Ph.D. Thesis

Oral squamous cell carcinoma in North-Eastern Hungary

Etiological and prognostic factors

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1. Introduction

In Hungary the prevalence of oral and pharyngeal tumors is displaying the most dynamic growth among malignancies and the prognosis of epitehelial cancers of this region is still very unfavorable. The cancer of the lip, oral cavity and pharynx is the sixth most common malignancy causing approximately 3% of all cancer deaths in our country. The mortality rate of lip and oral cavity cancers for males is 13,6, for females 1,9/100.000 person per year.

The incidence of oral cancer in males is higher than in women worldwide, in Hungary the male:female ratio is 6:1. The tumors occur mainly in the sixth or seventh decade of life, but the proportion of patients under 45 years is increasing. The overall 5-year survival of oral cancer patients is around 40%. The site of tumor, the clinical stage at the diagnosis and the therapy are the most important factors in the survival the patients.

Squamous cell carcinoma (SCC) accounts for over 90% of all lip and oral cavity malignancies. Oral SCCs (OSCC) develop from precancerous epithelial dysplasia. The most important steps of the multi-step process of carcinogenesis are the activation of oncogens and the loss of tumor suppressor genes.

It is generally agreed that tobacco, betel quid, and alcohol consumption are the major environmental risk factors for developing OSCC. However, some patients develop OSCC without exposure to these thre risk factors. This fact suggests that additional causes, such as genetic predisposition, diet, or oncogenic viruses, may also help cells to override or escape the physiological mechanisms of proliferation control.

In spite of the developments in diagnostic and therapeutic modalities in the last decades the prognosis of OSCCs are still very unfavorable, especially they are diagnosed in advanced clinical stages. Characterization of malignant diseases by histological, biological and molecular markers is expected to improve our understanding of variations in the clinical course of individual patients and help to estimate their prognosis.

2. Objectives

In my work I aimed to:

1. Analyze a case series of patients with squamous cell carcinoma of the lip and oral cavity. Determine the distribution of SCCs according to age, gender, clinicipathological parameters, treatment, recurrence, and survival with statistical methods. Find significant associations between epidemiological, clinical data and survival.

2. Determine the prevalence of risk factors (tobacco and alcohol abuse, dental status, urban-rural differences) among our OSCC patients. Find associations between risk factors and prognosis with statistical methods.

3. Determine the prevalence of high risk human papillomaviruses in OSCC samples by polymerase chain reaction (PCR). Investigate the expression of p53, Rb és $p16^{INK4A}$ proteins by immunohistochemistry. Find correlations between immunophenotype and clinical parameters in HR-HPV positive and negative cases with statistical methods.

4. Determine the expression of $p21^{WAF1/CIP1}$, p53, Ki-67 and cyclin D1 in lip and oral SCCs by immonohistochemistry. Investigate the prognostic significance of $p21^{WAF1/CIP1}$ expression, its relationship to p53 accumulation, proliferation-associated proteins Ki-67 and cyclin D1 in relation to survival and clinicopathological features in SCCs of the lip and oral cavity.

3. Material and methods

3.1. Patients and sample collection

Patients with primary OSCC treated between 1996 and 2001 in the Department of Oral Surgery, Faculty of Dentistry, University of Debrecen, Hungary, were studied. The formalin-fixed, paraffin-embedded blocks were retrieved from the surgical pathology archives of the Department of Pathology. All tumors were classified according to the International Union Against Cancer (UICC) TNM classification. Histological grading was done according to WHO classification. Clinicopathological information on each case, including age, gender, tumor size, nodal status, location, treatment, presence or absence of tumor recurrence and survival was obtained from patient record files. From the risk factors we investigated smoking and alcohol intake history, oral status and urban vs. rural residence. Serial 4 μm thick sections were cut from the tissue blocks and mounted on silanized slides. One section was stained with hematoxylin-eosin and examined to confirm the original diagnosis and tumor grade.

