PCR assays used, found 50-100% positivity. For further investigation of the role of EBV, we examined the expression of the viral protein LMP-1 by means of immunohistochemistry, but the LMP-1 protein was not demonstrable in any EBV-positive tumour tissue. Similar findings were also reported earlier by a group, which found EBV DNA in the majority of their patients, but the LMP-1 protein was never detected. Another group examined several viral transcripts and proteins (EBER, EBNA-1, EBNA-2, LMP-1, LMP-2, BHRF-1, BARF0 transcripts, EBNA-1, LMP-1 and ZEBRA proteins), but none of them were found in any samples. They concluded that the virus was inactive transcriptionally, therefore it did not have any role in the process of oral carcinogenesis. The local immunosuppression caused by the tumour may lead to higher virus production, but this does not represent causal relationship with the disease.

To assess the role of EBV in OSCC more precisely, our working group was the first to compare the presence of EBV in the healthy mucosa of OSCC patients as well as in the lesions and healthy mucosa of patients with premalignant oral lesions. In OSCC patients, the prevalence in the tumour samples and in the mucosa was comparable (73.8% vs. 66.2%, respectively). When the patients were divided into EBV positive and negative groups, the EBV carriage in the healthy mucosa in the patient group with EBV negative tumour was significantly higher than in the controls and was similar to the prevalence of patients with EBV positive tumours in their healthy mucosa. Thus presence of EBV DNA is not only characteristic of the tumour lesion but it is also abundant in the healthy mucosa, and is rather the consequence of the immunocompromised status induced by the tumour and may be a general characteristic of the malignant disease. This assumption is supported by the earlier findings of the workgroup with another tumour virus, human papillomavirus (HPV). HPV prevalence in the healthy mucosa of OSCC patients with HPV-negative tumour was



comparable to that found in the control group and was significantly lower than that found in the healthy mucosa of patients with HPV-positive tumour. In this case, it was concluded that presence of HPV is linked to the malignant lesion, supporting the aetiological role of HPV. In contrast, EBV prevalence is high both in the tumour tissue and in the healthy mucosa regardless of the EBV status of the tumour, which suggests that the aetiological role of the virus is improbable and its high prevalence is a consequence of the tumour-related immunological changes. High virus prevalence in the tumour tissue may also be explained by the infiltration of the tumour by EBV-positive lymphocytes and macrophages, as well as by the putative increased susceptibility of keratinocytes to EBV caused by the immunological alterations, which may in turn lead to appearance of the virus in the healthy mucosa of the tumour patients.

EBV prevalence data derived from investigation of premalignant lesions also seem to support the abovementioned. In the lesions of the OLP patient group the prevalence of EBV was close to 50%, while it was significantly lower in OL lesions. Compared to the healthy controls, OLP lesions were significantly more frequently EBV positive (similar to OSCC lesions), while the prevalence in OL was comparable to that seen in healthy individuals. Similar findings, i.e. significantly higher EBV prevalence in OSCC and OLP than in controls, were reported earlier.

This difference between the two oral precanceroses may be explained by their different pathogenesis. OLP is a chronic inflammation of the oral mucosa, most probably caused by a T-cell mediated autoimmune process. Overproduction of certain cytokines, mainly INF- $\gamma$  and TNF- $\alpha$  leads to apoptosis of the epithelial cells, which is triggered by activated CD8+ cells. It is thus conceivable that the autoimmune processes and altered cytokine profile in OLP provides a microenvironment which may favour reactivation and shedding of EBV. In contrast, OL is mediated mainly by mechanical or chemical irritation, and the immunological changes, which may aid EBV shedding, are not characteristic to OL. Nevertheless, the etiological role of EBV in the pathogenesis of OSCC cannot be ruled out altogether, as it may contribute to carcinogenesis in some of the tumours. It is hard to prove the etiological role, as the results available are biased by the fact that most studies, including the present, investigate small populations, which are not sufficient to draw firm conclusions.

Anomalies of the p14<sup>ARF</sup> and p16<sup>INK4A</sup> genes coded in the INK6A/ARF locus located in the short arm chromosome 9 have been observed in multiple tumour types. Inactivation of the p14<sup>ARF</sup> and p16<sup>INK4A</sup> tumour suppressor genes is mediated by deletion, mutation or

promoter methylation. The prevalence of these inactivating events varies between different studies, in a paper summarizing several studies reports frequencies of methylation of the p14<sup>ARF</sup> promoter between 14% and 34%, while promoter of p16<sup>INK4A</sup> was methylated in 5-68% of the samples.

