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MMP-2 and MMP-9 gene polymorphisms and risk of head and neck carcinomas

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Abstract

***Background**: Head and neck carcinomas (HNC) account for a majority of ear, nose and throat tumours. They account for 6.3% of all incident malignancies and 6.2 % of all deaths from cancer in Romania in 2020, the fifth most common cancer in this Eastern Europe country. Aim of the study: The aim of our study was to investigate the association between two MMP-2 and MMP-9 promoter gene polymorphisms and head and neck cancer.* ***Methods**. We enrolled 142 subjects, 65 cancer patients, and 77 control subjects and tested them for MMP-2 -735 C/T and MMP-9 -1562 C/T polymorphisms by PCR-RFLP.* ***Results**. Comparison between cancer patients and controls demonstrated the presence of MMP-2 -735 C/T and MMP-9 -1562 C/T in head and neck malignant tumours, with OR = 2.206 (95% CI 1.058-4.599, P = 0.03) for MMP-2 and OR = 2.748 (95% C.I. 1.262-5.981, P=0.009) for MMP-9 gene polymorphism. This means that the presence of T allele could be a risk factor for head and neck cancer development. The analysis included a stratification of studied groups by age and gender.* ***Conclusions**. Both genotypes were associated with a significant risk for head and neck carcinomas in case of the presence of the T allele. MMP-2 -735 C/T (rs2285053) and MMP-9 -1562 C/T (rs3918242) gene polymorphism could be an important genetic marker for head and neck cancer susceptibility. This finding could be useful for genetic screening in head and neck carcinomas.*

***Keywords**: risk, polymorphism, MMP-9, MMP-2, head and neck squamous cell carcinomas*

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Introduction

Head and neck carcinomas (HNC) account for a majority of ear, nose, and throat tumours (1). They have variate cell origins, but most of them (up to 90%) represent squamous cell carcinomas in adult population (2,3). Head and neck squamous cell carcinoma (HNSCC), a malignancy with primary sites in the oral cavity, lip, pharynx, larynx, and paranasal sinuses (4) is one of the most common cancers worldwide. With 6239 new cases and 3384 deaths from HNC, it

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accounts for 6.3% of all incident malignancies and 6.2 % of all deaths from cancer in Romania in 2020, being the fifth most common cancer in this Eastern Europe country (5).

HNSCC has highly invasive properties demonstrated by locoregional recurrence and lymph node metastasis with an expected 5-year survival rate of 50% in advanced disease (6). Apart from already established risk factors including cigarette smoking, alcohol intake, their combination (7), or human papilloma virus (HPV), which is mainly linked to oropharyngeal cases, some authors suggest a genetic susceptibility of individuals to head and neck carcinomas (8,9).

Cancer occurs following genetic alterations, leading to tumour progression and metastasis as a result of local interplay of growth factors, chemokines and matrix metalloproteinases (MMPs). This altered panel of aberrant expression leads to transformed tumour cells and degraded tissue microenvironment. Extracellular matrix environment remodelling in neoplasia could play an important role in tumour progression and metastasis. These processes are enhanced by defective MMPs function.

MMPs are a large group of zinc-dependent secreted proteinases or transmembrane proteins that are involved in extracellular matrix remodelling. There are numerous evidences showing MMPs overexpression in tumour tissue correlated with local invasion, metastasis and poor survival (10). They are also associated with apoptosis, angiogenesis, cell differentiation, and immune response (11). MMPs dissolve the basement membrane and degrade interstitial stroma thus facilitating invasion and metastasis, mainly by proteolysis mediated by gelatinases like MMP-2 and MMP-9. MMP-2 expression is higher in metastatic tumours than in those non-metastatic, therefore MMP-2 can be a predictive marker of metastasis in oral cancer (10, 12). The levels of gene expression and enzymatic activity are higher for these two gelatinases in tumours (colorectal, ovarian, breast, melanoma and oral cancer) compared to normal tissues (13). Moreover, MMP-9 was associated to invasiveness and a shorter disease-free survival in oral cancer (14). MMPs involvement in oral squamous cell carcinoma was extensively reviewed by Suvi-Tuuli Vilen et al. (15)

Continuing investigation about the MMPs as tumour markers, most studies focused on MMP-2 and 9, looking for association between these enzymes and tumour invasiveness or dissemination. Some hypotheses regarding the relationship between tumour development and MMP have been proposed, and angiogenesis has been the most favoured one (16, 17).

