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Selected *CDKN2A* and *MDM2* polymorphisms in oral cavity cancer

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The head and neck squamous cell carcinoma (HNSCC) is an aggressive human malignancy diagnosed in more than 800 000 new cases worldwide, and mostly arises in the oral cavity, oropharynx, larynx, hypopharynx, and nasopharynx. The study presented here aimed to determine a possible association of rs11515 and rs3088440 gene polymorphisms in the *CDKN2A* gene (cyclin dependent kinase inhibitor 2A), as well as rs769412 and rs937283 in the *MDM2* gene (murine double minute 2), with oral cavity cancer in a sample of Polish population; *CDKN2A* is crucial in regulating the cell cycle while *MDM2* is an oncogene involved in regulating cell proliferation and apoptosis. The study included 95 primary tumor samples following surgical resection from patients, and the control group consisted of 100 healthy individuals. DNA samples were genotyped by employing the 5′ nuclease assay for allelic discrimination using TaqMan SNP Genotyping Assays (Applied Biosystems, USA). There was no significant association between any of the polymorphism (rs11515, rs3088440, rs769412 and rs937283) and the oral cavity cancer risk. We found that the AA homozygotes for rs3088440 were significantly more frequent in the control group (OR=0.046, *p*<0.0001). In addition, the GG genotype of rs769412 was not found in any group. We found no influence of the examined genotypes on clinicopathological parameters, such as T, N and grading values in patients with oral cavity cancer. The results of this study indicate that none of the investigated polymorphisms were associated with the risk of oral cavity cancer in the examined sample of the Polish population.

Key words: head and neck squamous cell carcinoma, oral cavity cancer, *MDM2*, *p16*, single nucleotide polymorphism, cancer risk

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Abbreviations: HNSCC, head and neck squamous cell carcinoma; OSCC, oral squamous cell carcinoma; HPV, human papillomavirus; SNP, single-nucleotide polymorphisms; CDKN2A, cyclin dependent kinase inhibitor 2A; MDM2, murine double minute 2

INTRODUCTION

The head and neck squamous cell carcinoma (HNSCC) is an aggressive human malignancy diagnosed in more than 800 000 new cases worldwide (Bray *et al.*, 2018; Canning *et al.*, 2019). Due to high morbidity approximately 50% of patients die annually (Leemans *et al.*, 2018). The most common sites for the arising of HNSCC are the oral cavity, oropharynx, larynx, hypopharynx, and nasopharynx (Argiris *et al.*, 2008; Marur & Forastiere, 2016), however the most prevalent subsite is the oral cavity and thus the oral squamous cell carcinoma (OSCC) accounts for about 3% of all cancers worldwide (Yanamoto *et al.*, 2012; Weckx *et al.*, 2019). The list of risk factors associated with HNSCC includes alcohol and tobacco use, diet, infection with high-risk types of human papillomavirus (HPV), geographical location, habits, as well as the genetic background (Vigneswaran & Williams, 2014). Dysregulation of numerous genes, such as the tumor suppressor genes, oncogenes, and DNA repair genes, as well as loss of the genome integrity, are widely accepted causes of HNSCC (Gollin, 2014). Single-nucleotide polymorphisms (SNPs) are reported to be important factors in oral cancer susceptibility, and SNPs which are involved in DNA repair, cell cycle control, and carcinogen metabolism may especially be the pivotal factors for this malignancy (Shridhar *et al.*, 2016).

CDKN2A (cyclin dependent kinase inhibitor 2A) is a tumor suppressor gene involved in cell cycle regulation, and it is commonly mutated in HPV-negative patients with the head and neck cancer (Ausoni *et al.*, 2016). *CDKN2A* is located on chromosome 9 and encodes two products, p14-ARF and p16-INK4A (Gallagher *et al.*, 2005). p14-ARF interacts with MDM2 (Murine double minute 2) and as a result leads to ubiquitination of p53 (Gallagher *et al.*, 2005; Ausoni *et al.*, 2016), while p16-INK4A blocks phosphorylation of pRB and negatively regulates the cell cycle by arresting cells in the late G1 phase (Ausoni *et al.*, 2016).

