ACTA ABP BIOCHIMICA POLONICA

Vol. 65, No 1/2018
87–92
https://doi.org/10.18388/abp.2017_1613

Regular paper

The role of *MGMT* polymorphisms rs12917 and rs11016879 in head and neck cancer risk and prognosis

Paweł Kiczmer[1], Alicja Prawdzic Seńkowska[1], Joanna Katarzyna Strzelczyk[1], Błażej Szydło[1], Krzysztof Biernacki[1], Tadeusz Osadnik[2,3], Łukasz Krakowczyk[4] and Zofia Ostrowska[1]

[1]Department of Medical and Molecular Biology, School of Medicine with the Division of Dentistry, Medical University of Silesia in Zabrze, Katowice, Poland; [2]2nd Department of Cardiology and Angiology, Silesian Center for Heart Disease, Zabrze, Poland; [3]Genomics Laboratory, Kardio-Med Silesia Science and Technology Park, Zabrze, Poland; [4]Department of Oncological and Reconstructive Surgery, Maria Sklodowska-Curie Memorial Cancer Center and Institute of Oncology, Gliwice Branch, Gliwice, Poland

Head and neck squamous cell carcinoma (HNSCC) is one of the leading cancers by incidence worldwide. The risk of these cancers is strictly associated with alkylation factors present in tobacco smoke. The crucial role in preventing DNA alkylation is played by O^6-methylguanine-DNA methyltransferase (MGMT). Dysfunction or lack of MGMT is associated with an increased risk of cancer. The aim of the study was to assess the influence of *MGMT* polymorphisms: rs12917 and rs11016879 on HNSCC risk and course. The study consisted of 69 HNSCC patients and 242 healthy individuals. Case samples were taken from resected tumour tissue. The control group comprised samples of epithelial cells collected from mucous membranes using swabs. DNA samples were genotyped by employing the 5′ nuclease assay for allelic discrimination using TaqMan SNP Genotyping Assays. The significance between distributions of genotypes and alleles was tested using Pearson's χ^2 test analysis. Our results indicated that the *MGMT* rs12917 TT genotype increases the risk of HNSCC. The *MGMT* rs11016879 AG genotype and A allele were associated with increased HNSCC risk. We noted higher risk of nodal metastasis in rs11016879 AA homozygotes. Mechanisms leading to MGMT enzymatic defect are unknown and hence further studies need to be carried out. Our data suggest that the examined polymorphisms may be considered as potential prognostic factors for HNSCC risk and outcome. Further studies are necessary to verify our results.

Key words: *MGMT,* head and neck cancer, polymorphism, rs12917, rs11016879

Received: 17 May, 2017; revised: 31 October, 2017; accepted: 18 December, 2017; available on-line: 25 January, 2018

✉e-mail: PKiczmer@wp.eu

Abbreviations: HNSCC, head and neck squamous cell carcinoma; MGMT, O6-methylguanine-DNA methyltransferase; HPV, human papilloma virus; SNP, single nucleotide polymorphism

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) has received limited attention in recent years. Almost 540,000 new cases of HNSCC occur worldwide each year. The WHO predicts an increase of approximately 16% in the number of HNSCC cases by the year 2020. The risk of contracting this disease in both sexes is the highest after the age of 45, and its incidence is 2- to 5-fold higher in men (Majchrzak *et al.,* 2014).

HNSCC treatment is determined based on tumour stage characterization using the TNM scale. For tumours in I–II stage (T1/2, N0), treatment consists of radiotherapy or surgery (Krzakowski & Warzocha, 2013). The outcome at early stages varies, and a cure rate between 60% and 90% is observed. More advanced disease (T3/4 and/or N1-3) requires chemoradiotherapy or operation and radiotherapy and the outcome is poor, estimated as a 30% cure rate (Kordek *et al.,* 2013).

Throughout their lives, humans are exposed to chemical substances, physical interactions or biological factors which may be classified as mutagens leading to carcinogenesis. There is a common belief that most HNSCC cases are caused by tobacco smoking and alcohol consumption. Cigarette smokers are 5- to 25-fold more prone to develop HNSCC compared to non-smokers (Marur & Forastiere, 2008). Alcohol may influence the development of HNSCC as an independent factor as well as synergistically with tobacco use. On the other hand, an increased incidence of HNSCC in non-smokers and non-drinkers under 45 years of age has been observed. This phenomenon is attributed to human papillomavirus (HPV) (Majchrzak *et al.,* 2014). Oncogenic HPV DNA has been observed in approximately 22% of oropharyngeal cancers (Ndiaye *et al.,* 2014).