3.2. Detection of high risk human papillomaviruses

3.2.1. DNA extraction

Five 10 μm thick sections from each paraffin block were placed into 2 ml microcentrifuge tubes and dewaxed with sequential washes of xylene, absolute and 95% ethanol. Pellets were dried and digested overnight at 56°C with 25ug/ml proteinase K in 50 mM Tris-Cl (pH 8.0), 1 mM EDTA-Na and 0.5% Tween 20. The protease was then inactivated at 95°C for 10 min and the resulting DNA solution was stored at –80°C. Procedures to prevent carryover of PCR products were rigorously observed. A new microtome blade was used for each block.

3.2.2. PCR amplification

Each batch of samples included negative controls containing proteinase K buffer and positive control DNA from HPV 16 positive cervical carcinomas. The degenerate MY09/MY11 primer set was used to generate a PCR product of 450 bp. The amplification mixture consisted of 1 μl of sample DNA solution, 1x PCR Taq polymerase buffer (Stratagene), 2.5 U Taq DNA polymerase (Stratagene), 200 μM of

each dNTP, 25 pmol of MY09 and MY11 primers, and 1.5 mM $MgCl_2$ in 50 μl volume. 40 amplification cycles were completed in a Biometra T1 thermocycler as follows: one minute at 94°C, one minute at 56°C and one at 72°C. The initial denaturation step was 5 min at 95°C, and the final extension step was 6 min at 72°C. 0.5 μl of the MY09/MY11 amplification product was carried over to another amplification mixture of similar composition, and was cycled 23 times using the previous temperature profile, with MY11 and HPV-type specific primers (25 pmol each) amplifying high-risk HPV types 16, 18, 31, 33, 45, 51, 52 and 58 respectively. The oligonucleotide sequences of type-specific primers were exactly as published. After amplification, the reaction products were electrophoresed on 3% agarose and visualized by ethidium bromide staining. The two consecutive semi-nested PCR amplifications generated amplimers of ~340 bp if the corresponding HPV genome was detected in the sample.

3.2.3. Analysis of the physical state of HPV 16 genome

2 μl of the DNA from HPV16 positive samples was amplified by PCR using the above amplification mixture and 40 cycles of the above temperature profile with a primer pair (100 pmol each) specific for the E2 gene region, which is disrupted by the integration of the virus into the host genome. Entire, (infective) episomal HPV genome results in amplimers of 351 bp by these procedures, which were detected by agarose electrophoresis as before.

3.3. Immunohistochemistry

Immunohistochemical studies were performed on 4 μm tissue sections mounted on silanized slides using commercially available monoclonal antibodies; anti-$p16^{INK4A}$ (clone 6H12), anti-Rb (clone IF8), anti-$p21^{WAF1/CIP1}$ (clone 4D10) and anti-cyclin D1 (clone P2D11F11) obtained from Novocastra (Newcastle upon Tyne, UK), anti-p53 (Clone DO-7) from DAKO (Glostrup, Denmark), and anti-Ki-67 (MIB1) from BD PharMingen (San Diego, CA, USA). Immunohistochemical staining was conducted using DAKO LSAB2 alkaline phosphatase system according to the manufacturer's instructions. Briefly, dewaxed sections were heated in a microwave oven for 2x5 min in 10 mM citrate-Na (pH 6.0). After incubation with blocking serum for 20 min, sections were incubated with primary antibodies described above for 1 hour at room

temperature with an antibody dilution of 1:50 for anti- $p16^{INK4A}$, anti-cyclin D1, and Ki-67, 1:100 for anti-p53 and anti-Rb, 1:40 for anti-$p21^{WAF1/CIP1}$. After further incubation with biotynilated link antibody and peroxidase-labeled streptavidin, the staining was developed by reaction with New Fuchsin chromogenic substrate solution under microscopic control. In each experiment, a negative control, in which the primary antibodies were replaced by preimmune mouse IgG, and positive control slides were included. Nuclear staining was considered positive for p53, Rb, $p16^{INK4A}$, cyclin D1, Ki-67 and $p21^{WAF1/CIP1}$. A tumor was recorded positive if >10% of the tumor cells showed immunoreactivity. The staining characteristics were compared with adjacent non-neoplastic squamous epithelium.