Another important gene in carcinogenesis is the *MDM2* oncogene, a negative regulator of p53. *MDM2* takes part in regulation of cell proliferation and apoptosis through inhibition of the p53 activity (Denaro *et al.*, 2011), which is performed in the ubiquitination pathway (Yang *et al.*, 2013). Also, *MDM2* is a negative regulator of many other molecules, such as pRb, P21, p14 or ribosomal proteins (Rayburn *et al.*, 2005). Up-regulation of *MDM2* has been reported in various cancers, and may influence a decreased cellular response to radio- or chemo-therapy (Rayburn *et al.*, 2005). *MDM2* polymorphisms may be located in the promoter region, such as rs937283, and in consequence influence the transcription level of that gene (Feng *et al.*, 2016). The purpose of the study presented here was to determine a possible association of rs11515 and rs3088440 polymorphisms in the *p16* gene, as well as rs769412 and

Table 1. Types of polymorphism and primers used

SNP ID	Codon change	Amino acid change	Context Sequence [VIC/FAM]
rs11515	–	–	TCGGTGACTGATGATCTAAGTTTCC[C/G]GAGGTTTCTCAGAGCCTCTCTGGTT
rs3088440	–	–	GGTGGGTTGTGGCGGGGGCAGTTGT[A/G]GCCCTGTAGGACCTTCGGTGACTGA
rs769412	GAA, GAG	E,E	AACTGGAAAACTCAACACAAGCTGA[A/G]GAGGGCTTTGATGTTCCTGATTGTA
rs937283	–	–	GTGCCCTGGCCCGGAGAGTGGAATG[A/G]TCCCCGAGGCCCAGGGCGTCGTGCT

rs937283 in the *MDM2* gene, with HNSCC in a sample of the Polish population.

MATERIALS AND METHODS

Patients and samples. The study presented here included 95 primary tumor samples obtained from Polish patients following surgical resection at the Department of Otorhinolaryngology and Oncological Laryngology, Faculty of Medical Sciences in Zabrze, Medical University of Silesia, Maria Sklodowska–Curie Memorial Cancer Centre and Institute of Oncology (Gliwice, Poland). All of 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). All samples were quickly frozen at –80°C until DNA extraction. These tumor samples were histologically confirmed by pathologists and were classified as primary tumors. Tumor staging was based on the American Joint Committee on Cancer (AJCC, version 2007) (Brunner *et al*., 2014; Rodrigues *et al*., 2014). Patients included in this study had no history of preoperative radio- or chemo-therapy. The control group consisted of 100 healthy individuals. All of the patients and controls were Caucasians who lived in the area of Poland. The study was approved by the Ethics Committee of the Medical University of Silesia (Katowice, Poland; approval no. KNW/022/KB1/49/16), and the Institutional Review Board on Medical Ethics of the Maria Sklodowska–Curie Memorial Cancer Centre and Institute of Oncology in Gliwice (Poland; approval no. KB/493-15/08 and KB/430-47/13).

DNA extraction. Genomic DNA was extracted from each tumor sample (20 mg) by DNeasy Blood & Tissue Kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions, after tissue homogenization in a FastPrep®-24 instrument using Lysing Matrix A tubes (MP Biomedicals, Solon, CA, USA). In the control group, the DNA was extracted from swabs taken from oral mucous membranes using a Swab-Extract DNA Purification Kit (EURx, Gdansk, Poland) according to the manufacturer's instructions. After that, high-quality cellular DNA was eluted in low salt buffer containing 10 mM Tris-HCl, pH 8.5. Qualitative and quantitative analysis of all isolated DNA was performed by spectrophotometry in a Biochrom WPA Biowave DNA UV/Vis Spectrophotometer (Biochrom, Cambridge, UK) according to the manufacturer's instructions.

