Expression profiles of selected genes in tumors and matched surgical margins in oral cavity cancer: Do we have to pay attention to the molecular analysis of the surgical margins?

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Abstract

Background. Head and neck squamous cell carcinomas (HNSCCs) are associated with an interplay between genetics and the environment; they account for 3% of all diagnosed malignant tumors in men and 2% of those in women.

Objectives. The aim of the study was to analyze the significance of *TIMP3*, *SFRP1*, *SFRP2*, *CDH1*, *RASSF1*, *RORA*, and *DAPK1* gene expression in head and neck squamous cell carcinoma tumors, and in matching surgical margin samples. We also analyzed the association between clinical parameters and the expression of the selected genes.

Material and methods. Following surgical resection, 56 primary HNSCC tumors and matching surgical margin samples were collected from patients at the Clinic of Oncological and Reconstructive Surgery of Maria Skłodowska-Curie Memorial Cancer Center and the Institute of Oncology in Gliwice, Poland. The gene expression levels were analyzed by quantitative reverse transcription (qRT)-PCR.

Results. *SFRP1* gene expression was statistically significantly lower in the tumor samples than in the surgical margins (0.30 ± 0.36 vs 0.62 ± 0.36; $p < 0.01$). No correlation was found between gene expression and clinical parameters, except *DAPK1*, where low expression correlated with alcohol abuse (0.85 ± 1.19 vs 1.97 ± 3.22; $p = 0.074$). Moreover, patients with G3 grade tumors, i.e., poorly differentiated tumors, had significantly higher values of *DAPK1* gene expression than the G1 (well-differentiated tumors) and G2 (moderately differentiated) groups.

Conclusions. There are many different reasons and concepts for altered gene expression in tumors and surgical margin tissue. Tumor heterogeneity and its microenvironment are undoubtedly linked to the biology of HNSCC. In order to understand specific tumor behavior and the microenvironment, further studies are needed. To find markers connected with cancer development and to provide insight into the earliest stages of cancer development, attention should also be focused on molecular analysis of the surgical margins.

Key words: gene expression, surgical margin, oral cavity, head and neck cancer

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Introduction

Head and neck squamous cell carcinomas (HNSCCs) account for 3% of all diagnosed malignant tumors in men and 2% of those diagnosed in women.[1] Head and neck cancer is associated with an interplay between genetics and environment. It is associated with abnormalities in proliferation, apoptosis, differentiation, cell cycle regulation, DNA repair, signal transduction, and angiogenesis.[2] The instability of the genome, chromosomal rearrangement and loss of DNA fragments are associated with carcinogenesis of HNSCC.[3] Califano et al. proposed a model for the dynamics of chromosomal damage in the course of cancers of the head and neck.[4] Through progression, an increased amount of chromosomal loss takes places. The conversion from normal mucosa to invasive cancer is linked with an accumulation of genetic changes in tumor suppressor genes and proto-oncogenes. Furthermore, adjacent areas share similar genetic alterations. For HNSCC, the list of environmental risk factors includes alcohol consumption, tobacco use, poor oral hygiene, chronic exposure to certain industrial agents, and infection with specific subtypes of human papillomavirus (HPV).[1] Slaughter et al. first proposed the idea of "field cancerization" as the "preconditioning of an area of the epithelium to cancer growth by a carcinogenic agent", which means that an area with genetically altered cells could play a crucial role in the carcinogenic process based on many molecular findings.[5,6] The initiation of field cancerization is associated with various types of molecular damage, e.g., altered gene expression. The residual field in the region adjacent to the tumor can cause local recurrences and 2nd primary tumors after surgical intervention for the primary carcinoma. Actually, there are many theories interpreting oral field cancerization.[7] The "cancer field effect" has been explained by the presence of cells with genetic alterations; however, involvement of epigenetic alterations in field cancerization has also been shown. Epigenetic changes are defined as alterations in gene expression that are not coded in the DNA sequence. Among epigenetic mechanisms, such as DNA methylation at CpG sites or histone modifications, aberrant DNA methylation has been frequently analyzed in various types of cancer. Hypomethylation leads to the activation of cancer-associated genes, whereas hypermethylation of promoter CpG islands is associated with the silencing of various tumor-suppressor genes. Several environmental factors could induce epigenetic modifications.[8] In this study, we have analyzed the gene expression of *TIMP3, SFRP1, SFRP2, CDH1, RASSF1, RORA*, and *DAPK1* in primary HNSCC tumors and matching surgical margin samples. We selected genes involved in degradation of the extracellular matrix, cellular proliferation, migration, and apoptosis. Disruption of these processes can lead to carcinogenesis. The characteristics of these genes are shown in Table 1.[9–15]