Apart from the environmental factors, we must consider molecular and genetic aspects of development of HNSCC. Human cells have evolved a large number of mechanisms to protect themselves from mutagenic agents, one example being the functioning of O^6-methylguanine-DNA methyltransferase (MGMT). This enzyme prevents DNA alkylation by removing a methyl group from the O-6 position in guanine and transferring it to its own cysteine. Dysfunction or lack of MGMT is associated with an increased risk of contracting diseases including cancer (Shawney *et al.,* 2012). The enzymatic activity may vary according to polymorphisms in its gene. The major *MGMT* polymorphism type is the single nucleotide polymorphism (SNP). In this study, we focused on two polymorphisms: rs12917 and rs11016879. rs12917 genotype involves conversion from cytosine to thymine. A Leu to Phe change at position 84 in the MGMT protein alters the enzyme substrate affinity and thus has been associated with a higher risk of cancer (Molina *et al.,* 2013). The second polymorphism, rs11016879, is a conversion from adenine to guanine which is a synonymous substitution. Despite the enzyme structure

not changing, synonymous SNPs may affect mRNA structure leading to allele-specific biological consequences (Shen *et al.,* 1999). Some studies suggest that the synonymous substitution of one nucleotide may alter mRNA folding and decrease half-life resulting in downregulation of protein synthesis (Czech *et al.,* 2010). On the other hand, it is suggested that replacement of certain synonymous codons could lead to the alteration of ribosome traffic on the mRNA at a selected position. Decreased protein synthesis may also be caused by asymmetric tRNA abundance leading to a delay in the rate of translation (Komar *et al.,* 1999).

MATERIALS AND METHODS

Study population. This study was approved by the Institutional Review Board on Medical Ethics of the Maria Sklodowska–Curie Memorial Cancer Centre and Institute of Oncology in Gliwice (No KB/493-15/08 and KB/430-47/13). We obtained written informed consent from all patients. We studied 69 patients with a primary tumour in the location of the oral cavity. Tissue samples were collected during surgical operation from patients with a previously untreated squamous cell carcinoma at the Department of Oncological and Reconstructive Surgery, Maria Sklodowska–Curie Memorial Cancer Centre and Institute of Oncology, Gliwice, Poland. There were 49 men and 20 women, with a mean age of 56.07±9.81 years (range: 29–73). Tumour staging was determined in accordance with TNM classification (Krzakowski & Warzocha 2013). Nine of the patients (13.04%) were in T1 stage, 14 (20.29%) were in T2 stage, 16 (23.19%) were in T3 stage and 30 (43.47%) were in T4 stage. Moreover, 30 patients (43.47%) were in N0 stage, 18 (26.09%) were in N1 stage, 21 (30.43%) were in N2 stage. 12 subjects (17.39%) had G1, 49 (68.12%) had G2, and 7 had G3 (8.70%). None of the patients had received preoperative radio- or chemotherapy. The 5 year-survival rate was 59.09% among >II TNM stage patients and 80% among patients in TNM stages I and II.

In the study group, 54 (78.26%) subjects were smokers (current), 43 (62.32%) reported alcohol intake. 38 (55.07%) subjects reported both smoking and drinking and 23 (33.33%) had previously had a cancer episode among first degree relatives.

The control group consisted of 242 (134 women and 108 men) healthy individuals. The mean age was 34.68 ± 12.6 years (range 18–87). 72 (29.75%) of them were smoking and 64 (26.44%) reported alcohol intake. Epithelial cells were taken using swabs from the oral mucosa.

The Review Board on Medical Ethics of the Medical University of Silesia approved the study protocol (No KNW/0022/KB1/49/16). Informed consent was obtained from all participants.

DNA extraction. Genomic DNA was extracted from each sample of tumour tissues (20 mg) using the DNeasy Blood & Tissue Kit (Qiagen, USA) according to manufacturer's instructions, following homogenization of tissues using the FastPrep®-24 instrument and Lysing Matrix A tubes (MP Biomedicals, USA).

We used the GeneMATRIX Swab-Extract DNA Purification Kit (EURx, Poland) to extract DNA from the control group individuals. Thereafter, the DNA was eluted in low salt buffer that contained 10 mM Tris-HCl, pH 8.5.

Qualitative and quantitative analysis of the isolated DNA was performed by spectrophotometry. We used the NanoPhotometer™Pearl Spectrophotometer (Implen, Germany) to measure absorbance.