Single nucleotide polymorphism (SNP) analyses. Genotyping was conducted with a QuantStudio 5 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The reaction solution contained 5 μg DNA, 12.5 μl TaqMan Genotyping Master Mix (Applied Biosystems, , Foster City, CA, USA), and 1.25 μl TaqMan Genotyping Assay (Applied Biosystems, Foster City, CA, USA). The types of polymorphisms and the primers used are displayed in Table 1. SNP calling was read out automatically in QuantStudio Design and Analysis Software v1.5.1 (Applied Biosystems, Foster City, CA, USA).

Statistical analysis. The significance between distributions of genotypes and alleles, T, N and grading were tested using Pearson's χ^2 test. Logistic regression modelling was performed to analyse the oral cavity cancer risk, including the examined SNPs, gender, and age. *P* values <0.05 were considered as statistically significant. The statistical software STATISTICA 13 for Windows (TIBCO Software Inc., Palo Alto, CA, USA) was used to perform all analyses.

RESULTS

Patient characteristics

A total of 95 patients with oral cavity cancer were included in this study; their clinical parameters are shown in Table 2. The average age was 62 years (range: 15–78 years). There were 69 (73%) men and 26 (27%) women: 65 (68%) of the patients were smokers; 64 (67%) of them reported alcohol consumption; 51 (54%) were both smokers and alcohol consumers. The control group consisted of 100 healthy individuals. There were 20 smokers (20%) and 68 drinkers (68%), and 20 were consumers of both tobacco and alcohol (20%). The average age of the controls was 52.92 years (range: 18–69 years). There were 22 (22%) men and 78 (78%) women.

Demographic characteristics and oral cavity cancer risk

We estimated differences in demographic characteristics, such as gender and age, between cancer patients and controls. There was no significant association of the

Table 2. Clinical parameters of patients with oral cavity cancer

Clinical parameters	Patients, n (%)
Histological grading	
G1 (Well differentiated)	16 (17)
G2 (Moderately differentiated)	65 (68)
G3 (Poorly differentiated)	14 (15)
T classification	
T1	13 (14)
T2	23 (24)
T3	22 (23)
T4	37 (39)
Nodal status	
N0	43 (45)
N1	24 (25)
N2	26 (27)
N3	2 (2)

Table 3. Comparison of genotype distributions in patients with oral cavity cancer and in controls

Variable	HNSCC (%)	Control (%)	OR (95%CI)	p-value
rs11515				
GG	72 (75.79)	78 (78.00)	1	–
CG	22 (23.16)	20 (20.00)	1.19 (0.60-2.36)	>0.05
CC	1 (1.05)	2 (2.00)	0.54 (0.05-6.10)	>0.05
CG + CC	23 (24.21)	22 (22.00)	1.13 (0.58-2.21)	>0.05
rs3088440				
GG	75 (83.33)	65 (71.43)	1	–
AG	14 (15.56)	6 (6.59)	2.02 (0.73-5.57)	>0.05
AA	1 (1.11)	20 (21.98)	0.046 (0.01-0.33)	<0.0001
AG + AA	15 (16.67)	26 (28.57)	0.5 (0.24-1.02)	>0.05
rs769412				
AA	84 (89.36)	83 (83.84)	1	–
AG	10 (10.64)	16 (16.16)	0.62 (0.26-1.44)	>0.05
GG	0 (0.00)	0 (0.00)	–	–
AG + GG	10 (10.64)	16 (16.16)	0.62 (0.26-1.44)	>0.05
rs937283				
AA	30 (31.91)	30 (30.00)	1	–
AG	50 (53.19)	55 (55.00)	0.91 (0.48-1.71)	>0.05
GG	14 (14.89)	15 (15.00)	0.93 (0.38-2.27)	>0.05
AG + GG	64 (68.09)	70 (70.00)	0.91 (0.50-1.68)	>0.05

demographic status with the rs1115 and rs3088440 polymorphisms in the *CDKN2A* gene in these patients, nor with rs769412 and rs937283 in the *MDM2* gene. Furthermore, all examined SNPs were also not associated with smoking or the alcohol status.