The main aim of the study was to provide more information concerning the molecular mechanism of oral malignancy based on gene expression, which could provide valuable information for a better understanding of the oral carcinogenesis process.

Material and methods

Patients and tissue samples

We collected 56 primary HNSCC tumors and 56 matching surgical margin samples following surgical resection from patients at the Clinic of Oncological and Reconstructive Surgery of Maria Skłodowska-Curie Memorial Cancer Center and the Institute

Table 1. The characteristics of selected genes

Symbol	Name	Location	Function	Reference
TIMP3	tissue inhibitor of metalloproteinases 3	22q12.3	The proteins encoded by this gene family are inhibitors of the matrix metalloproteinases, a group of peptidases involved in the degradation of the extracellular matrix (ECM).	9
SFRP1	secreted frizzled-related protein 1	8p11.21	This gene encodes secreted frizzled-related protein 1, a negative modulator of the Wnt signaling pathway.	10, 11
SFRP2	secreted frizzled-related protein 2	4q31	This gene encodes secreted frizzled-related protein 2 (SFRP2), a negative modulator of the Wnt signaling pathway.	10, 11
CDH1	cadherin 1 also known as E-cadherin	16q22.1	This gene encodes a calcium-dependent protein. E-cadherin plays an important role in the maintenance of cell differentiation and the normal architecture of epithelial tissues.	12
RASSF1	Ras association (RalGDS/AF-6) domain family member 1	3p21.3	This gene encodes RASSF1A. It inhibits cell cycle progression at the G1/S transition by preventing the accumulation of cyclin D1.	13
RORA	RAR-related orphan receptor A	15q22.2	This gene encodes RORA. The protein encoded by this gene is a member of the NR1 subfamily of nuclear hormone receptors. These receptors are critical in the regulation of a number of physiological processes. RORA has also been suggested to be involved in lipid metabolism and to possess immunomodulatory activity.	14
DAPK1	death-associated protein kinase 1	9q21.33	This gene encodes calmodulin-dependent serine-threonine kinase. *DAPK1* is involved in programmed cell death.	15

of Oncology in Gliwice, Poland. After resection, these specimens were submerged in the tissue storage and ribonucleic acid (RNA) stabilization solution, RNA*later*® (Sigma-Aldrich, Saint Louis, USA), then frozen at –80°C until RNA extraction. All the tumors collected during surgery were oral cavity cancers (comprising the maxilla, mandible, floor of the mouth, tongue, and cheek, with the highest number being mandible and tongue cases). Representative tumor samples were histologically confirmed by pathologists from Maria Skłodowska-Curie Memorial Cancer Center and the Institute of Oncology in Gliwice, and were classified as primary HNSCC tumors. Oral mucosal biopsy specimens were taken from the surgical margin at least 10 mm from the tumor border and were histologically confirmed as being free of cancer. Intraoperative histopathological examinations were performed whenever positive margins were suspected.

The Institutional Review Board on Medical Ethics of Maria Skłodowska-Curie Memorial Cancer Center and the Institute of Oncology in Gliwice approved the study protocol (nos. KB/493-15/08 and KB/430-47/13). An informed consent form was obtained from all patients. None of the patients included in this study had preoperative radio- or chemotherapy. The average age of the patients was 56.05 years (range: 29–77 years, median: 58.5 years). There were 37 men (66%) and 19 women (34%); 80% of the patients (45/56) were smokers; 73% of them (41/56) reported alcohol consumption; 64% (36/56) were both smokers and alcohol consumers. Tumor staging was based on the pathology findings and then revised according to the 2007 version of the American Joint Committee on Cancer (AJCC) staging system for analysis.[16,17] The clinical parameters of the patients are shown in Table 2.