Genotyping. DNA samples were genotyped by employing the 5' nuclease assay for allelic discrimination using TaqMan® SNP Genotyping Assays. Purified DNA in a final concentration of 5 ng/μl was added to a MicroAmp™ Optical 96-Well Reaction Plate (Applied Biosystems, USA). Each assay contained 11.25 μl DNA; 12.5 μl TaqMan® Genotyping Master Mix (Applied Biosystems, USA) and 1.25 μl TaqMan® Genotyping Assays (Applied Biosystems, USA). The genotyping was conducted in Cobas z480® Analyzer (Roche, USA). In 10% of the samples we repeated the procedure to verify the results.

In samples which were identified as homozygous and heterozygous (six samples representing each genotype) additionally we performed Sanger sequencing to confirm the genotype detected by TaqMan® SNP Genotyping Assays.

DNA sequencing. The sequence analysis of the selected PCR amplified fragments of the *MGMT* gene was carried out using ABI PRISM® BigDye® Terminator v3.1 CycleSequencing Kit (Thermo Fisher, USA). The cycle sequencing was performed in SimpliAmp™ ThermalCycler (Thermo Fisher, USA). The separation of the PCR amplified fragments was conducted in 3130 Genetic Analyzer Applied Biosystems (Thermo Fisher, USA). The obtained data were managed in Applied Biosystems Sequencing Analysis Software (Thermo Fisher, USA) and further analysed in Thermo Fisher Applied Biosystems Variant Analysis (VA) Module. The analysis of *MGMT* gene fragment sequences in some of previously chosen samples confirmed the existence of the SNPs in the examined fragments.

Statistical analysis. The significance between distributions of genotypes and alleles was tested using Pearson's χ^2 test analysis. We used non-parametric ANOVA analysis to examine the association of *MGMT* polymorphisms with grading, T, and N parameters of the TNM classification. Logistic regression modelling was performed to analyse the 5-year survival including examined SNPs, age, grading, sex, tobacco, alcohol consumption and cancer episodes in family, regarding them as independent and coexisting factors. P values <0.05 were considered as statistically significant. The statistical software STATISTICA 12 for Windows (STATSOFT, USA) was used to perform all analyses.

RESULTS

Allele and genotype distributions

The *MGMT* rs12917 TT genotype was observed significantly more frequently in the HNSCC patients (OR= 6.17; 95% CI 1.98-19.26) (Table 1). Rs12917 was not correlated with the risk of nodal infiltration (Table 2). We observed no association between rs12917 and tumour staging.

We found a significant association between the *MGMT* rs11016879 A allele and HNSCC risk (OR=2.00; 95% CI 1.05–3.83). Moreover, rs11016879 AG genotype significantly influenced HNSCC risk (OR=2.07; 95% CI 1.04–4.13) (Table 3). We noted that the rs11016879 AA genotype was more common

Table 1. *MGMT* rs12917 polymorphism genotype frequency among groups

MGMT rs12917	Cases (n=69)		Controls (n=239)			
Genotypes	n	%	n	%	*P* value	OR (95% CI)
CC	49	71.01	168	70.29	–	1.00 (Reference)
CT	11	15.94	66	27.62	0.12	0.57 (0.28–1.17)
TT	9	13.04	5	2.09	0.0005	6.17 (1.98-19.26)
CT + TT	20	28.99	71	29.71	0.91	0.97 (0.54-1.74)

Table 2. Association between *MGMT* rs12917 polymorphism genotypes and risk of nodal metastases

MGMT rs12917	Nodes not involved (n=30)		Nodes involved (n=39)			
Genotypes	n	%	n	%	*P* value	OR (95% CI)
CC	22	73.33	27	69.23	–	1.00 (Reference)
CT	4	13.33	7	17.95	0.61	1.43 (0.37-5.51)
TT	4	13.33	5	12.82	0.97	1.02 (0.24-4.26)
CT + TT	8	26.67	12	30.77	0.71	1.23 (0.42-3.52)

Table 3. *MGMT* rs11016879 polymorphism genotype frequency among groups

MGMT rs11016879	Cases (n=66)		Controls (n=234)			
Genotypes	n	%	n	%	*P* value	OR (95% CI)
GG	14	21.21	82	35.04	–	1.00(Reference)
AG	34	51.52	96	41.03	0.04	2.07 (1.04-4.13)
AA	18	27.27	56	23.93	0.11	1.88 (0.87-4.09)
AA + AG	52	78.79	152	64.96	0.03	2.00 (1.05-3.83)

in patients with nodal metastases (Table 4); this association was statistically significant (OR=4.67; 95% CI 1.01–21.65). Additionally, patients with rs11016879 A allele had significantly higher N parameter value (Fig. 1). We observed no association between rs11016879 and tumour staging.