MMP-2 (gelatinase A) gene is located on chromosome 16q12.2 with 13 exons and 12 introns (18). *MMP-2* promoter contains regulatory elements like cAMP response element-binding protein, AP-1, AP-2, PEA3, C/EBP, and Sp1. The expression of this gelatinase is subjected to regulation by transcription factors (19). In the *MMP-2* gene promoter region there were described several polymorphisms. Among them, the most extensively studied is a C-T transition at -1306 position shown to disrupt a Sp1-type promoter motif (CCACC box) with the direct consequence of lower promoter activity with the T allele (20). Another C-T transition at -735 position (rs2285053) in *MMP-2* gene promoter was recently described and seems to have a similar effect by destroying a Sp1 binding element and being associated with significant lower promoter activity and distant metastasis in oesophageal carcinomas (21).

MMP-9 (gelatinase B) gene is located on chromosome 20, q11.2-13.1 region. *MMP-9* -1562 C/T (rs3918242) polymorphism causes a lower binding of the transcription repressor proteins to the promoter and results in a higher transcriptional activity. The presence of the T allele (TT or TC genotype) showed a 2-fold higher expres-

sion than CC genotype in severe coronary atherosclerosis (22).

Some studies demonstrating overexpression of MMPs in association with head and neck cancers suggested their involvement in tumour development (23) while other authors demonstrated MMP-9 association with lymph node tumour spreading (24).

In order to have an early identification of a malignancy, we tested two functional SNPs (single nucleotide polymorphisms) in promoter of *MMP-2* and *MMP-9* genes to find any possible associations with susceptibility for head and neck carcinomas, tumorigenesis, local progression and metastasis.

Materials and methods

A group of 65 patients diagnosed with head and neck carcinomas and 77 control subjects were enrolled after they gave their informed consent. The study was approved by the Ethical Committee of the Institute (27/01/2015).

Patients admitted to the hospital with head and neck cancers were investigated by CT scan or MRI imaging before surgery. A histological diagnosis after surgery and a detailed record of the possible risk factors (mainly smoking status) for cancer development were recorded for each patient.

The exclusion criteria were: lack of confirmation of the disease by histological examination or previous surgical or other therapeutic intervention before admission. The histological examination of all removed tissues (tumour and lymph nodes) at surgery allowed us to assess the stage of the disease.

Genomic DNA extraction from whole blood was performed with Promega Wizard Genomic DNA Purification kit (Promega Inc, USA) according to manufacturer's protocol.

MMP-2 -735C/T and *MMP-9* -1562 C/T gene polymorphisms were determined by PCR-RFLP. 50 ng DNA were amplified using previously reported primers (25): MMP-2F: 5'-ATAGGGTAAACCTCCCCACATT-3', MMP-2R: 5'-GGTAAAATGA GGCTGAGACCTG-3', MMP-9F: 5′-GCCTGGCACATAGTAGGCCC-3′, MMP-9R: 5′-CTTCCTAGCCAGCCGGCATC-3′. PCR amplification protocol for *MMP-2* was: denaturation 5 min., 95 °C; 35 cycles of 45 s at 94 °C, 45 s at 62 °C and 60 s at 72 °C; 10 min. at 72 °C. *MMP-9* amplification protocol was: denaturation 10 min., 95 °C; 35 cycles of 30 s at 94 °C, 30 s at 59 °C and 30 s at 72 °C; 10 min. at 72 °C. *MMP-2* amplicons were enzymatically treated with 10 U of restriction enzyme *Hinf*I (New England Biolabs) and *MMP-9* amplicons were treated with 10 U of *SphI* (New England Biolabs) for 3h at 37 °C. Restriction products, as presented in Table 1, were analysed in 2% agarose gel (Life Technologies) (Figure1, 2).

Statistical analysis

Pearson's Chi-square test was applied to analyse the distribution of the *MMP-2* -735C/T and *MMP-9* -1562C/T genotypes among the subgroups and to test for Hardy-Weinberg equi-