3.4. Statistical analysis

The data were stored and analyzed by means of SPSS.11 software (SPSS Inc., Chicago IL, USA). Chi square test was used for univariate analysis of categorical data whereas a t-test was used for continuous data. Correlation among variables was estimated by Spearman Rank correlation coefficient. Survival curves were generated using Kaplan-Meier method and compared using the log-rank test. Tests were considered significant when their P values were less than 0.05.

4. Results

4.1. Demographical characteristics, clinicopathological parameters, treatment, recurrence and survival in the OSCC patient group

A total of 119 patients were included for assessment with a median age of 57.4 years. The male:female ratio was 5.2:1. The tissue samples were of the following sites: 33 mouth floor (27.7%), 32 lip (26.9%), 27 tongue (22.7%), 9 palate(7.6%), 8 gingiva (6.7%), 6 retromolar region (5%) and 4 other oral sites. The median age of lip cancer patients was 66.4 years, which is significantly higher than the other patients' age ($p<0.001$).

Analyzing the size of the tumors 70.6 percent of the cases belonged to the favorable group (T1, T2). We found regional lymph node involvement in 28.4%, distant metastases in 3.4% of the cases. 65.6 percent of the lip SCCs were diagnosed with stage I disease while in case of the tongue only 25.9%, and in case of the mouth floor only 24.2% of the cases belonged to this group ($p<0.001$). Stage IV patients were 10,6 years younger than patients with stage I disease ($p=0.002$).

Histologically, 43.0 percent of SCCs was well differentiated, 48.6% was moderately, and 8.4% was poorly differentiated. The degree of differentiation was significantly associated with neither clinical parameters nor survival though in case of well differentiated tumors we found better 5-year overall survival rates (43% vs. 30%).

The overall survival rate was 55.5% after 2 years, 38.7% after 5 years. The size of the tumor, the regional lymph node involvement and the clinical stage significantly influenced the overall survival. We found the strongest association with clinical stage (Spearman correlation coefficient -0.423).

Surgical treatment alone was the treatment for 51% of the cases. Surgery combined with radiation was provided in 22.6%. In the rest of the cases: radiotherapy alone 13.2%, chemotherapy and radiation 2.8%, chemotherapy-surgery-radiation 3.8%, cryotherapy 6.6%. 20.7 percent of the cases experienced a recurrence of disease during the follow-up period. We found significant association between recurrence and histological differentiation of the tumor ($p=0.025$).

4.2. Risk factors and their influence on survival

At the diagnosis of SCC 65.5 percent of the patients were tobacco users, 41.2% smoked 20 cigarettes or more per day. Among smokers we found male dominance (71% vs. 36.8%, p=0.004). Above 65 years of age the ratio of smokers was 38.3%, under the age of 45 it was 86.4%. In advanced clinical stages (III and IV) the ratio of smokers was significantly higher than in early stages (83.3% vs. 52.4%, p=0.044). We found significant association between smoking and drinking habits ($p<0.001$).

Seventy-five percent of the patients reported alcohol consumption auto-anamnestically. The daily intake was 50 g pure alcohol or more in 41.2%. Among males the ratio of abstainers was 19 percent, among women 52.6% (p=0.002). We found significant association between alcohol consumption and the site of the tumor (p=0.033).

Dental status was significantly associated with the age of the patient ($p<0.001$), smoking ($p<0.001$) and alcohol consumption (p=0.005). Toothless patients were usually older than 65 years, were non-smokers and non-drinkers.

Sixty percent of the patients were urban, 40% were rural residents. We found no significant urban-rural differences.