The rs11515, rs3088440, rs769412, and rs937283 polymorphisms and the oral cavity cancer risk

Frequencies of the four polymorphisms in the oral cavity cancer cases and cancer-free controls are shown in Table 3. Upon analysis of the *CDKN2A* polymorphisms, we did not find an association between the rs11515 genotype or allele and the risk of oral cavity cancer. We found that the AA homozygotes for rs3088440 were significantly more frequent in the control group (OR= 0.046, $p<0.0001$). Differences in the frequency of *MDM2* rs769412 and rs937283 genotypes between groups were nonsignificant. Interestingly, we did not find any cases of a GG genotype of rs769412 in either group.

Association between the rs11515, rs3088440, rs769412, and rs937283 polymorphisms and clinicopathological parameters in patients with oral cavity cancer

These genetic polymorphisms were analyzed in the light of the clinical status of each patient, including the lymph node metastasis. There were no significant associations of the clinicopathological parameters, such as the T, N and grading values, with the *p16* rs11515 and rs3088440 polymorphisms or with the *MDM2* rs769412 and rs937283 polymorphisms in these patients (Table 4).

DISCUSSION

CDKN2A is an important tumor suppressor gene involved in cell cycle regulation (Ausoni *et al*., 2016) and *MDM2* plays a crucial role during carcinogenesis as an oncogene (Denaro *et al.,* 2011). We observed that neither of the *p16* polymorphisms, rs11515 or rs3088440, were associated with the risk of oral cavity cancer. However, we found that the AA homozygotes for rs3088440 were significantly more frequent in the control group (OR= 0.046, $p<0.0001$). Our results are similar to those of Pinheiro and coworkers (Pinheiro *et al*., 2014) who found no significant association between rs11515 and HNSCC, and moreover that none of the genotype variants of this SNP were correlated with clinicopathological parameters in HNSCC patients. In contrast, another study investigating the *CDKN2A* rs15115 polymorphism for its connection with oral cancer and oral lesions among a North Indian population (Tripathi *et al*., 2018) reported that the GG genotype decreased the risk for oral disease in comparison to the CC and CG genotypes which were associated with higher risk, and suggested that the G allele may play a protective role against oral lesions and oral cancer.

In studies related to this polymorphism in other cancers, Royds and others (Royds *et al*., 2016) showed that the CG heterozygotes in rs11515 were significantly more frequent in breast cancer cases and the CG genotype was correlated with an older age and lymph node involvement, in comparison to individuals with the CC genotype. Nevertheless they found that the frequency of rs3088440 in breast cancer patients was not significantly different from controls. Chansaenroj and coworkers (Chansaenroj *et al.,*2013) investigated the genotype distributions of both *CDKN2A* polymorphisms: rs11515 and rs3088440, in HPV-infected women and showed that neither was significantly associated with cervical cancer. According to Liu and others (Liu *et al*., 2017), rs3088440 was not associated with disease-free survival in patients

Table 4. Association between *p16* and *MDM2* polymorphisms and T, N and grading in patients with oral cavity cancer

