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 O6-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 (Ohla et al., 2009; Kordi-Taman-dani 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 O6 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 (Ohla 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...
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 H2O 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 H2O (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^-ΔΔCt 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 test.
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).

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 Rodriguez 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 compared 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 chemotheraphy (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).

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mean RQ±SD</th>
<th>Tumor</th>
<th>Surgical Margin</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGMT</td>
<td>0.86 ± 0.78</td>
<td>1.07 ± 0.78</td>
<td>0.157</td>
<td></td>
</tr>
<tr>
<td>p16</td>
<td>3.69 ± 9.96</td>
<td>1.35 ± 9.95</td>
<td>0.104</td>
<td></td>
</tr>
<tr>
<td>APC</td>
<td>0.91 ± 0.49</td>
<td>0.94 ± 0.49</td>
<td>0.790</td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mean RQ±SD</th>
<th>Tobacco +</th>
<th>Tobacco -</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGMT</td>
<td>0.87 ± 0.68</td>
<td>1.34 ± 1.10</td>
<td>0.065</td>
<td></td>
</tr>
<tr>
<td>p16</td>
<td>1.78 ± 3.77</td>
<td>2.40 ± 4.53</td>
<td>0.509</td>
<td></td>
</tr>
<tr>
<td>APC</td>
<td>0.91 ± 0.50</td>
<td>1.01 ± 0.43</td>
<td>0.366</td>
<td></td>
</tr>
</tbody>
</table>

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-Ordóñez 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 p16⁴⁰⁸⁴⁸⁴⁴ 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 (Redd 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 blockade of tumor suppressor 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, Szukala 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.

REFERENCES