4.3. The role of high risk human papillomaviruses in the etiology of oral cancer in our study population

HR-HPV positive patients were diagnosed 0.9 years earlier (mean 55.3 years) then HPV-negative patients (mean 56.2 years), this difference is statistically insignificant (p=0.72). HR-HPV DNA was detected in 42% of the cases. 82% of HR-HPV positive tumors contained HPV16, but other HR types were also detected in 30 percent of the positive cases. Dual infections with HR-HPV types were noted in 12% of the positive cases. Of the HPV16 positive patients, only 7% showed positive for harboring the tested 351 base pair long sequence of the E2 HPV gene, which is indicative of episomal, self-replicable, potentially infective virus genome. No significant association was found between the presence of HR-HPV DNA and

particular anatomical sites of tumor origin. HPV positivity was not found to correlate with tumor stage, histological differentiation or other factors examined. Neither was a significant association of HR-HPV detectability seen with smoking-and-drinking patients, nor with abstainers.

P53, p16^{INK4a} and Rb protein expression

61% of HR-HPV positive and 54% of HR-HPV negative OSCC blocks were immunopositive for p53. The overexpresion of p53 showed the characteristic nuclear location with variations in the staining intensity and in the number of positive cells. Diffuse nuclear staining with some cytoplasmic positivity for p16^{INK4a} was seen in 13% of HR-HPV positive and 20% of HR-HPV-negative patients. 81% of HR-HPV positive and 84% of HR-HPV negative OSCC samples exhibited Rb nuclear staining.

The association of p53 immunopositivity with p16^{INK4a} and Rb protein expression, HR-HPV status or carcinogenic oral habits was not stronger than what might result from common chance, only smoking and p53 expression exhibited significant correlation (p=0,042).

4.4. The expression of p21$^{WAF1/CIP1}$, p53, Ki-67 and cyclin D1 proteins in SCCs of the lip and oral cavity

P21$^{WAF1/CIP1}$ nuclear staining was present in 61.3% of the specimens. The nuclear staining was most frequent in the suprabasal region, less in the upper region, and least in the basal region. P53 expression could be detected in 60.0% of the cases. The proliferation antigen Ki-67 (MIB1) was present in 76.5% of OSCC samples. The frequency of cyclin D1 positivity was 45.7%.

Expression of p21$^{WAF1/CIP1}$ and clinicopathological parameters

The higher expression of p21$^{WAF1/CIP1}$ in OSCCs was significantly associated with the larger tumor size (T3 and T4 tumors, p=0.005), positive lymph node metastasis (p=0.002), advanced cancer stages (stages III and IV, p<0.001), and the location of the tumor at the tongue and the retromolar region (p=0.002). Furthermore, survival of p21$^{WAF1/CIP1}$ immunopositive cancer patients was markedly less immediately after diagnosis and this difference was significant after 2 years

(p=0.018). However, there was no significant correlation between $p21^{WAF1/CIP1}$ expression and age, gender, alcohol and tobacco use, dental status or histological grade of the OSCC.

Co-expression of $p21^{WAF1/CIP1}$, p53, Ki-67 and cyclin D1 in OSCC

The $p21^{WAF1/CIP1}$ protein expression in OSCC cases significantly and positively correlated with the expression of Ki-67 (p=0.010) and cyclin D1 (p<0.001). Expression of $p21^{WAF1/CIP1}$ was not associated with the expression of p53. None of the investigated proteins exhibited significant correlation with p53 immunopositivity. As expectable, Ki-67 expression showed strong association with cyclin D1 immunopositivity (p<0.001)

5. Discussion

Oral cancer is mostly affecting males, the male:female ratio changes between 2:1 and 16:1 worldwide. In our series we found 5.2:1 ratio, which was consistent with other OSCC reports in Central-Europe. The median age of the patients was 57.4 (37-94) years, which is somewhat lower than in other regions. This fact can be explained with the high (18.5) percent of the younger (≤45 years) patients in our study population.

The most frequent tumor localizations were the mouth floor and the lips. In developed western countries the tongue, while in Asian countries the bucca is the most common anatomical site of OSCC. This difference can be explained with different etiological factors. In Europe alcohol and tobacco consumption, in Asia betel quid chewing are the leading risk factors.