No association between the coexistence of rs12917 and rs11016879 polymorphisms and HNSCC risk was observed. Neither *MGMT* rs12917 nor rs11016879 demonstrated an association with the T parameter and grading.

Due to the small number of I–II tumour stage cases, survival analysis was performed only for >II stage patients. We observed no influence of rs12917 or rs11016879 on the 5-year survival rate. A test for interaction between the examined SNPs, age, grading, sex, tobacco, alcohol consumption and cancer episodes in family, regarding them as independent and coexisting factors, using the logistic regression model for survival rate, was not significant.

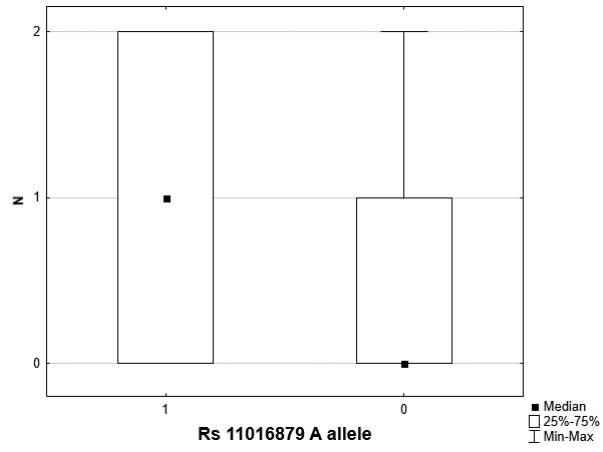

Figure 1. Association of the rs11016879 A allele genotype with the N parameter (p=0.048, Mann-Whitney U test)

DISCUSSION

Head and neck cancers are an important clinical issue. These lesions are difficult to cure, especially because of their location. Surgical operations are limited by the complexity of important anatomical structures in the regions of the cancer. Furthermore, proximity to the central nervous system and eyes limits radiotherapy (Bartkowski 1996; Ziółkowska 2011).

MGMT rs12917 T allele is commonly associated with an overall increased risk of cancer. It has also been suggested that the T allele may increase the risk of non-small cellular lung cancer (Du *et al.,* 2013). However, its role in HNSCC remains unclear; Zhang *et al.,* found no correlation between rs12917 and HNSCC risk (Zhang *et al.,* 2010). In our study *MGMT* rs12917 polymorphism may be considered as a risk

Table 4. Association between *MGMT* rs11016879 polymorphism genotypes and risk of nodal metastases

MGMT rs11016879	Nodes not involved (n=26)		Nodes involved (n=40)			
Genotypes	n	%	n	%	*P* value	OR (95% CI)
GG	8	30.77	6	15.00	–	1.00 (Reference)
AG	14	53.85	20	50.00	0.31	1.90 (0.54-6.71)
AA	4	15.38	14	35.00	0.04	4.67 (1.01-21.65)
AA+AG	18	69.23	34	85.00	0.13	2.52 (0.76-8.39)

factor for HNSCC. We observed a significant association between rs12917 TT genotype and risk of HNSCC. In contrast, a different study has demonstrated that the T allele may decrease head and neck cancer risk (Hall *et al.,* 2007). Moreover, the same researchers have suggested a protective role of another interesting polymorphism, rs2308321, whereas most other studies have determined a negative influence of this SNP. It is commonly known that rs12917 is associated with an increased risk of lung cancer and may be responsible for the increased risk of oesophageal cancer (Doecke *et al.,* 2008; Du L *et al.,* 2013; Qui *et al.,* 2014).

