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Association Between *MMP8* Gene Polymorphisms and Laryngeal Squamous Cell Carcinoma

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Abstract. *Background/Aim: Matrix metalloproteinases (MMPs) are a family of proteins which are involved in breakdown of the extracellular matrix in embryonic development, tissue remodeling and in some diseases. MMP8 has both cancer-promoting and anticancer properties. However, the contribution of MMP8 to laryngeal squamous cell carcinoma (LSCC) has not been elucidated. In this study we aimed to test the contribution of two MMP8 polymorphisms, located in the gene promoter region, to the development of LSCC. Materials and Methods: This case–control study involved 569 DNA samples which were genotyped for two single nucleotide polymorphisms using real-time polymerase chain reaction method. Statistical analysis was performed with SPSS Statistics 20 software. Results: Regression analysis adjusted by age showed that for MMP8 rs11225395 each minor A allele copy significantly reduced the odds for LSCC development (odds ratio=0.49, 95% confidence intervaI=0.04-2.19, p=0.048). MMP8 rs11225395 AA genotype was associated with smaller laryngeal tumour size (p=0.023). Smoking habit also correlated with laryngeal tumor size. Conclusion: MMP8 rs11225395 and smoking habits have a prominent interface with LSCC tumour size.*

Laryngeal squamous cell carcinoma (LSCC) is the second-most common type of head and neck cancer worldwide. It comprised 1.0% of all new cancer cases and 1.0% deaths in 2018 (1). In terms of histopathology, the dominant cancer form in this region is invasive laryngeal squamous cell carcinoma (2). The disease is typically diagnosed in the male patients, usually between the fifth and seventh decade of life (3), and is extremely rare in adolescents (4, 5).

Tobacco smoking and alcohol consumption are the major risk factors for LSCC in developed countries (6). Both factors affect epigenetic reprogramming and genetic instability which induce oncogenesis (7). Tobacco smoking has a major impact on the onset of the disease and current-smokers have around a 20-fold higher risk of LSCC than never-smokers (6, 8). Heavy alcohol consumption is also an independent risk factor for LSCC, however, it has a weaker effect than smoking (increasing disease risk by 2- to 5-fold) (9). Both of these factors are considered to act synergistically. Combined alcohol and tobacco consumption confer multiplicative risk for heavy drinkers and smokers (6, 7). Other risk factors such as radiation, low vegetable intake, gastroesophageal reflux disease, viral infections with human papilomavirus oncogenes mutations and genomic instability are related in the mechanisms of development of LSCC (8-10).

Matrix metalloproteinases (MMPs), also known as matrixins, are a family of zinc-dependent proteolytic enzymes that can degrade all protein components of the extracellular matrix (ECM) and basement membranes (11, 12). MMPs are involved in various physiological processes *e.g.* in tissue morphogenesis and repair, ovulation, and angiogenesis (13, 14). MMPs also play role in pathological conditions caused by excessive degradation of the ECM such as rheumatoid arthritis, periodontitis, cardiovascular disease, as well as tumor invasion and metastasis (13, 15, 16). The role of *MMP2* and *MMP9* in LSCC tumorigenesis has been investigated in several studies and overexpression of these MMPs has been associated with the development of LSCC (17-20). The results

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of a recent study revealed that combination of smoking and 6A/6A genotype of MMP3 *(-1171 5A/6A)* polymorphism are associated with significantly increased odds of LSCC, thus confirming a presumably important role of MMP3 (*-1171*) 6A/6A genotype in LSCC carcinogenesis (21).

MMP8 (neutrophil collagenase II) is produced mainly by neutrophils, in the course of inflammatory conditions (13, 14). In recent years, several studies showed that MMP8 might play suppressive roles in cancer and metastasis (22, 23). Firstly, knockout of *Mmp8* caused increased aggressive and undifferentiated grade III fibrosarcoma formation in a male mouse model (23). Overexpression of MMP8 in melanoma (B16F10) and Lewis lung carcinoma cell lines reduced metastasis and invasive capacity, and increased cell adhesion (22). The single nucleotide polymorphisms (SNPs) at the *MMP8* gene promoter region rs11225395 C-799T and rs2155052 C+17G, which are in strong linkage disequilibrium with the entire *MMP8* coding region, provided higher promoter activity and were associated with early-stage diseases (14, 24-27).

