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Regular paper

Expression profiles of *MGMT*, *p16,* and *APC* genes in tumor and matching surgical margin from patients with oral squamous cell carcinoma

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The aim of the study was to assess the expression of *MGMT, p16,* and *APC* genes in tumors and matching surgical margin samples from 56 patients with primary OSCC. We also analyzed the association of the clinical variables with the expression of the studied genes. After RNA isolation and cDNA synthesis gene expression levels were assessed by quantitative reverse transcription (qRT)–PCR. Two-sided parametrical Student's *t*-test for independent groups with equal/unequal variances showed no statistically significant differences in genes' expression in tumor compared to margin samples. No association was found between the genes' expression and clinical parameters, except for *MGMT,* whose low expression was probably associated with smoking (0.87 *vs* 1.34, *p*=0.065). 'Field cancerization' is an area with genetically or epigenetically altered cells and at the same time a risk factor for cancer. Disturbances in gene expression could also be the source of damages leading to cancerization. In conclusion, it is important to mention that the field remaining after a surgery may pose an increased risk of cancer development. It may be suggested that the diagnosis and treatment of cancers should not be concentrated only on the tumor itself, but also on the cancer field effect. Therefore, further molecular analysis on surgical margins and additional research regarding their assessment are required.

Key words: HNSCC, gene expression, surgical margin, *MGMT*, *p16*, *APC*

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INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is arising in the oral cavity, pharynx, larynx, nasal cavity, and paranasal sinus. Oral squamous cell carcinoma (OSCC) is the most common malignant tumor of the head and neck, and the number of patients diagnosed with HNSCC has been constantly growing in the past years (Bagan & Scully, 2008; Kang *et al.*, 2015). The list of risk factors associated with HNSCC includes geographical location, habits, diet, alcohol consumption, tobacco use, infection with specific subtypes of human papilloma virus (HPV) and genetic background (Vigneswaran & Williams, 2014). HNSCC develops as a consequence of deregulation of many genes (such as oncogenes, tumor suppressor genes, DNA repair genes) and loss of genome integrity. Different molecular subtypes were proposed for HNSCC (Gollin, 2014). Several cancers are known to have 'field effect' regions outside tumor border that harbor histological or molecular changes associated with cancer (Risk *et al.*, 2010). The aim of the study was to assess the expression of O^6-methylguanine-DNA methyltransferase (*MGMT*), Cyclin-Dependent Kinase Inhibitor 2A (*p16*) and Adenomatous Polyposis Coli (*APC*) genes in primary OSCC (Oral Squamous Cell Carcinoma) tumors and matching surgical margin samples. Alteration of the expression of these genes was found to be connected with cancerogenesis in various types of human cancer (Ohta *et al.*, 2009; Kordi-Tamandani *et al.*, 2010; Pérez-Sayáns *et al.*, 2012). *MGMT* gene is responsible for encoding enzyme involved in the repair of the DNA. It removes alkyl substituents located at the O^6 position in guanine (Kordi-Tamandani *et al.*, 2010). *p16* gene encodes two cell cycle regulatory proteins, p14ARF and p16INK4A. The latter inhibits cyclin D1 dependent phosphorylation of RB protein, thereby preventing cell cycle transition at the G1/S checkpoint (Ohta *et al.*, 2009). *APC* gene encodes tumor suppressor protein that acts as an antagonist of the Wnt signaling pathways. It is also involved in other processes including cell migration and adhesion, transcriptional activation, and apoptosis (Pérez-Sayáns *et al.*, 2012).