			T1	T2	T3	T4	N0	N1	N2	N3	G1	G2	G3
rs11515	GG	n	9	21	17	25	33	16	22	1	14	45	12
		%	12.50	29.17	23.61	34.72	45.83	22.22	30.56	1.39	19.44	62.50	16.67
	CG	n	4	2	5	11	10	9	3	0	2	18	2
		%	18.18	9.09	22.73	50.00	45.45	40.91	13.64	0.00	9.09	81.82	9.09
	CC	n	0	0	0	1	0	1	0	0	0	1	0
		%	0	0	0	100	0	100	0	0	0	100	0
p			0.354				0.297				0.46149		
			T1	T2	T3	T4	N0	N1	N2	N3	G1	G2	G3
rs3088440	GG	n	11	17	18	29	37	22	15	1	12	52	10
		%	14.67	22.67	24.00	38.67	49.33	29.33	20.00	1.33	16.00	69.33	13.33
	AG	n	2	3	4	5	5	3	6	0	4	7	3
		%	14.29	21.43	28.57	35.71	35.71	21.43	42.86	0.00	28.57	50.00	21.43
	AA	n	0	0	0	1	0	1	0	0	0	1	0
		%	0	0	0	100	0	100	0	0	0	100	0
p			0.916				0.418				0.607		
			T1	T2	T3	T4	N0	N1	N2	N3	G1	G2	G3
rs769412	AA	n	12	19	18	35	38	24	21	1	13	59	11
		%	14.29	22.62	21.43	41.67	45.24	28.57	25.00	1.19	15.48	70.24	13.10
	AG	n	1	3	4	2	5	2	3	0	3	5	2
		%	10.00	30.00	40.00	20.00	50.00	20.00	30.00	0.00	30.00	50.00	20.00
	GG	n	0	0	0	0	0	0	0	0	0	0	0
		%	0	0	0	0	0	0	0	0	0	0	0
p			0.439				0.894				0.409		
			T1	T2	T3	T4	N0	N1	N2	N3	G1	G2	G3
rs937283	AA	n	2	10	7	11	17	6	7	0	7	18	4
		%	6.67	33.33	23.33	36.67	56.67	20.00	23.33	0.00	23.33	60.00	13.33
	AG	n	10	11	11	18	19	17	14	0	8	34	8
		%	20.00	22.00	22.00	36.00	38.00	34.00	28.00	0.00	16.00	68.00	16.00
	GG	n	1	2	3	8	6	3	4	1	1	11	2
		%	7.14	14.29	2143	57.14	42.86	21.43	28.57	7.14	7.14	78.57	14.29
p			0.427				0.320				0.688		

with hepatocellular carcinoma. In another study, no correlation was found between rs3088440 and type 2 diabetes (Xiao *et al*., 2016).

In our study, we investigated two *MDM2* polymorphisms, rs937283 (also known as SNP G2164A) which causes an adenine to guanine change in position 2164 in the promoter region of the gene (Cao *et al*., 2018), and rs769412. We found no significant difference in the frequency of rs937283 between the oral cavity cancer patients and controls. The relationship between *MDM2* polymorphism and squamous cell carcinoma has been rarely investigated; according to Chen and coworkers (Chen *et al*., 2010) the AG genotype (OR, 2.20), as well as the combined AG/GG genotype (OR, 2.05), was significantly associated with a higher risk of oral squamous cell carcinoma, and further analyses based on OSCC risk in relation to HPV16 L1 led the authors to suggest a connection between the HPV16 status and the *MDM2* polymorphism in the head and neck cancer. That study also revealed a significantly increased risk of OSCC in individuals with the AG or GG genotypes and HPV16 L1 seronegativity (OR, 1.85), while in HPV16 L1 seropositive cases it was higher in connection with the AA genotypes (OR, 2.29), and AG or GG genotypes (OR, 6.88) in comparison to cases with AA genotypes and HPV16 L1 seronegativity. In addition, examination of interaction between the HPV16 L1 seropositivity and the combination of *MDM2* rs2279744 and rs937283 polymorphisms showed a borderline significance (Chen *et al*., 2010). In contrast, another study revealed no association between any genotype of rs937283 with Caucasian HNSCC cases (Yu *et al*., 2011). Meta-analysis based on 3 studies of rs937283 *MDM2* polymorphism showed no significant association between that SNP and the oral cancer susceptibility (Feng *et al*., 2016). Similarly, Yu and coworkers (Yu *et al*., 2011) reported a meta-analysis that no rs937283 polymorphisms were associated with the squamous cell carcinoma susceptibility.