Table 2. Clinical features of patients

Clinical parameters	Patients, n (%)
Histological grading	
G1 (well-differentiated)	5 (8.9)
G2 (moderately differentiated)	44 (78.6)
G3 (poorly differentiated)	7 (12.5)
T classification	
T1	3 (5.4)
T2	6 (10.7)
T3	13 (23.2)
T4	34 (60.7)
Nodal status	
N0	21 (37.5)
N1	22 (39.3)
N2	13 (23.2)

Homogenization and ribonucleic acid extraction

The tissue samples were fixed in RNA*later*® Stabilisator Reagent (Sigma-Aldrich, Saint Louis, USA) and frozen to –80°C, then thawed slowly at room temperature and homogenized with FastPrep®-24 homogenizer (MP Biomedicals, Santa Ana, USA) using tubes with ceramic beads for tissue homogenization, Lysing Matrix D (MP Biomedicals, Santa Ana, USA). The RNA was isolated using an RNeasy® Mini Kit (Qiagen, Hilden, Germany). In addition to the standard procedure, DNase I digestion was performed on the extracted RNA (RNase Free DNase Set, Qiagen, Hilden, Germany) to remove the residual genomic DNA.

The RNA was quantified by measuring the UV absorbance at 260/280 nm (NanoDrop™ ND, 1000 Spectrophotometer, Thermo Fisher Scientific, Waltham, USA) and the integrity was assessed by electrophoresis on 1.2% agarose gel stained with ethidium bromide (Serva, Heidelberg, Germany). Additionally, the RNA integrity number (RIN) was derived with an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, USA) using an Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, USA); this helped to ensure RNA quality.

Complementary DNA (cDNA) synthesis

The total RNA from each tumor and surgical margin sample was reverse-transcribed into cDNA using a High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Foster City, USA). The Total RNA (30 ng) was reverse-transcribed into cDNA. The reaction was performed with a volume of 20 μL according to the manufacturer's instructions and using Mastercycler® Personal (Eppendorf, Hamburg, Germany). To avoid multiple thawing, cDNA samples were divided into a number of portions, which were sufficient for all subsequent quantitative polymerase chain reactions (Q-PCR); these portions were frozen at –80°C.

Quantitative polymerase chain reaction

The gene expression analysis was performed by quantitative reverse transcription (qRT)-PCR using specific TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, USA). Q-PCR was performed for 7 genes: *TIMP3* (Hs00165949_m1), *SFRP1* (Hs00610060_m1), *SFRP2* (Hs00293258_m1), *CDH1* (Hs01023894_m1), *RASSF1* (Hs00200394_m1), *RORA* (Hs00536545_m1), and *DAPK1* (Hs00234489_m1).

The Q-PCR for all genes was performed in a volume of 20 μL using 1 μL of cDNA, 10 μL of TaqMan® Gene Expression Master Mix (Applied Biosystems, Foster City, USA), 1 μL of primer and probe mix (TaqMan® Gene Expression Assays), and 8 μL of H_2O (Qiagen, Hilden, Germany). All assays were carried out in 96-well plates (Applied Biosystems, Foster City, USA) on a 7300 Real-Time PCR System and were analyzed by SDS v. 1.4 software (Applied Biosystems, Foster City, USA). The glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*, Hs99999905_m1) was used as an internal control. The expression levels of all these genes were normalized to those of *GAPDH*. In all amplification reactions, a negative control was also included: water free

of DNase, RNase, and protease (5Prime, Hilden, Germany) was used in place of cDNA. Thermal cycling for all analyzed genes and *GAPDH* was 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min. The comparative threshold cycle (Ct) method $2^{-\Delta\Delta Ct}$ was used to determine the relative gene expression levels (relative quantification – RQ) for each of the 7 target genes. Five surgical margin samples were used as a calibrator. Relative mRNA expression was determined from the tumor and surgical margin samples using mRNA expression from the calibrator.