MATERIALS AND METHODS

The study group consisted of 56 patients with primary OSCC who underwent surgery at the Clinic of Oncological and Reconstructive Surgery, Maria Sklodowska-Curie Memorial Cancer Center and Institute of Oncology in Gliwice, Poland. Samples of tumors and matching surgical margins were collected. All tumors collected during the surgery were oral cavity cancers, including cases of the maxilla, mandible, floor of the mouth, tongue, and cheek cancers. Matching, morphologically normal-appearing oral mucosa biopsy specimens were obtained from regions located more than 10 mm from the tumor

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Abbreviations: OSCC, Oral Squamous Cell Carcinoma; HNSCC, Head and neck squamous cell carcinoma; MGMT, O^6-methylguanine-DNA methyltransferase; p16, Cyclin-Dependent Kinase Inhibitor 2A; APC, Adenomatous Polyposis Coli; GAPDH, Glyceraldehyde - 3-phosphate dehydrogenase; (qRT)–PCR, Quantitative Reverse Transcription Polymerase Chain Reaction; HPV, Human Papillomavirus; Ct: Threshold cycle; RQ, Relative Gene Expression levels; LOH, Loss of heterozygosity; SCC, Squamous Cell Carcinoma

Table 1. Clinical characteristics of patients

Clinical parameters	Patients n (%)
Sex	
Male	37 (66)
Female	19 (34)
Age (years)	
Range	29-77
Median	58.5
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)
Risk factor	
Smoking	45 (80)
Alcohol	41 (73)
Smoking and alcohol	36 (64)

lesion margins and microscopically verified by pathologists as normal. The Review Board on Medical Ethics of the Maria Sklodowska–Curie Memorial Cancer Center and Institute of Oncology in Gliwice approved the study (No KB/493-15/08 and KB/430-47/13). All patients signed and provided written informed consent. Clinical characteristics of the patients are shown in Table 1. None of the patients received chemotherapy or radiotherapy prior to the surgery, as the surgery was the primary treatment in all cases. Selective neck dissection was performed in patients at N0 stage, because the risk of occurrence of occult lymph nodes metastases oscillated around 30% in this group (Shah, 1999). The rationale for the selective neck dissection was based on known patterns of metastases occurrence from each site. Currently, selective neck dissection is oncologically safe. It is performed as a standard surgical procedure for patients at N1 stage and for clinically negative necks, because of the risk of micrometastasis occurrence in up to 30% (Schmitz *et al.*, 2009; Robbins *et al.*, 2013). Typically, unilateral or bilateral dissection of levels II, III, and IV was performed. In all cases of N+ and in advanced tumors III-IV microvascular free flaps were applied.

Homogenization, RNA extraction and complementary DNA (cDNA) synthesis. Immediately after surgery the specimens were submerged in tissue and RNA stabilization solution RNA*later* (Sigma-Aldrich, USA), and stored at –80°C. Before RNA extraction, samples were thawed slowly and then using Lysing Matrix D ceramic beads (MP Biomedicals, USA) homogenized in FastPrep®-24 homogenizer (MP Biomedicals, USA). RNA was isolated using the RNeasy Mini Kit (Qiagen, Germany). Additionally, digestion with DNase I was performed on the extracted RNA (RNase Free DNase Set, Qiagen, Germany) to remove the residual genomic DNA. RNA was quantified by measuring the UV absorbance at 260/280 nm (NanoDrop ND – 1000 Spectrophotometer, Thermo Fisher Scientific, USA) and the integrity was assessed by electrophoresis in 1.2% agarose gel stained with ethidium bromide (Serva, Germany). Additionally the RIN value (RNA Integrity Number) was derived with the Agilent Bioanalyzer 2100 (Agilent Technologies, USA) using Agilent RNA 6000 Nano Kit (Agilent Technologies, USA).

Total RNA from each tumor and matching surgical margin sample was reverse-transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, USA). 30 ng of total RNA was reverse transcribed into cDNA according to manufacturers' instructions. The reaction was performed in 20 µl volume containing: 2 µl of 10x Buffer RT; 0.8 µl of 25x dNTP mix (100 mM); 2 µl of 10x RT Random Primers; 1 µl of MultiScribe™ Reverse Transcriptase; 1 µl of RNase inhibitor; 3.2 µl of nuclease free H_2O and 10 µl of previously isolated RNA. The reaction was carried out in Mastercycler personal (Eppendorf, Germany) with the following thermal profile: 25°C for 10 min, 37°C for 120 min, 85°C for 5 min, 4°C – ∞.