There is only one publication describing the association between the rs11016879 polymorphism and cancer risk, specifically oesophageal squamous cell carcinoma. The study demonstrated that increased oesophageal cancer risk is related to the rs11016879 A allele (Ma *et al.,* 2010). Other studies suggest no association between this SNP and renal cancer risk (Lee Moore *et al.,* 2009). According to our knowledge, our study is the first to describe the influence of rs11016879 on HNSCC risk. We found a significant association between AG genotype and A allele and HNSCC risk. Moreover, this is the first study examining the influence of the rs11016879 genotype on the risk of nodal metastases. Our study provides information about the association between the AA genotype of this polymorphism and increased risk of nodal metastases. Involved lymph nodes are a prognostic factor for HNSCC recurrence (Burusapat *et al.,* 2015; Leemans *et al.,* 1994). Patients with nodal metastases require adjuvant treatment. Analysis of nodal metastases risk factors may improve the survival rate. The *MGMT* rs11016879 genotype may be considered as a nodal infiltration risk factor. Further studies are needed to determine the clinical utility of this SNP as an independent risk factor of nodal metastases. Our results demonstrated for the first time that rs11016879 may be associated with a poor prognosis of head and neck cancers, however, we observed no influence of rs11016879 on the 5-year survival rate. These results are compatible with those from the study by Wibom and coworkers which suggest no association between this SNP and glioblastoma survival (Wibom *et al.*, 2012).

Rs12917 and rs2308321 *MGMT* polymorphisms are situated near the enzyme active site, thus affecting molecular activity (Egyhazi *et al.,* 2002; Sharma *et al.,* 2009). Some researchers suggest that rs12917 may influence Zn^{2+} binding ability leading to changes in MGMT enzymatic activity (Sharma *et al.,* 2009). Some SNPs, such as rs11016879, are silent mutations that do not change the amino acid sequence. However, this may affect MGMT activity. Dysfunction of MGMT enzyme may lead to carcinogenesis. MGMT activity may be affected by the methylation of its promoter, thereby lowering its expression (Sharma *et al.,* 2009). Smoking is suggested to be one of the inducing factors of low transcription of *MGMT* at the epigenetic level (Strzelczyk *et al.,* 2016). Activity of this enzyme may vary according to its polymorphisms (SNPs). Moreover, some polymorphisms influence the expression of *MGMT* by increasing promoter methylation (Fogli *et al.,* 2016). Sawnhey *et al.,* observed that increased risk of nodal infiltration is associated with *MGMT* methylation in oral cancer (Sawhney *et al.,* 2012). Additionally, Yu and coworkers demonstrated that *MGMT* methylation increases the risk of nodal metastases in gastric cancer (Yu *et al.,* 2016).

Nonetheless, lowered MGMT activity may have a positive influence on cytostatic treatment. This is because of the mechanism of action of alkylating drugs that damage DNA leading to cell death. Previous studies have reported a higher survival rate in glioblastoma patients with lower *MGMT* expression caused by methylation during temozolomide treatment (Rapkins *et al.,* 2015). However, some studies suggest that *MGMT* hypermethylation has no influence on the chemotherapy response in laryngeal cancer (Onerci Celebi *et al.,* 2016). Other researchers reported that MGMT activity does not act as a resistance factor for radiochemotherapy (Jacob *et al.,* 2010).

Modifiable environmental factors, such as alcohol or smoking, strongly affect the HNSCC risk (Hashibe *et al.,* 2009; Sharma *et al.,* 2009). It is worth mentioning that tobacco and alcohol jointly have a greater than multiplicative effect on HNSCC risk (Maier *et al.,* 1992). Tobacco smoke contains large amounts of harmful substances, the individual effects of which are difficult to assess at the molecular level because this complex mixture of carcinogens, tumour growth promoters, and cocarcinogens manifests its effects upon chronic exposure (Pfeifer *et al.,* 2002). Alkylating factors are an example of cancerogenous agents present in tobacco smoke. These substances damage DNA through the addition of alkyl groups (Jacob *et al.,* 2010). Humans have developed several mechanisms for protection against alkylating factors. The primary role in this defence is played by MGMT (Shawney *et al.,* 2012).

In our study, we observed no significant interactions between modifiable environmental factors, cancer episodes in family, staging, grading and the examined SNPs for the 5-year survival in the logistic regression model. It should be emphasized that we were the first to compare these parameters with rs12917 and rs11016879. However, our study was limited by small sample size.

In conclusion, *MGMT* rs12917 polymorphism may be associated with increased risk of HNSCC. However, this SNP does not affect prognosis. On the

other hand, the *MGMT* rs11016879 polymorphism is both related to cancer risk and may affect prognosis. To determine the clinical consequences of examined SNPs on HNSCC risk and prognosis and to confirm our results, further studies, comprising greater sample sizes, are needed to be performed. Mechanisms leading to MGMT enzymatic defect remain to be clarified. We believe, that analysis of *MGMT* gene polymorphism may provide novel insights for cancer prediction in the near future.

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