Regarding head and neck squamous cell carcinomas, *MMP8* expression is linked with prolonged survival in human tongue cancer and reduced susceptibility to tongue squamous cell carcinoma in mouse models (28). However, to the best our knowledge, the associations between MMP8 and LSCC have never been investigated before.

In this study we aimed to reveal the relationship of *MMP8* rs11225395 and rs215052 polymorphisms on the risk of LSCC development and its clinical and morphological manifestation.

Materials and Methods

Study population. A group of 226 of patients diagnosed with LSCC at the Department of Otorhinolaryngology of the Lithuanian University of Health Sciences (LUHS) was recruited to this study. Participants completed a questionnaire including questions on history of alcohol consumption, smoking habit and body mass index (BMI). We also included information about tumor stage, tumor differentiation grade, size of the primary tumor and the degree of spread to regional lymph nodes from pathologist's records according to the TNM classification of malignant tumours (29).

The reference group consisted of 343 random research sample of Kaunas city (Lithuania) residents who were selected during the international Health, Alcohol and Psychosocial Factors in Eastern Europe project (HAPPIE) study (30). The sample was collected by the Population Study Laboratory at the Institute of Cardiology of LUHS by carrying out a selection based on the lists of residents of Kaunas city. The LSCC patient and reference groups were matched by age and gender (p>0.05) (Table I).

The present study was approved by Kaunas Regional Biomedical Research Ethics Committee (Protocol No. BE-2-34) and informed consent was obtained from all the participants prior to inclusion in the study.

MMP8 genotyping. Genotyping of *MMP8* rs11225395 and rs2155052 was carried out at the Laboratory of Genetics of the Institute of Biology Systems and Genetic Research of LUHS. Venous blood samples for DNA extraction were collected in ethylenediaminetetra-acetic acid tubes. Genomic DNA from peripheral blood leucocytes was extracted using genomic DNA purification kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's recommendations. SNP rs11225395 in *MMP8* gene was estimated by using a commercially available

Table I. *Demographic characteristics of the study population*.

Characteristic	Group		p-Value
	LSCC (n=226)	Control (n=343)	
Gender, n (%)			
Male	210 (92.9%)	316 (92.1%)	0.727
Female	16 (7.1%)	27 (7.9%)	
Age, years			
Mean±SD	62.68±10.09	62.20±9.45	0.140

LSCC: Laryngeal squamous cell carcinoma; SD: standard deviation.

Table II. *Patient characteristics (n=226)*.

Characteristic	Number of patients with LSCC*, n (%)
Alcohol consumption	
Consumer	147 (65.0)
Cigarette smoking	
Smoker	190 (84.1)
>20 Cigarettes per day	42 (22.1)
BMI	
>30 kg/m^2	45 (44.6)
Tumor stage (n=136)	
I	32 (23.6)
II	41 (30.1)
III	40 (29.4)
IV	23 (16.9)
Tumor grade (n=153)	
1	88 (57.6)
2	40 (26.1)
3	25 (16.3)
Tumor T-stage (n=153)	
Tis	2 (1.3)
T1	43 (28.1)
T2	25 (16.3)
T3	27 (17.7)
T4a	51 (33.3)
T4b	5 (3.3)
Tumor N-stage (n=145)	
N0	88 (60.7)
N1	30 (20.7)
N2	27 (18.6)
N3	0 (0.0)

BMI: Body mass index; LSCC: laryngeal squamous cell carcinoma; Tis: carcinoma *in situ*. *LSCC group consisted of 226 patients, however, some on tumor information is missing.

Table III. *Distribution of genotypic and allelic frequencies for single nucleotide polymorphisms at matrix metalloproteinase 8 (MMP8) gene promoter regions rs11225395 (C-799T) and rs2155052 (C+17G) in the laryngeal squamous cell carcinoma (LSCC; n=226) and control (n=343) groups.*

MMP SNP			Control, n (%)	*p*-Value HWE	LSCC, n (%)	*p*-Value HWE	*p*-Value
rs11225395	Genotype	GG	100 (29.2%)	0.759	74 (32.7%)	0.313	0.300
		GA	173 (50.4%)		117 (51.8%)		
		AA	70 (20.4%)		35 (15.5%)		
	Allele	G	373 (54.4%)	-	265 (58.6%)	-	0.157
		A	313 (45.6%)		187 (41.4%)		
rs2155052	Genotype	GG	294 (85.7%)	0.629	192 (85.0%)	0.175	0.172
		GC	46 (13.4%)		46 (14.1%)		
		CC	3 (0.9%)		2 (0.9%)		
	Allele	G	624 (92.3%)	-	430 (89.6%)	-	0.107
		C	52 (7.7%)		50 (10.4%)		

HWE: Hardy–Weinberg equilibrium.