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT–PCR). The glyceraldehyde – 3-phosphate dehydrogenase (*GAPDH*) gene was used as an internal control. qRT-PCR was performed using specific TaqMan® Gene Expression Assays (Applied Biosystems, USA): *MGMT* (Hs01037698_m1), *p16* (Hs00923894_m1), *APC* (Hs01568269_m1) and *GAPDH* (Hs99999905_m1) with 7300 Real-Time PCR System (Applied Biosystems, USA). The reaction was performed in 20 µl volume using 1 µl of cDNA, 10 µl of TaqMan® Gene Expression Master Mix (Applied Biosystems, USA), 1 µl of primers and probe mix (TaqMan Gene Expression Assays), and 8 µl of H_2O (Qiagen, Germany). Negative control was used in all reactions (DNase-, RNase-, Protease-free water, 5Prime, Germany). Thermal cycle for all analyzed genes was: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min. All assays were carried out in 96-well plates (Applied Biosystems, USA) and were analyzed by the SDS 1.4 software (Applied Biosystems, USA). The gene expression level of all investigated genes was normalized according to *GAPDH* level. The comparative threshold cycle (Ct) method $2^{-\Delta\Delta Ct}$ was used to determine the relative gene expression levels (RQ) for each target genes. Five samples of surgical margins were used as a calibrator. Relative mRNA expression in tumor and margin samples was determined using mRNA expression pattern from calibrator. Each sample, calibration and negative control were analyzed twice.

Statistical analysis. Statistical analysis was performed using STATISTICA 10.0 PL (StatSoft, Cracow, Poland). Statistical significance was set as p value below 0.05. All tests were two-tailed. Imputations were not done for missing data. Nominal and ordinal data were expressed as percentages, whilst interval data as mean value ± standard deviation. Distribution of variables was evaluated by the Shapiro-Wilk's test and homogeneity of variances was assessed by the Levene's test. The Student's t-test for independent samples was used for comparison of data between the two groups. For comparison between groups of different grade one-way analysis of variance (ANOVA) was used with Dunnett's post-hoc

Table 2. RQ values for *MGMT, p16,* and *APC* in tumor vs. surgical margin in patients with OSCC

Gene	Mean RQ±SD		*p*
	Tumor	Surgical Margin	
MGMT	0.86 ± 0.78	1.07 ± 0.78	0.157
p16	3.69 ± 9.96	1.35 ± 9.95	0.104
APC	0.91 ± 0.49	0.94 ± 0.49	0.790

test or, in the case of tumor and surgical margins analysis, two-way ANOVA was used with contrast analysis.

RESULTS

We found no statistically significant differences in the expression level of *MGMT*, *p16,* and *APC* genes in tumor samples compared to margin samples. The relative levels of the expression (RQ values) of *MGMT, p16,* and *APC* are shown in Table 2. We also assessed the correlation of the expression levels of selected genes with clinical characteristics of the patients. The expression of *p16* and *APC* genes was not correlated with any of the clinical or pathological parameters, whereas *MGMT* expression was inversely related to cigarette smoking. The results for *MGMT* expression tend to be statistically significant (Table 3).