Factors affecting survival are numerous. In our study population age and gender had no significant effect on survival. The overall 5-year survival rate of lip cancers was more than 20 percent higher than tongue's and the mouth floor's, though this difference wasn't significant. In our series histological differentiation of tumors, similar to other studies, didn't influence the prognosis. We found significant correlation between overall 5-year survival and tumor size, regional lymph node involvement, clinical stage and the type of the treatment. The tumor size and the lymph node positivity are significant prognostic factors alone, but together they give stronger prognosis ($p<0.001$). With the advancement of the clinical stages the overall 5-year survival is rate decreasing (68.3% vs. 11.1%). In our patient group the overall 5-year survival rate was 38.7%, which is consistent with the literature.

The treatment of the lip and oral SCC patients were performed according to the nationwide accepted „oncotherapeutic protocol". The treatment modality is influenced by the site and size of the tumor, the size and topography of the regional metastases, the presence of distant metastases and the general heath of the patient. Surgical treatment alone was limited to patients with early stages of disease without regional lymph node involvement. In advanced stages patients received surgical therapy in combination with postoperative radiotherapy. In special cases when the preoperative down staging of the tumor was required we applied neoadjuvant

radiotherapy. If the patient was un-operable or tumor was un-resecable the treatment was primary radiotherapy, in case of residual tumor in combination with chemotherapy. Treatment modality significantly influenced survival. Consistent with the literature the 5-year overall survival rate of patients treated with surgery alone was higher (57.5%) than those of with other treatment options (26.9%, $p<0.001$).

In our study, we found the most significant association between the 5-year overall survival of OSCC patients and the severity of the clinical stage. Unfortunately, 41% of the cases were diagnosed with advanced stages, when the diameter of the tumor is larger than 4 cm and/or regional lymph node involvement is present.

The proportion of the younger patients (under the age of 45) in our study population was extremely high (18.5%), if we compare with the international data. In this group the number of the mouth floor tumors and stage IV diseases was significantly higher than in the other age groups ($p=0.001$ and $p=0.023$). These tumors were nor really larger than the tumors of the older population but were more aggressive and metastatic ($p=0.028$). Their histological differentiation was poorer, more recurrence and reduced survival was found, though these differences are not statistically significant.

Seventy-one percent of the OSCC patients were tobacco users and 79% consumed alcohol. At the same time in the average Hungarian population 35 percent smoked and 60 percent drank regularly. Twenty-seven percent of the patients were not only serious alcohol abuser but smoked 20 cigarettes or more daily. These self-destructive behaviors are widespread in Hungary. According to official statistics 55 percent of the tobacco users smoked 20 cigarettes or more daily, and the ratio of alcoholics in the adult population was 11% in the examination period. It's well known that with the increase of the number of daily smoked cigarettes increases the risk of oral cancer development. In smokers the most common tumor site was the mouth floor, though we found no significant association between smoking and site of the tumor. The consumption of alcoholic drinks increases the risk of oral cancer development. The risk increases with the amount and the concentration of the consumed alcohol. In our patient group 34.5 percent was light ("social") drinker, 41.1% drank regularly over the recommended level. Under the age of 45 the ratio of the heavy smokers and drinkers was significantly higher than in the other age

groups. Some studies found, that alcohol and tobacco consumption is associated not only with the risk of oral cancer development but with poorer prognosis too. In our OSCC group we found reduced survival rate in the smoking-drinking group but this association wasn't significant.

Poor oral hygiene and untreated, carious, periodontotic teeth increase the risk of oral cancer development. In the investigated OSCC group only 12.6 percent of the patients had acceptable dental status. We couldn't find significant correlation between dental status and the site of the tumor; though like Lockhart at al. we found the highest proportion of toothless patients in case of mouth floor tumors. Dental status alone is a weak risk factor, but in combination with alcohol consumption they multiply their effect. In our study population 37% of the patients were heavy drinkers with poor dental status.

In the etiology of lip cancers rural life-form (open-air agricultural work) was found to be associated with increased risk. As a high proportion of our cancer patients lived and worked in small, rural villages we wanted to sum up urban-rural differences. We couldn't find any significant differences between the two groups, nor in the prevalence of lip cancers, nor in other clinicopathological parameters. This means that in our region residence is not an independent risk factor in the etiology of lip- or oral cavity cancers. We can suppose that environmental risk factors are similar in the urban and rural areas of North-Eastern Hungary.