Statistical analysis

Statistical analysis was performed using STATISTICA v. 10.0 PL (QUEST, Tulsa, USA). Statistical significance was set at a p-value of less than 0.05. All tests were two-tailed. Imputations were not performed for missing data. Nominal and ordinal data were expressed as percentages, while interval data were expressed as means ±standard deviation. The distribution of variables was evaluated by the Shapiro-Wilk test and the homogeneity of variances was assessed by the Levene test. For comparison of data between 2 groups, the Student's t-test was used for independent data. For comparison between different histological grades, one-way analysis of variance (ANOVA) was used with an LSD post-hoc test.

Results

When gene expression levels were compared between the tumor samples and the margin samples, a statistically significantly lower gene expression of *SFRP1* was found in the tumor samples. The RQ values of the selected genes are reported in Table 3.

Table 3. Relative quantification (RQ) values in tumor vs surgical margin in patients with HNSCC

Gene	Tumor mean RQ ±SD	Margin mean RQ ±SD	p-value
TIMP3	0.62 ±0.80	0.97 ±0.80	0.113
SFRP1	0.30 ±0.36	0.62 ±0.36	<0.01
SFRP2	0.60 ±0.66	0.54 ±0.66	0.609
CDH1	0.70 ±0.45	0.68 ±0.45	0.846
RASSF1	0.71 ±0.49	0.64 ±0.49	0.465
RORA	0.46 ±0.66	0.54 ±0.66	0.423
DAPK1	1.07 ±1.44	1.24 ±1.45	0.654

SD – standard deviation.

We also analyzed the correlation of the clinical parameters with the expression levels of selected genes. No association was found between the gene expression levels and clinical parameters, except *DAPK1*, in which a low gene expression level statistically correlated with alcohol abuse (Table 4). Moreover, the one-way ANOVA showed

Table 4. Relative quantification (RQ) values of *DAPK1* in the group of patients with HNSCC with/without alcohol abuse

Alcohol	*DAPK1* (mean RQ ±SD)	p-value
Alcohol +	0.85 ±1.19	0.074
Alcohol –	1.97 ±3.22	

SD – standard deviation.

a significant influence of the histological stage. The LSD post-hoc test showed that the patients with high-grade G3 tumors – i.e., poorly differentiated – had a significantly higher gene expression level of *DAPK1* than the patients with low-grade G1 (well-differentiated) tumors ($p < 0.05$) or G2 (moderately differentiated) tumors ($p < 0.01$). These results are shown in Fig. 1.

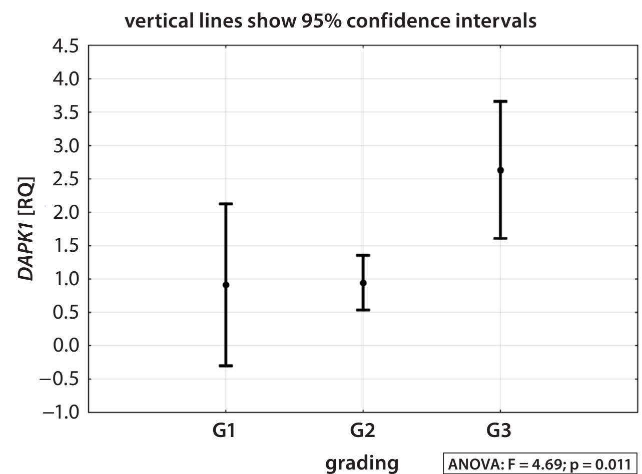

Fig. 1. Relative quantification (RQ) values of *DAPK1* in the group of patients with HNSCC according to G1, G2 and G3 grading stage

Discussion

In the literature, the expression profiles of many genes have been reported in different types of human cancer,[18–21] including HNSCC.[12,22–27] The majority of the research has compared tumor tissue from patients with normal tissue from healthy individuals or normal human cells obtained commercially.[12,18,20–22,28,29] Simultaneous analysis of gene expression levels in the tumor and surgical margins has rarely been performed.[23,26,30] Cancer development is the result of the accumulation of genetic and epigenetic alterations. Because changes in certain genes occur in the very early stages of tumorigenesis, the detection of preneoplastic alterations in the surgical margin could facilitate the detection of cancer. A molecular approach to the matching margin can contribute to cancer prevention and control; therefore, we studied the tumors and matched surgical margins from the patients. Moreover, in our study, the expression levels of the *TIMP3, SFRP1, SFRP2, CDH1, RASSF1*, and *RORA* genes did not

correlate with any of the clinical-pathological parameters, whereas *DAPK1* correlated with both the histological grade of the tumor and alcohol consumption. In addition, in some findings there was a significant correlation between clinical and pathological parameters and gene expression, but in others there was no significant association.[22,25–28,31–33]