Table 1. Fragment length analysis by genotype following enzyme digestion

Polymorphism	Restriction enzyme	Genotype	Fragment length (bp)
-735C/T *MMP-2*	*Hinf*I	CC	300 bp
		TT	254bp+ 46bp
		CT	254bp+46bp+300bp
-1562C/T *MMP-9*	*Sph*I	CC	435 bp
		TT	247 bp+188 bp
		CT	247 bp+188 bp+435 bp

bp: base pairs

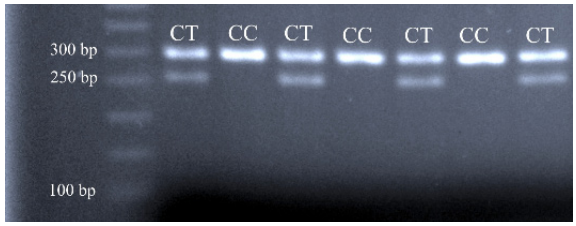

Fig. 1. ***PCR-RFLP electrophoresis image for MMP-2 -735 C/T polymorphism***

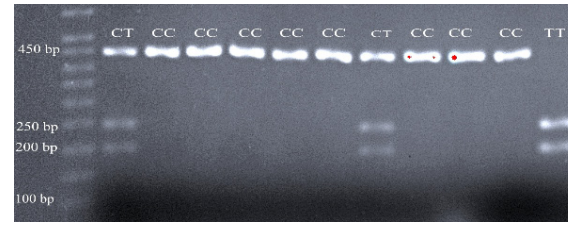

Fig. 2. ***PCR-RFLP electrophoresis image for MMP-9 -1562 C/T polymorphism***

librium. The association between these MMPs genotypes and risk of head and neck cancer was estimated by computing odds ratios (ORs) and their 95% confidence intervals (C.I.) from logistic regression analysis. We also performed additional analyses on the population stratified based on age and sex (Cochran-Mantel-Haenszel test). Tests for deviation from Hardy-Weinberg equilibrium and tests for association were assessed with an internet-based calculator (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). All statistical analyses were conducted with MedCalc® Statistical Software version 20.022 (MedCalc Software Ltd, Ostend, Belgium; https://www.medcalc.org; 2021).

Results

The age of the patients showed a higher incidence of the disease in aged people. The mean age of cancer group was 58.04±8.29 yrs. (mean±SD) vs. 51.47±11.62 yrs. (mean±SD) in control group. In order to test the association of MMPs polymorphisms with the stage of the disease, histologic differentiation grading and presence of node metastases, we divided the patients according to early disease stage or late disease stage, histologic differentiation grading and presence of metastasis, as presented in Table 2. Almost 70% of the patients presented in stage III and IV at admission. Histology elicited in some patients more advanced cell proliferation and local spread than suspected after imaging and clinical exam.

Table 2. Disease stage, histologic differentiation grading and metastasis presence in cancer group

Groups	N	%
Gender		
male	54	83.07
female	11	16.9
Age		
>50 y	52	80
<50y	13	20
Environment		
urban	36	55.3
rural	29	44.7
Smoking status		
Yes	44	67.7
No	21	32.3
Disease stages		
I, II	20	30.7
III, IV	45	69.3
Histologic differentiation grading		
G1	33	50.7
G2	17	26.1
G3	15	23.2
Metastasis		
yes	33	50.7
no	32	49.3

N: number of patients; I, II: early stages, III, IV: advanced stages

Staging of the disease was performed in accordance with the international TNM criteria and scores, taking into consideration the imaging performed before surgery and the histology of the removed tissues. The pathology assessed the origin of the tumour, except for 3 cases with lymph nodes metastasis in which no primary tumour could be revealed.

The *MMP-2* -735C/T and *MMP-9* -1562C/T promoter genotypes are presented in Table 3. There is a clear difference in genotype and allele distribution between the cancer patient group compared to controls. The percentage of T allele is 1.71 times higher in the cancer group for *MMP-2* and 2.29 times higher for *MMP-9*.

We did not find any association between genotype and disease stage, proliferating cell grading or the presence of metastasis (data not shown).

Multiple logistic regression analysis returned an OR=2.206 (1.058-4.599 95% CI, p=0.03) for *MMP-2* - 735 C/T and OR = 2.748 (1.262-5.98195% C.I., p=0.009) for *MMP-9* -1562C/T gene polymorphisms. This means that the presence of T allele could be a risk factor for head and neck cancer development (Table 4).

In order to evaluate if the results are affected by the heterogeneity of the study population, we stratified the groups based on age (below and over 50 years old) and gender (male and female). Cochran-Mantel-Haenszel (CMH) test results showed that age distribution between the control and cancer group did not affect results analysis for *MMP-9* -1562 C/T polymorphism (p= 0,098). Instead, *MMP-2* -735 C/T polymorphism is significantly different between age groups in controls and cancer group (p=0.002). Moreover, gender influenced the results for *MMP-9* -1562 C/T polymorphism (p=0,043), but not for *MMP-2* -735 C/T polymorphism (p=0,1). All results for CMH test are presented in Table 5.