Although no connection between rs937283 and cancer risk was observed in cases of the esophageal squamous cell carcinoma among a Chinese Han population (Yang *et al*., 2013), Wang and others (Wang *et al*., 2015) utilized RFLP-PCR to examine the association between *MDM2* rs937283 polymorphism and the risk of laryngeal cancer in another Chinese Han population. The analysis of rs937283 showed that the GG homozygotes, as well as individuals with a G allele, had a significantly higher risk of laryngeal cancer when compared to patients with the AA genotype. Furthermore, they observed that rs937283 was correlated with an increased larynx cancer risk among drinkers. The connection between rs937283 polymorphism and susceptibility to a variety of cancers has been analyzed in a few meta-analyses. For liver cancer, Chen and coworkers (Chen *et al*., 2018) revealed that the G allele and the G variant genotype of rs937283 were significantly associated with a higher risk, while the G allele and GG genotype were connected with an increased susceptibility for breast cancer; no associations were found with the rectal, colon or cervical cancers. However, that result was significant in an Asian population but not in a Caucasian group (Chen *et al*., 2018). For retinoblastoma, Cao and coworkers (Cao *et al*., 2018) revealed in a meta-analysis that the rs937283 homozygote (AA vs GG) was connected with a decreased risk when compared to a fixed-effects variant and a recessive model (AA vs AG+GG). In contrast, Jiao and others (Jiao *et al*., 2016) showed that the AG and GG genotypes of rs937283 were associated with a higher risk and moreover, that patients with the GG genotype and the G allele had poor survival.

The *MDM2* rs769412 polymorphism (SNP E354E), which leads to a change of A to G in the 354 codon but does not cause an amino acid change (Ahmad *et al*., 2015), was not associated with the HNSCC risk in Caucasians in our study. Moreover, we did not observe any GG genotype cases. There have been only a few studies on rs769412 and cancer risk, and only one connected with the head and neck cancer (Wang & Ma, 2015) where rs769412 was not significantly associated with the larynx cancer risk in a Chinese population, although further exploration revealed that it was associated among drinkers, suggesting that the *MDM2* polymorphism may interact with environmental factors and in consequence lead to cancer development. Our study was performed on a Caucasian population, while Wang and others (Wang *et al*., 2015) reported this association in a Chinese population, possibly reflecting an influence of ethnicity. However, two other reports based on the Chinese populations showed a lack of association between *MDM2* polymorphisms and cancer risk; rs769412 was not significantly connected with prostate cancer risk (Xue *et al*., 2016) and no genotype variants of SNP354 showed a significant connection with retinoblastoma risk (Jiao *et al*., 2016). On another hand, Pine and others (Pine *et al*., 2006) found no association between any genotype of rs769412 and lung cancer risk among Caucasian and African-American populations. Ahmad and coworkers (Ahmad *et al*., 2015) investigated the correlation of rs769412 with smoking-related risk of lung cancer in a Saudi Arabian population and observed that rs769412 A>G was significantly associated with non-smokers. In contrast, Rajamaran and others (Rajamaran *et al*., 2007) reported that individuals with the G variant of rs769412 (Ex12+162A>G) had a significantly decreased risk of glioma.

In conclusion, the results of study presented here indicate that none of the *CDKN2A* or *MDM2* polymorphisms examined (rs11515, rs3088440, rs769412 or rs937283) were associated with the oral cavity cancer risk in a Polish population.

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Authors' contributions

KG, research concept and design; KMO, ŁK, collection of tumor samples; JG, KG, JKS, collection of control group samples; JG, KG, DNA extraction and SNP analyses; JG, KG, PK, data analysis and interpretation; JG, KG, writing of the article; MM, ZO, critical revision of the article; All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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