Exon deletion was found in only one OSCC sample affecting the p16<sup>INK4A</sup> gene (exon 1 $\alpha$ ), but in LSCC exon deletion may be important inactivation mechanism in case of both genes, as exon deletion in p14<sup>ARF</sup> and p16<sup>INK4A</sup> was significantly more frequent in LSCC than in controls or in OSCC. Exon deletion in p16 may also affect survival in LSCC, however, the survival difference was not statistically significant (p=0.054). An earlier report found p16<sup>INK4A</sup> deletion in 68.6% of 140 LSCC tissue samples; they also found significantly poorer tumour-free survival in patients with deletion.

Interestingly, deletions affecting either genes were also found in healthy controls. This may indicate that these healthy individuals have a genetic predisposition to development of malignancies, but the lack of amplimers indicating probable deletions may also be caused by less important genetic alterations (polymorphisms, mutations or small deletions) affecting the primer binding site.

Mutations seem to play a minor role in tumourgenesis; only two mutations were detected both in the OSCC group. Both affected p16 exon 1 $\alpha$ , one was found in the coding, the other in the non-coding region. The detected polymorphisms were identical to previously described polymorphisms; some of these were implicated in the development of certain tumours.

Similarly to our data, a paper reports three mutations in 32 oral and maxillofacial squamous cell cancer samples; these affected p16<sup>INK4A</sup> exon 1 $\alpha$  and exon 2. Two further groups found similarly low mutation rates and concluded that mutations may not play an important role in tumourgenesis. In our present study groups, the polymorphisms affecting p16<sup>INK4A</sup> seem to be unimportant as all were heterozygous.

Based on the methylation-specific PCR, promoter methylation in case of the p16<sup>INK4A</sup> gene may represent an important mechanism of inactivation, while in case of p14<sup>ARF</sup> promoter methylation may be less important. Similarly to the results of two other groups, several samples showed partial methylation, i.e. signal characteristic both to methylated and unmethylated promoters were found simultaneously. This may result not only from partial methylation of the promoter, but also from histological heterogeneity of the tumour tissue. As the methylation specific PCRs are very sensitive, even small amounts of normal tissue with

unmethylated promoter may trigger the unmethylated signal. Present data show significantly higher frequency of hypermethylation of the p16<sup>INK4A</sup> promoter in OSCC and LSCC than in the healthy controls, and survival analysis revealed poorer tumour-free survival in patients with hypermethylated p16<sup>INK4A</sup> promoter, though this was not statistically significant (p=0.108). Others reported more frequent hypermethylation of the p16<sup>INK4A</sup> promoter in oral epithelial dysplasias, where the risk of malignant transformation was higher. Based on these findings, hypermethylation of the p16<sup>INK4A</sup> promoter seems to be a promising biomarker in assessment of risk of malignant transformation in oral dysplasia. Methylation was also detected in case of the p14<sup>ARF</sup> promoter, but statistical difference was found only in case of OSCC. We can conclude that the hypermethylation of the critical CpG islands of the promoters of tumour suppressor gene may be important or moderately important inactivation mechanism in head and neck as well as in other cancers.

The presented data draw attention to the differences in the importance of the genetic alterations and in the frequency of hypermethylation of the p14<sup>ARF</sup> and p16<sup>INK4A</sup> promoters in case of head and neck cancer. Exon mutations seem to be unimportant both in oral and laryngeal cancer. In OSCC, promoter methylation, especially in case of the p16<sup>INK4A</sup> promoter, proved to be the most frequent among the genetic and epigenetic alterations investigated, while in LSCC promoter methylation and exon deletion may be important in gene inactivation. These alterations were detected in the healthy population with low frequencies. Though the effect of the genetic and epigenetic alterations on survival was not significant statistically, they are close to the limit of significance. Therefore, more accurate assessment of these issues warrants investigation of larger cohorts.

## SUMMARY

In Europe, Hungary ranks first in both the morbidity and mortality lists regarding head and neck cancer, and the mortality due to head and neck tumours increased nearly five-fold in the past 35-40 years. Despite the development of diagnostic and therapeutic options, the prognosis of oral cancers is unfavourable. Moreover, the various environmental and cellular processes underlying in the background of the development of tumours are not exactly known. To our knowledge, published information on the EBV prevalence of oral tumours and premalignant lesions or the aberrations of p14<sup>ARF</sup> and p16<sup>INK4A</sup> genes in head and neck cancers in the Hungarian population is not available.