Table 3. Comparison of the *MGMT* gene expression in the group of patients with OSCC in relation to smoking habits and alcohol consumption

Gene	Mean RQ±SD		*p*
	Tobacco +	Tobacco –	
MGMT	0.87 ± 0.68	1.34 ± 1.10	0.065
p16	1.78 ± 3.77	2.40 ± 4.53	0.509
APC	0.91 ± 0.50	1.01 ± 0.43	0.366
	Alcohol +	Alcohol –	
MGMT	0.91 ± 0.71	1.12 ± 0.99	0.205
p16	2.01 ± 4.14	1.60 ± 3.30	0.623
APC	0.89 ± 0.49	1.02 ± 0.48	0.230

DISCUSSION

MGMT

DNA repair gene (*MGMT*) acts as a regulator of genome integrity (Marini *et al*., 2006). Many authors indicated the role of *MGMT* in tumorigenesis (Corderio *et al*., 2012; Schena *et al*., 2012; McCormack *et al*., 2013). Kordi-Tamandani *et al*. (2010) observed significantly higher mRNA expression of *MGMT* in the group of patients with oral cavity cancer compared to healthy control group. On the other hand, no correlation was found in the study comparing the expression level of *MGMT* in solid tissues and blood of patients with diagnosed HNSCC (Schena *et al*., 2012). Similarly, our studies showed no correlation regarding *MGMT* expression between tumor and matching surgical margin tissue in patients with OSCC. Nevertheless, we observed down-regulated expression of *MGMT* gene in the group with smoking habits (Table 2 and 3). Similar results, where statistically significant relationship between smoking and the loss of MGMT protein expression, were shown in oral squamous cell carcinomas by Rodriquez *et al*. (2007). Such results were also obtained by Jacob *et al*. (2010) whose data showed that in non-cancer control group MGMT activity in the pharyngeal mucosa was down-regulated in smokers comparing to non-smokers. It can indicate that smoking depletes MGMT activity (Jacob *et al*., 2010). Data regarding impact of smoking on *MGMT* expression are not consistent. There is some evidence that agents found in tobacco can induce increased MGMT protein expression or activity in tumor and normal tissues (Christmann & Kaina, 2012). As it was mentioned above, according to the results of our research, *MGMT* expression appears to decrease as a result of smoking. It could be related to the mutation of this gene; therefore, our results may reflect a mechanism associated with gene mutation which occur more frequently after tobacco exposure. Because *MGMT* is one of DNA-repair genes its decreased repair ability in cancer cells could be advantageous for proper response to chemotherapy (Schena *et al*., 2012; Cordeiro *et al*., 2012). Another theory is involves methylation of the promoter region of this gene as a mechanism responsible for its lower expression. Smoking is suggested to be one of the inducing factors of *MGMT* low transcription on the epigenetic level. A significantly increased frequency of tumors with methylated *MGMT* promoter in smokers comparing to non-smokers in different types of cancers was reported (Ushijima, 2007; Christmann & Kaina, 2012; Cordeiro *et al*., 2012). According to available data there is a link between *MGMT* polymorphisms and the risk of cancer resulting from smoking. In humans, several polymorphisms of *MGMT* were described. Furthermore, its regulation by different transcription factors was also reported (Christmann & Kaina, 2012).

p16^{INK4A}

In head and neck cancer *p16* also known as *INK4A* is often inactivated either by homozygous deletion, promoter methylation or point mutations (Miracco *et al*. 1999; Perez-Ordoñez *et al*., 2006; Lee *et al*. 2011). Moreover, inactivation of this gene is described to be second, after p53 inactivation, most frequent incident in different types of human cancer (Sailasree *et al*., 2008). Demokan *et al*. (2011) observed that *p16^{INK4A}* gene expression was down-regulated in tumor samples compared to adjacent normal tissue in head and neck cancer. Other researchers observed a decrease or lack of *p16* mRNA and protein expression in oral squamous cell carcinoma lines and in primary tumor tissues of HNSCC (Reed *et al*., 1996; Yakushji *et al*., 2001; Ai *et al*., 2003). Interestingly, high *p16* expression was correlated with better outcome and lower recurrence rate in patients with oropharyngeal squamous cell carcinoma (Huber *et al*., 2011). High p16 protein expression was also reported to be a predictor of better response to chemoradiotherapy in patients with stage IVa/b of head and neck squamous cell carcinoma (Chen *et al*., 2011). We did not observe any statistically significant correlation regarding expression of this gene, but we found that it was higher in tumors compared to margins (3.69 *vs*. 1.35; Table 2). These findings could be associated with HPV infection, as this particular group of patients also showed a high risk of developing HPV-related HNSCC (Majchrzak *et al*., 2014). However, it remains speculative as we were not provided with any information on patients' HPV status. We are currently in the process of investigating on this matter. Moreover, some studies of oropharyngeal carcinoma reported corre-