TIMP3

The expression of *TIMPs* in oral squamous cell carcinoma (OSCC) shows a trend that is higher in tumors than in normal tissue, and which correlates with an increased risk of metastasis and regional lymph node involvement, although some deviations from this have been noted.[34] There are suggestions that *TIMP3* protects against tumor development by suppressing angiogenesis, tumor growth and metastasis through inferred apoptosis.[9]

Coskunpinar et al. analyzed the expression levels of 88 genes in laryngeal carcinoma using a PCR array and showed an altered expression of 16 genes when compared to paired cancer-free tissue.[30] One of the altered genes in which expression was significantly higher in the tumor tissue was the *TIMP1* gene. *TIMP1* protein expression was significantly higher in laryngeal squamous cell carcinoma than in adjacent non-cancerous tissues.[19] Similarly, an increased expression of *TIMP2* has been observed in tumor tissues compared with normal tissues.[26] Burduk et al. described oropharyngeal cancer without lymph node metastasis showing lower *TIMP1* and *TIMP2* protein expression in cancer cells and stroma compared to patients with lymph node metastasis.[35] On the other hand, it has been reported that a downregulated expression of the *TIMP3* protein in esophageal adenocarcinoma is associated with enhanced tumor invasiveness and reduced patient survival[9] and that the downregulation of *TIMP3* stimulates growth and invasion in endometrial cancer cell lines.[36] The present study of *TIMP3* gene expression in tumors and surgical margin tissues and the correlation between gene expression and clinical-pathological parameters in oral cavity cancers indicated no significant differences (Table 3).

SFRP1 and *SFRP2*

Research has shown that the expression of *SFRP* genes is often downregulated in many cancers, indicating that *SFRP* functions as a tumor suppressor gene.[37–39] In our study, we examined *SFRP1* and *SFRP2* gene expression levels and found a statistically significantly lower expression of *SFRP1* in tumor samples compared to margin samples, although the difference was not significant for the *SFRP2* gene (Table 3). Sogabe et al. proved that in OSCC cell lines, the silencing of *SFRP* genes and their loss of function lead to cell proliferation during oral carcinogenesis.[38] Similarly, Xiao et al. showed that *SFRP2* mRNA expression was downregulated in tumor samples of OSCC compared to tumor-adjacent normal tissue, and that the loss of expression was connected with hypermethylation of the gene promoter.[39] Reduced *SFRP1* expression was also detected by immunohistochemical staining in a group of patients diagnosed with mucoepidermoid carcinoma of the salivary glands.[40] The *SFRP1* protein can act as a Wnt signaling inhibitor by attaching extracellular Wnt ligands or directly binding to the transmembranous receptor FZD. Its role as a potential progression marker was clarified in a study by Rogler et al. which used cell cultures and tumor samples of bladder cancer.[37] They demonstrated that the function of *SFRP1* in the process of oncogenesis is more complicated, considering the non-canonical Wnt- and mitogen-activated protein kinase (MAPK) signaling pathways.

CDH1

A dysfunction of cadherin 1 is involved in carcinogenesis, and a loss of function has been demonstrated to promote tumor invasion and metastasis in different cancer models.[41] It has been reported that the loss of protein expression of *CDH1* is also associated with an increased invasive potential in head and neck cancer.[42] In a study conducted on clinical samples collected from patients with tongue squamous cell carcinoma, a significantly lower *CDH1* mRNA expression level than in the corresponding non-cancerous tissues was shown.[31] In our study, no significant differences were found between the *CDH1* gene expression levels of the tumors and the surgical margin samples. Similar results were obtained by Kordi-Tamandani et al., who found no significant differences between the mRNA expression of the *CDH1* gene of patients with oral cavity cancer and that of a healthy control group.[12]