Finally, we can say that smoking and alcohol consumption are not the only risk factors in the etiology of lip and oral cavity SCCs, but in North-Eastern Hungary their importance is the most significant. The proportion of heavy smoking and drinking is the highest under the age of 45. The widespread of these risk factors can partly explain the high frequency of OSCC in this younger age group, but genetic predisposition, diet, oncogenic viruses and other environmental risk factors may also help the development of oral squamous cell carcinoma.

In a lesser part of the patients OSCC develops without exposure to alcohol and tobacco. This fact suggests that additional causes, such as oncogenic viruses, may also involved in the etiology of oral cancers. Numerous studies provide evidence for casual association between human papillomaviruses and a subset of head and neck cancers, similar to cases in the anogenital region.

Our study population of OSCC patients was markedly older than the age typical for intensive sexual activity. Even if excessive tobacco and alcohol consumption might be indicative of some (oral) hedonistic exorbitance, there is no hint to suppose that our OSCC patients were more likely to have been exposed to sexual habits than what is expected for age and dental (~socioeconomic) status. Thus, it is likely that the high prevalence of HR-HPV DNA in oral tumors was acquired long before the manifestation of tumorand persisted in the dividing basal cells by integration into the genome. Indeed, we found potentially infective (episomal/circular) HPV DNA harboring uninterrupted E2 gene sequence in only 7% of HPV16-positive OSCC samples.

The presence of HR-HPV DNA was detected in 42% of OSCC cases, 12% of which were infected with more than one HR-HPV type. This frequency is in approximate agreement with previous reports and is higher than what was reported in normal oral mucosa and precancerous lesions. This is a strong and notorious argument for expecting a cancerogenic role for HR-HPV types in oral cancer. However, our statistical analyses failed to identify any significant difference in the variables studied, between HR-HPV-positive and HR-HPV-negative OSCC patients.

Previous studies have also indicated unclear associations between viral infection and other known causes of oral cancer, such as tobacco, alcohol and betel quid. The lack of correlation between HR-HPV positivity and clinical parameters (tumor size, nodal status, tumor grade) in non-tonsillar head-and-neck squamous carcinomas has already been reported by previous studies. In our patients the prevalence of tobacco and alcohol abuse was very high in both HR-HPV-positive and negative patients, but HR-HPV was not significantly more frequent in the patients which were reported free of these established mutagenic risk factors.

In the course of malignant transformation, early HPV proteins E6 and E7 interact with the cellular antioncogene proteins. In particular, E6 can complex the p53 protein and E7 binds to Rb, enhance their degradation, and thereby alter the normal control of cell growth. The bypassing of the p53 and Rb checkpoints is necessary for any malignant tumor, and therefore the developing tumor is not expected to alter its early oncogenic mechanisms in consecutive phases of tumor development, once inactivation of these proteins was successfully accomplished by

HPV oncogene in the early phase of tumor development. In our OSCC specimens, we found that the overexpression of p53, $p16^{INK4a}$ and Rb proteins does not associate with the presence of HR-HPV types. The independence between the presence of HR-HPV DNA and p53 overexpression agrees with previous data reported in these tumors. The association of overexpression of $p16^{INK4a}$ with oncogenic HPV infections was reported in head and neck cancers in several recent reports; however this association was mostly detected in oropharyngeal and tonsillar carcinomas, or in the rare verrucous oral carcinomas. No association between HR-HPV status and $p16^{INK4a}$ was reported in Indian betel quid chewers.

Although *in vitro* experimental evidence links HPV infection with p53, Rb and $p16^{INK4a}$ alterations, and these findings were found with cervical and anal carcinomas, our data indicate that there are clear molecular differences between anogenital and oral squamous cell carcinomas in tumorgenetic effects and mechanisms. Clearly, HPV detection by PCR in OSCC is not equivalent to potent E6/E7 activity, i.e. a causal and direct involvement of the infection in the carcinogenesis process.