lation between *p16* protein overexpression in tumor cells and HPV viral detection; what is more, *p16* presence was proposed as a reliable prognostic marker of HPV infection status in HNSCC (El-Naggar *et al*., 2002; Majchrzak *et al*., 2014).

APC

APC may be subjected to certain types of mutations, loss of heterozygosity (LOH) or alterations of epigenetic mechanisms. All of them may result in reduction of the expression of this gene and blockage of tumor suppressant action of *APC,* therefore, contributing to progress of Oral Squamous Cell Carcinoma (OSCC) (Pérez-Sayáns *et al*., 2012). Uesugi *et al*. (2005) found significant down-regulation of *APC* expression in OSCC patients. The same gene was also the most frequently hypermethylated gene in HNSCC cell lines (Worsham *et al*., 2006). We found no differences regarding this gene expression in our study group.

The presented results showing lack of statistically significant differences between tumor and matching margin expression levels may be difficult to interpret. To make a potent conclusion more studies are required. One of the ideas led us to the hypothesis of 'field cancerization'. Exposure of epithelial tissue to carcinogenic substances is connected with the idea of 'field cancerization' by Slaughter *et al*. (1953). Braakhuis *et al*. (2003) defined the field as an area with genetically altered cells and a risk factor for cancer. Nowadays the concept of field effect includes many, not only genetic but also epigenetic, disturbances (Shen *et al*., 2005; Ushijima, 2007). One of the elements triggering field cancerization effect could be connected with disturbances in gene expression (Angadi *et al*., 2012). Primary tumors are thought to be the consequence of 'field cancerization' (Argiris *et al*., 2004). Interestingly, Szukała *et al*. (2004) examined larynx cancer positive patients and found LOH on chromosomal regions 13q14 and 13q34 not only in tumor tissues but also in safe margins, with the frequency range 12-31%. Surprisingly, they observed LOH in distant tumor-free regions, too.

In our analysis patients were divided into two groups. The first consisted of individuals with smoking habit (80%) whereas in the second subjects who never used tobacco were included. Most of smoking patients smoked in the past and still smoked more than one pack of cigarettes a day (>20 cigarettes/day) at the moment of conducting the study. The survey also included alcohol consumption and, according to patients' answers, 73% drank alcohol. Occasional alcohol consumption was declared by 69% of the study population and 4% declared abusing the substance (high-frequency alcohol consumption). Moreover, 64% of patients declared both tobacco use and alcohol consumption. It is well known that cigarette smoking and alcohol drinking are main risk factors for this group of patients (Guha *et al*., 2007). Hashibe *et al*. (2009) corroborated that joint action of tobacco and alcohol use had increased effect on head and neck cancer risk, particularly oral cancer. Future perspectives to diminish clinical implications of oral field cancerization aim at more precise surgery, chemotherapy, radiotherapy and new tools of molecular genetics (Angadi *et al*., 2012). Many molecular markers with the potential to predict the development of local relapse in HNSCC were described but their clinical usefulness in routine diagnostics has not yet been assessed (Braakhuis *et al*., 2010). In conclusion, it is important to mention that the field remaining after a surgery may pose an increased risk of cancer development. It may be suggested that the diagnosis and treatment of cancers should not be concentrated only on the tumor itself, but also on the cancer field effect. Therefore, further molecular analysis on surgical margins and additional research regarding their assessment are required.

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Competing interests

The authors declare that they have no competing interests.

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