Table IV. *Model selection according to Akaike information criteria (AIC) for matrix metalloproteinase 8 (MMP8) gene promoter regions rs11225395 and rs2155052.*

MMP8 SNP	Model	OR (95% Cl)	*p*-Value	AIC
rs11225395	Dominant (GG *vs*. GA+AA)	1.399 (0.896-2.185)	0.140	764.341
	Recessive (AA *vs*. GA+GG)	0.719 (0.458-1.116)	0.140	764.341
	Overdominant (GA *vs*. GG+AA)	1.036 (0.741-1.450)	0.835	766.528
	Additive	0.199 (0.938-1.531)	0.148	762.613
rs2155052	Dominant (GG *vs*. GA+AA)	1.399 (0.896-2.185)	0.140	764.314
	Recessive (AA *vs*. GA+GG)	0.715 (0.458-1.116)	0.140	764.341
	Overdominant (GA *vs*. GG+AA)	1.056 (0.655-1.732)	0.800	764.507
	Additive	0.950 (0.615-1.466)	0.950	768.517

OR: Odds ratio; Cl: confidence intervals.

genotyping kits C_1366493_10 (rs11225395) and C_16139909_10 (rs2155052) (Applied Biosystems, Foster City, CA, USA). Applied Biosystems 7900HT Real-Time Polymerase Chain Reaction System (Applied Biosystems, Foster City, CA, USA) was used for SNP detection. The cycling program started with heating at 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 s and at 60˚C for 1 min. Finally, allelic discrimination was performed using SDS 2.3 software provided by Applied Biosystems.

Statistical analysis. Statistical analysis was performed using IBM SPSS Statistics 20 software (IBM Corp., Armonk, NY, USA). The results are presented as total number, percentages, mean and standard deviation (SD). Student's *t*-test was used to compare mean values between two groups when data values were normally distributed. Distribution of SNP genotypes in patients and reference groups was evaluated by Hardy–Weinberg equilibrium (HWE) using chi-squared test. The association between the *MMP8* polymorphisms and LSCC was estimated by computing odds ratios (ORs) and their 95% confidence intervals (Cls) from logistic regression in five inheritance models: Recessive (wild-type homozygous with heterozygous *vs*. minor allele homozygous), dominant (wild-type homozygous *vs*. heterozygous with minor allele homozygous), overdominant (wild-type homozygous with minor allele homozygous *vs*. heterozygous) and additive inheritance model. Akaike information criterion was used to choose the model that best fitted the data.

Polymorphisms rs11225395 and rs2155052 are both located on chromosome 15, therefore haplotype analysis was carried out. Estimation of haplotype frequencies and haplotype association with frequencies of at least 5% were carried out using PLINK software version 1.07 (31). Results were considered statistically significant when the *p*-value was less than 0.05.

Results

LSCC patient characteristics according to smoking and alcohol consumption habits, BMI, cancer stage, grade, T-stage and N-stage are given in Table II.

MMP8 rs11225395 and rs2155052 genotypes and allelic frequencies among the reference group and patients with LSCC are presented in Table III. The distribution of both SNPs in LSCC and control groups was consistent with the HWE. The analysis of *MMP8* gene polymorphisms did not show any statistically significant differences in the distribution of genotypes between the patients with LSCC and the controls (*MMP8* rs11225395 genotypes GG, GA and

Table V. *Haplotype association of single nucleotide polymorphisms (SNPs) at matrix metalloproteinase 8 (MMP8) gene promoter region rs11225395 (C-799T) and rs2155052 (C+17G) with laryngeal squamous cell carcinoma.*

SNP rs11225395-rs2155052	Frequency				
Haplotype	Cases	Controls	Chi-square	Degrees of freedom	*p*-Value
G-G	0.570	0.530	1.744	1	0.187
G-C	0.016	0.014	0.139	1	0.708
A-G	0.350	0.394	2.214	1	0.137
A-C	0.063	0.062	0.006	1	0.939

AA: 32.7%, 51.8% and 15.5% *vs*. 29.2%, 50.4% and 20.4%, respectively; $p=0.300$; and *MMP8* rs2155052 genotypes GG, GC and CC: 85.0%, 14.1% and 0.9% *vs*. 85.7%, 13.4% and 0.9%, respectively; $p=0.172$) (Table III). Moreover, no statistically significant differences were found between the distribution of the *MMP8* rs2155052 and rs11225395 genotypes according to tumor grade.