RASSF1

Another gene that plays an important role in human cancer cell growth and progression is *RASSF1*, and an abnormal expression of *RASSF1* could be an important step in oncogenesis.[21] In the case of the *RASSF1* gene, we found no significant differences in our study. A downregulated expression of *RASSF1A* transcripts and protein in tumor tissues in esophageal and nasopharyngeal carcinomas were observed by Lo et al.; moreover, a reduced expression correlated with the histological grade of the tumor.[33] The mRNA expression of *RASSF1A* was also downregulated in primary tumors in a group of patients with cutaneous melanoma and lacrimal gland carcinoma compared to healthy groups, and was also downregulated in lung and breast cancer cell lines.[18,21,43] Furthermore, aberrant methylation of *RASSF1A* has been observed in several cancer types[43] and a few reports have been focused on the methylation of this gene in HNSCC.[44,45] It is well known that hypermethylation is one of the important epigenetic mechanisms responsible for inactivation of the gene, in addition to genetic alterations of gains and losses.[12,43]

RORA

RORA is a steroid hormone receptor involved in cellular processes, including metabolism, angiogenesis, inflammation, and the circadian rhythm. There are several diseases where *RORs* are integral to the onset and progression, such as autoimmune diseases, inflammation, osteoporosis, and cancer, and it has been proven that in tumor cell lines and cancerous tissues, *ROR* mRNA levels are often downregulated.[46] *RORA* expression was analyzed in colorectal adenocarcinomas, where *RORA* mRNA expression was downregulated in comparison to normal colonic tissue.[47]

In this study, we found no significant differences in *RORA* gene expression, and to our knowledge there have been insufficient studies on the expression of this gene in patients with head and neck cancer. Sørensen et al. analyzed gene expression in human squamous cell carcinoma cell lines and observed a higher level of *RORA* gene expression in the hypoxia group compared to the control group; this was one of the genes induced at low oxygen independent of pH.[29] Genes upregulated by low oxygen have been considered endogenous markers for tumor hypoxia.

DAPK1

DAPK1 is a regulator of apoptosis; suppression of this gene is thought to be critical in the development of tumors. Lower mRNA and protein expression of *DAPK1* was observed in a group of patients with primary gastric cancer compared with adjacent non-tumor tissues.[48] Mariano et al. detected that even with losses of copy numbers for the *DAPK1* gene, the immunohistochemical reaction showed protein expression of this gene in a group of patients with salivary gland neoplasms.[49] Our study showed no significant differences in *DAPK1* gene expression between the tumors and the surgical margin tissues, but there was a significant association between *DAPK1* gene expression and tumor grade. Patients with G3 tumors had significantly higher RQ values of *DAPK1* than patients with G1 and G2 tumors. Figure 1 shows the histological findings of the tumors as related to the gene expression of *DAPK1.* It is often difficult to compare findings between studies because of the different populations and methods used, but our results regarding *DAPK1* are not compatible with other studies. Another study revealed that the methylation of the *DAPK1* gene was associated with the progression of HNSCC.[44,45] Aberrant promoter DNA methylation of this gene has been examined in other types of cancer, including breast cancer; furthermore, tumors with an advanced T-category revealed a higher frequency of *DAPK1* methylation.[50] Surprisingly, the data of Brait et al. on *DAPK1* promoter methylation showed the frequencies of methylation for *DAPK1* in normal thyroid samples to be higher than the frequencies in cancer samples.[51] According to the research, these results limit the usefulness of this gene as a diagnostic marker; additionally, the hypermethylation in the tumors in neoplastic relevance is questionable. Our understanding of the physiological role of the gene is still at an early stage. Furthermore, our studies of the selected tumor histological grade were done on a small population; we must carry out further studies on a larger population in order to verify this result.