In conclusion, our data indicate that the presence of HR-HPV DNA, in the overwhelming majority of OSCC, may be a sign of markedly unhealthy lifestyle or a consequence of genetic instability contributed by other environmental or host factors, but is unlikely to have a specific, independent and strong cancerogenic effect comparable to HPV-related anogenital malignancies.

In our OSCC patients, we found no correlation between the expression of $p21^{WAF1/CIP1}$ and p53 accumulation. This is in agreement with many of the previous reports from oral SCCs and other carcinomas. According to previous investigations, wild-type p53 is able to upregulate WAF1/CIP1 gene transcription, resulting in enhanced expression of $p21^{WAF1/CIP1}$ protein. This observation was supported by studies in non-OSCC cases, where authors found inverse correlation between $p21^{WAF1/CIP1}$ and p53 expression. This antagonism was not found by other reports. These findings suggest that the physiological control of $p21^{WAF1/CIP1}$ by p53 may be irrelevant for the immunophenotype of „common" OSCC, since tumor progression requires the "individual" inactivation of most of the key antioncogens.

$P21^{WAF1/CIP1}$ expression may be regulated by p53-independent and p53-

dependent pathways. This implicates that functional $p21^{WAF1/CIP1}$ might inhibit tumor growth even after mutations or deletions inactivate both functional p53 alleles, and tumor progression favors the loss of functional $p21^{WAF1/CIP1}$ expression. In our study 60.3% of the p53 positive tumors overexpressed the $p21^{WAF1/CIP1}$ antigen, which is not different from the frequency of p53 (60%) and $p21^{WAF1/CIP1}$ (61.3%) overexpression in the total sample pool. One can assume that the remaining tumors were immunonegative owing to other ways of antioncogen inactivation. Our result support the finding that both p53-dependent and p53-independent mechanisms are involved in the expression of $p21^{WAF1/CIP1}$ in OSCCs, and getting rid of active p53 protein does not completely abolish the antiproliferative activity of functional $p21^{WAF1/CIP1}$ expression.

In our series, $p21^{WAF1/CIP1}$ overexpression significantly associated with tumor size, lymph node metastasis, clinical stage, and tumor site, whereas p53 did not, the later is in agreement with numerous previous findings. Patients with OSCC overexpressing $p21^{WAF1/CIP1}$ had decreased 2-year survival. Twenty-seven percent of the study population was lip SCC, which is typically associated with lower stages and histological grades at the time of diagnosis, and therefore has better outcome than the SCCs of the oral cavity. Also, the importance of sun-induced DNA damage is thought to be more important for the genesis of lip SCCs than the role of mutagenic oral habits. We found a lower occurrence of p21 immunopositivity in lip SCC than in other locations, which is reflected by the association of p21 immunopositivity with the inner mouth tumors, and could be attributed to the lower tumor staging of the lip cases. However, the outcome and expression of neither p53, nor proliferation markers were found to be significantly different from the residual population of the same p21 status. This might indicate the similar effects of random physical and chemical mutagens on the evolution of malignant epithelial stem cells.

The fact that high $p21^{WAF1/CIP1}$ expression significantly correlates with poorer clinical outcome is in agreement with previous studies conducted on both OSCC and other carcinomas. Opposite results have also been reported from breast carcinoma, lung, laryngeal, and tongue SCC studies. These contradictory results indicate that association between $p21^{WAF1/CIP1}$ expression and prognosis is complex, tumor-type and possibly etiology-related. Some studies reveal that combined p53/$p21^{WAF1/CIP1}$

expression may be a predictive factor for reduced survival time when $p21^{WAF1/CIP1}$ or p53 alone is not a significant prognostic variable. Other studies reported that $p21^{WAF1/CIP1}$ expression in combination with p53 accumulation provides better prognostic information than evaluation of either individual variable alone. Our observation is that though p53+/p21+ immunohistochemical phenotype means significantly ($p=0.046$) worse prognosis than p53-/p21- or p53+/p21- phenotype, of which the prognostic value for $p21^{WAF1/CIP1}$ immunopositivity alone is stronger ($p=0.018$).