Associations between *MMP8* rs11225395 polymorphism and LSCC according to the inheritance models are presented in Table IV. The lowest Akaike information criterion (762.613) was for the additive model of *MMP8* rs11225395. Regression analysis demonstrated that the additive model was statistically significant in the group when age was 60 years or less (OR=0.54, 95% CI=0.05-2.26, $p=0.026$).

Inheritance models were also calculated to determine the link between LSCC and *MMP8* rs2155052 genotype (Table IV). The lowest Akaike information criterion (764.314) was found for the dominant model. However, the binominal logistic regression analysis adjusted by age in patient and control groups did not indicate any significant variables.

Association analysis between the risk of LSCC and haplotypes for *MMP8* rs11225395-r2155052 are shown in Table V. The linkage disequilibrium between these two polymorphisms (D' value) was 0.662. However, analysis did not shown any statistical significant results.

Analysis of the size of primary tumor and *MMP8* gene SNP rs11225395 genotypes showed that patients with wild-type GG genotype had larger primary tumors than AA genotype carriers ($p=0.023$; Table VI). However, no relation was found for *MMP8* rs2155052 genotypes. We also found that larger primary laryngeal tumor size was also significantly associated with smoking habit ($p=0.002$) and male gender ($p=0.027$) (Table VII).

Table VI. *The size of primary laryngeal tumor according to genotype of the matrix metalloproteinase 8 (MMP8) gene promoter region rs11225395 (sample size n=226).*

MMP8 rs11225395 genotype	Frequency	T-Stage, n (%)	
		Tis-2	3-4b
GG	74 (32.7%)	17 (32.7%)	35 (67.3%)
GA	117 (51.8%)	35 (47.9%)	38 (52.1%)
AA	35 (15.5%)	15 (60%)*	10 (40%)*

*Significantly different at $p<0.05$ for AA *vs*. GG.

Discussion

MMP8 is a major collagenase that cleaves collagen type I and can suppress tumor growth and invasion (32). It was thought that by using MMPs malignant cells are able to break down the basement membrane and degrade interstitial stroma, thus leading to tumor invasion or metastases (33). However, some new studies showed rather controversial results, *i.e*. MMP8 was associated with antitumor properties in tongue squamous cell carcinoma and a reduced number of lymph node metastases in human breast cancer (22, 32). Moreover, SNP of *MMP8* rs2155052 was associated with decreased risk of lung cancer, especially in high-risk patients such as ever-smokers (34). Three independent studies showed that *MMP8* rs11225395 was associated with better breast cancer survival, however, opposite findings were presented in studies with ovarian cancer and melanoma (32, 34).

It is known that increased expression of MMP2 and MMP9 is associated with glottis squamous cell carcinoma grade and reduced survival (35, 36). However, as far as we are aware, there are no studies yet to show the impact of polymorphisms in *MMP8* gene on laryngeal cancer development. In this study, we examined *MMP8* gene polymorphisms rs11225395 and rs2155052 in patients with LSCC.

Our results showed no statistically significant genotypic differences between LSCC patient and reference groups. These results concur with those of Hung *et al.,* where *MMP8* gene polymorphism rs11225395 was analyzed in patients with oral cancer; it was found this SNP may not play a major role in mediating personal risk of oral cancer (37). However, González-Arriaga *et al*. demonstrated that polymorphism +17 C/G in *MMP8* was associated with

Table VII. Associations between the primary tumor size and characteristic of patients with laryngeal squamous cell carcinoma.