Studies suggest that EBV contributes to the development and/or progression of OSCC. However, it is probable, that the role of EBV differs by geographical region, population and tumour type. In our study, we examined the presence of EBV in oral squamous cell carcinoma, premalignant oral lesions (oral leukoplakia, OL, oral lichen planus, OLP) and healthy individuals as a control group. In order to investigate the aetiological role of the virus in more detail, exfoliated cell samples from apparently healthy mucosa were also examined in case of all patients. Examining the EBV positivity of the lesions, patients suffering from OSCC and OLP carried more EBV DNA in their samples than controls. However, when we examined the apparently healthy mucosa of the OSCC patients, the tumour tissue and the apparently healthy mucosa showed comparable EBV prevalence rates. These data suggests that the EBV prevalence is not associated with the lesions itself, but rather with the general immunological status of the patients. The altered immunological process as the consequence of the malignancy may activate the B-lymphocytes and the high virus presence in tumour tissue, surrounding tissue and the saliva may be attributed to the infiltration by B-lymphocytes.

The frequencies of the genetic and epigenetic alterations of p14<sup>ARF</sup> and p16<sup>INK4A</sup> tumour suppressor genes were examined in patients with head and neck cancer (OSCC and LSCC). According to our data, p14<sup>ARF</sup> and p16<sup>INK4A</sup> tumour suppressor gene mutations and polymorphisms seem to be infrequent and consequently unimportant events in Hungarian population with head and neck cancer. However, the exon deletions may be important inactivation mechanism for both genes in LSCC. Exon deletions in case of LSCC may lead to poorer tumour free survival. Examination of the methylation status of the tumour suppressor

promoters, more frequent methylation of the p16<sup>INK4A</sup> promoter was found both in OSCC and in LSCC group than in healthy individuals. Nevertheless, altogether we detected lower promoter methylation rate than earlier studies. Our data suggest that in head and neck cancer methylation status affecting the p16<sup>INK4A</sup> promoter seems to be a moderately important inactivation mechanism, while less important in case of p14<sup>ARF</sup>. Genetic and epigenetic alterations of tumour suppressor genes during carcinogenesis may vary according to tumour types, geographical regions and therefore dietary, smoking and cultural habits as well as depends on the differences in the environmental damage suffered by the population.



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### List of publications related to the dissertation

1. **Kis, A.**, Tatár, T.Z., Gáll, T., Boda, R., Tar, I., Major, T., Redl, P., Gergely, L., Szarka, K.:  
Frequency of Genetic and Epigenetic Alterations of p14ARF and p16INK4A in Head and Neck Cancer in a Hungarian Population.  
*Pathol. Oncol. Res. Epub ahead of print (2014)*  
DOI: <http://dx.doi.org/10.1007/s12253-014-9775-9>  
IF:1.806 (2013)
2. **Kis, A.**, Fehér, E., Gáll, T., Tar, I., Boda, R., D. Tóth, E., Méhes, G., Gergely, L., Szarka, K.:  
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### List of other publications

3. Gáll, T., **Kis, A.**, Tatár, T.Z., Kardos, G., Gergely, L., Szarka, K.: Genomic differences in the background of different severity in juvenile-onset respiratory papillomatosis associated with human papillomavirus type 11.  
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*Orv. Hetil.* 147 (33), 1539-1544, 2006.

Total IF of journals (all publications): 24,29

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## **LIST OF PRESENTATIONS RELATED TO THE DISSERTATION**

**Andrea Kis**, Enikő Fehér, Etelka D. Tóth, Róbert Boda, Ildikó Márton, Ildikó Tar, Zoltán Beck, Krisztina Szarka: Investigation of EBV prevalence in oral squamous cell cancer and in oral leukoplakia.

Annual Meeting of the Hungarian Society for Microbiology, October 15-17, 2008, Keszthely, Hungary

**Andrea Kis**, Tamás Gáll, Csilla Péter, Attila Nochta, Krisztina Szarka: Genetic and epigenetic alterations in the INK4A/ARF tumour suppressor locus in head and neck cancer.

Annual Meeting of the Hungarian Society for Microbiology, October 13-15, 2010, Keszthely, Hungary