Interestingly, we also found a lower *DAPK1* gene expression in the group of patients with alcohol abuse (0.85 vs 1.97; Table 4). This finding could be explained by an epigenetic mechanism after exposure to a risk factor like alcohol consumption, as different lifestyle factors induce expression changes in different genes. Also, low transcription could be associated with methylation induction, as genes specific to a factor could be methylated.[8] In the development of upper aerodigestive tract cancer, alcohol and tobacco are 2 well-established associated risk factors.[52]

There are many different reasons and hypotheses for altered gene expression in tumors and surgical margin tissue. A tumor's heterogeneity and microenvironment are undoubtedly linked to the biology of HNSCC. The clinical application of genetic alterations and their role in HNSCC progression are still being discussed.[41] Different mutations of the *TP53* gene are most prevalent among the numerous observed mutations in HNSCC. An association between the specific mutations of this gene and the biological and clinical course of cancer has been found.[53] Moreover, epigenetic events, such as aberrant promoter gene hypermethylation, are often observed not only in tumor tissue, but also in surgical margins.[39,54,55] Abnormal DNA methylation patterns in promoter regions can inactivate genes and facilitate tumor formation and progression.[56] Environmental factors like exposure to alcohol and cigarettes can influence aberrant methylation patterns, too.[57] Apart from tobacco and alcohol consumption, HPV has been named as a causative agent in a subset of this cancer.[58] HPV infection leads to deregulation of the cell cycle and it is well-known that additional genetic changes are needed for HPV-mediated oncogenesis.[59] It was found that an overexpression of *p16* was connected with molecular events occurring after HPV infection and *p16* has been used as a surrogate marker for evaluation of HPV status.[60,61] Interestingly, HPV was also found to deregulate the methylation levels in individuals with HPV infection.[62] Recently, *Helicobacter pylori* was detected in samples collected from malignancies in the oropharyngeal area and its influence on carcinogenesis was also suggested.[63] It is essential to further study the methylation status in this group of patients, and papers on this subject are currently in progress. One limitation of this study was the fact that we did not investigate the patients' HPV status; we are also considering further investigation of this issue.

In our study population, 80% of the patients were current smokers (most of them smoked more than 1 pack per day); 73% of them reported alcohol consumption, and 64%

both smoked and consumed alcohol. Given the important role that environmental factors such as alcohol abuse and tobacco exposure play in the onset of cancer, it is clear that some genetic or epigenetic alteration occurs in non-cancerous tissues adjacent to the tumor tissue.[1] As many of the patients studied were exposed to these factors, they showed altered expression levels, and the lack of statistically significant differences between the tumor and the margin can also be interpreted in terms of "field cancerization." It is known that patients with head and neck cancer subsequently have increased morbidity and mortality and that subsequent primary tumors are the main reason for mortality in this group of patients.[64] It is well-known that the carcinogenic process involves a progressive accumulation of genetic abnormalities and that HNSCC is a diverse disease with complex molecular abnormalities. A special model of genetic alterations and the progression of squamous dysplasia to invasive carcinoma has been described, including the clonal growth of transformed cells with the formation of an abnormal genetic field.[65] Moreover, oral field cancerization suggests that oral cancer does not arise as an isolated cellular phenomenon but rather as an anaplastic tendency involving many cells at once and it results in the multifocal development of cancer at varied rates within the total field as a reaction to a carcinogen, particularly tobacco. This concept could explain the appearance of multiple primary cancers and recurrences despite the total excision of oral cancer.[7] Tabor et al. found genetically altered fields in the non-neoplastic mucosa surrounding the tumors of 10 out of 28 patients (36%) with HNSCC – the size of the fields was variable. Moreover, in 7 out of 10 patients the field extended beyond the surgical margin.[66]

In order to understand specific tumor behavior and the microenvironment, further studies are needed. As a lot of clinical procedures are limited predictors of tumor progression, many authors suggest that the detection of abnormalities in the field defect can be a useful diagnostic marker to help in assessing the risk of cancer.[67,68] It would be favorable to change the assessment of a safe margin extension or to expand the irradiation field.[69] The discovery of a marker of cancerization is important, but the oncogenesis process is very complicated and molecular techniques have limitations as well. Finding a marker for the development of cancer which provides insight into its earliest stages requires attention to also be focused on a molecular analysis of the surgical margin.

Conclusions

To find markers connected with cancer development and to provide insight into the earliest stages of cancer development, attention should be focused on a molecular analysis of the surgical margins. More investigation is required to completely understand all of the components.

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