Characteristic	Comparison	T-Stage		*p*-Value
		Tis-2	3-4b	
Gender	Female *vs*. male	6 (85.7%) *vs*. 62 (43.1%)	1 (14.3%) *vs*. 82 (56.9%)	**0.027**
Age	≤60 *vs*. >60 Years	16 (38.9%) *vs*. 52 (47.7%)	26 (61.9%) *vs*. 57 (52.3%)	0.287
Smoking habit	Non-smoker *vs*. smoker	55 (40.7%) *vs*. 13 (81.2%)	80 (59.3%) *vs*. 3 (18.8%)	**0.002**
Cigarettes/day	≤20 *vs*. >20	58 (47.9%) *vs*. 10 (33.3%)	63 (52.1%) *vs*. 20 (66.7%)	0.150
Alcohol	Consuming *vs*. non-consuming	53 (49.1%) *vs*. 15 (34.9%)	55 (50.9%) *vs*. 28 (65.1%)	0.114
BMI	≤25 *vs*. >25 kg/m^2	40 (41.7%) *vs*. 28 (50.9%)	56 (58.3%) *vs*. 27 (49.1%)	0.272

BMI: Body mass index. Statistically significant *p*-values are shown in bold.

reduced lung cancer risk (38). To the best of our knowledge, the present study is the first to investigate *MMP8* rs2155052 polymorphism in LSCC.

In Lithuania, LSCC is usually diagnosed in males above 60 years old (39). For this reason, we divided the study subjects into groups by age (analysis by gender would be inappropriate because female groups were smaller than those of men (n=16 *vs*. n=210 and n=5 *vs*. n=163). *MMP8* gene allelic frequencies were not significantly different between the two groups divided by age (<60 *vs*. ≥60 years old); however, logistic regression analysis revealed that for the *MMP8* rs11225395 SNP, each copy of the A allele reduced the risk of LSCC in group of those 60 years old or less (OR=0.49, 95% CI=0.04-2.19; p=0.048). To the best of our knowledge, the association between *MMP8* and LSCC has never been investigated before, therefore we cannot compare our results with similar studies. However, *MMP8* gene polymorphism rs11225395 was analyzed in other disorders and obtained opposite results. Chen *et al*. showed that in the additive inheritance model, minor A allele increased the risk of osteonecrosis in Chinese males (40). Debniak *et al*. also showed minor allele homozygous (AA) and heterozygous (GA) genotypes for *MMP8* rs11225395 polymorphism to be related to increased risk of malignant melanoma (41). Majumder *et al*. similarly showed that the minor A allele increased susceptibility to chronic periodontitis (42). However, Decock *et al*. showed that *MMP8* rs1122395 minor A allele was related to reduced risk of breast cancer and better patient survival (25).

Interestingly, in some studies, *MMP*8 gene activity was related to gender. Koroi and coworkers found that *MMP8* antitumor mechanisms in tongue squamous cell carcinoma may be estrogen related (32). Balbin *et al*. showed that ovarian elimination increased susceptibility to skin cancer in *Mmp8-null* mice (18). According to these studies, it would be interesting to use a larger group of females with LSCC and compare *MMP8* gene polymorphism distributions between male and female groups and calculate inheritance models according to gender.

It was shown in previous studies that *MMP8* expression levels correlated with tumor size and invasive properties (22). Decock *et al*. demonstrated that in *Mmp8*-wild-type and heterozygotic mice, mammary glands tumors were significantly smaller than in *Mmp8*-null mice (43). Results of our study are in concordance with these data, and show that *MMP8* rs11225395 AA genotype, with its higher promoter activity (44), are associated with smaller laryngeal tumor size than the wild-type GG genotype (p=0.023).

In addition to analysis of the impact of this genetic factor on laryngeal cancer development, an influence of the modifiable factors, smoking habit, alcohol consumption and BMI, were evaluated in the present study. It is known that all these factors increase laryngeal cancer risk and affect the course of the disease (6, 32, 45). In a study by Chen *et al*., patients who never smoked had significantly smaller tumors of non-small cell lung cancer than did smokers (46). In another study, O'Donnell *et al*. reported that smokers trended to have larger size germ-cell tumors than non-smokers (47). Our data agreed with these results and showed that smoking to be significantly related to the size of LSCC (p=0.002).

Our findings suggest that *MMP8* gene polymorphism rs11225395 and smoking habit have a prominent association with LSCC tumor size. The interaction between the effect of gene polymorphism on the growth of laryngeal cancer and gender should be further investigated.

Conflicts of Interest

None of the Authors had any conflicts of interest to declare.

Authors' Contributions

The Author(s) made the following declaration about their contributions: Study design: Alina Smalinskiene. Assembled study population: Vykintas Liutkevicius and Virgilijus Uloza. Performed experiments: Ruta Insodaite and Rosita Solovejutė. Analysed the data: Ruta Insodaite. Wrote the article: Ruta Insodaite and Alina Smalinskiene. Critical revision of the article: Vykintas Liutkevicius and Virgilijus Uloza.

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