We found significant correlation between $p21^{WAF1/CIP1}$ overexpression and the expression of Ki-67 or cyclin D1 in OSCCs, although the association of $p21^{WAF1/CIP1}$ positivity with histological grading was not significant. This observation argues for the higher diagnostical accuracy of checking possibly more than one proliferation-related antigen expression for the assessment of tumor dignity and suggests that the inactivation of $p21^{WAF1/CIP1}$ might be delayed to later stages of the tumor dedifferentiation, whereas p53 must be neutralized in the early steps of tumor progression, otherwise the tumor could not grow. Alternatively, the genomic mutations leading to the overproduction of both inactive p53 and $p21^{WAF1/CIP1}$ proteins may reflect a higher mutagenesis frequency and genomic instability, which makes such tumors grow and evolve faster than others. We find it noteworthy to mention that $p21^{WAF1/CIP1}$ positivity was significantly more common in advanced clinical stages ($p<0.001$), which association supports the previous assumption and might be reconciled with the lack of association with histological grading. In either case, however, the immunopositivity for $p21^{WAF1/CIP1}$ is a marker of higher tumor aggressivity and worse prognosis.

In conclusion, the overexpression of $p21^{WAF1/CIP1}$ was unrelated to the overexpression of p53, the main regulatory protein of $p21^{WAF1/CIP1}$ in cell cycle control. However, $p21^{WAF1/CIP1}$ immunopositivity was associated with higher cell proliferation as indicated by Ki-67 and cyclin D1 expression and larger, more metastatic tumors. Our results suggest that immunohistochemically detectable overexpression of $p21^{WAF1/CIP1}$protein has diagnostic value in the pathological assessment of oral SCCs.

6. Summary of the results, new findings

6.1. We characterized for the first time a population with lip and oral cavity SCC in North-Eastern Hungary in a retrospective clinical study.

6.2. We found significant correlations between tumor size, regional lymph node involvement, clinical stage and survival. Clinical stage is the strongest prognostic factor, mortality increases in relation to the clinical stage.

6.3. We reported for the first time that in North-Eastern Hungary the prevalence of OSCC is higher, the proportion of tumors with advanced stages is increased and the survival is reduced under the age of 45 than in the general trend.

6.4. We described for the first time, that in our region in the etiology lip and oral cavity SCCs tobacco smoking and alcohol consumption are the most important environmental risk factors. The occurrence of these risk factors is significantly higher under the age of 45.

6.5. We described for the first time that in North-Eastern Hungary (rural) residence is not a specific or independent risk factor in the etiology of lip - and oral cavity SCCs.

6.6. We examined for the first time the prevalence of high risk HPV types in OSCCs in North-Eastern Hungary.

6.7. We reported for the first time in our region that the presence of HR-HPV DNA in the overwhelming majority of OSCCs, may be a sign of markedly unhealthy lifestyle or a consequence of genetic instability, but is unlikely to have a specific, independent and strong cancerogenic effect.

6.8. We proved among the first ones the prognostic significance of $p21^{WAF1/CIP1}$ expression in lip and oral cavity SCCS. $p21^{WAF1/CIP1}$ immunopositivity is associated with higher cell proliferation and larger, more metastatic tumors.

7. Utilization of results

7.1 We are planning to use the results we obtained from the epidemiological studies on OSCC patients in our future case-control studies. We would like to sum up the odds ratio of specific risk factors in the etiology of oral squamous cell carcinoma.

7.2. The results of our previous studies remind us that the proportion of oral tumors with advanced clinical stages is very high. This fact underlines the necessity of screening, the best method of early detection with special reference to the younger age groups.

7.3. The extremely high prevalence of environmental risk factors underlines the importance of health education.

7.4. The results of our immunohistochemical studies, the prognostic significance of p21 $^{WAF1/CIP1}$ can be used in the treatment planning of the OSCC patients.

8. Literature

Publications in the topic of thesis:

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