Table 3. Genotype distribution and allele frequencies for *MMP-2* -735 C/T (rs2285053) and *MMP-9* -1562 C/T (rs3918242) in cancer compared to control group.

Polymorphism	Genotype or allele	Cases (%)	Controls (%)
MMP-2 -735 C/T	CC	40 (59.70%)	60 (77.92 %)
	CT	24 (35.82%)	16 (20.78%)
	TT	2 (2.98%)	1 (1.30%)
	C	104 (78.78%)	136 (88.31%)
	T	28 (21.22%)	18 (11.69%)
MMP-9 -1562 C/T	CC	43 (64.18%)	64 (83.11%)
	CT	21 (31.34%)	12 (15.59%)
	TT	4 (5.98%)	1 (1.30%)
	C	107 (78.67%)	140 (90.90%)
	T	29 (21.33%)	14 (9.10%)

Table 4. Logistic regression in cancer vs. control group

Polymorphism	Model	OR (95% C.I.)	P value
MMP-2 -735 C/T	Dominant CC+CT vs. TT	0.421 (0.037-4.751)	ns
	Recessive CC vs CT+TT	2.294 (1.105-4.763)	0.024
MMP-9 -1562 C/T	Dominant CC+CT vs. TT	0.211 (0.023-1.931)	ns
	Recessive CC vs CT+TT	2.862 (1.320-6.204)	0.006

OR, odds ratios; p<0,05 was considered statistically significant

Discussion and conclusions

Head and neck squamous cell carcinomas are very aggressive and relatively frequent malignancies in Romania.

Given the high incidence and multiple histopathology of this heterogeneous group of tumours, it is very important to identify early potential biomarkers of susceptibility to the occurrence and progression of this aggressive cancer.

In this case-control study, we investigated the role of two functional SNPs in *MMP-2* and *MMP-9* as having a potential role for the predisposition to HNSCCs. Our findings showed that *MMP-2* -735 CT+TT and *MMP-9* -1562 CT+TT genotypes were significantly associated with an increased risk of HNSCC.

The *MMP-2* -735 C/T (rs2285053) polymorphism was less investigated in cancer compared to *MMP-2* -1306 C/T. However, few studies showed that the rs2285053 T allele carriers had a reduced risk of breast cancer in Tunisian (26) and Iranian (27) populations. Instead, others did not find any association between rs2285053 and breast cancer risk in additive models (28).

In lung cancer patients, those who have the C-C haplotype made up by *MMP-2* rs243865 and rs2285053 polymorphisms have higher risk of lung cancer than those having the T-T haplotype. Tumour *MMP-2* gene expression was lower in -735 CC patients than in those with CT or TT genotypes (29).

MMP-2 -735 C/T polymorphism was associated with lower risk in lung and oesophageal cancers (30), increased risk of nasopharyngeal carcinoma (31), increased risk of gallbladder cancer (32) but no association in hepatocellular carcinoma (33).

Another study did not find any differential distribution in genotypic or allelic frequencies in oral cancer patients versus non-cancer patients in Taiwan, but CT and TT genotypes were associated with lower metastasis rates within 5 years in oral cancer patients (34).

Regarding head and neck carcinomas, studies have shown that *MMP-2* is overexpressed in HNSCC tissues with higher ability of invasion and metastasis (35).

Our results showed a significant difference of rs2285053 allele T distribution between cancer and non-cancer subjects.

The difference between our results and previous studies regarding -735 C/T *MMP-2* gene polymorphism could be related to the ethnicity of studied population, various sites of HNSCC considered, and a relatively small number of patients. It is worth specifying that MMP activity is regulated at the transcription level and transcription is differently regulated with each cell type (e.g., keratinocyte, melanocyte, and fibroblast) capable of displaying unique proteolytic phenotypes (36). Moreover, other authors (37) have obtained similar results to our study but on the analysis of rs243865 (-1306 C/T *MMP-2*) in HNSCC patients. It was demonstrated that the -1306 C/T and -735 C/T polymorphisms are in a linkage disequilibrium, indicating an interactive effect of these two SNPs on *MMP-2* transcriptional function (21).

MMP-9 -1562 C/T is one of the five SNPs discovered in the promoter of *MMP-9* gene (38), being extensively studied in relation to cancer occurrence and progression. Under physiological states, *MMP-9* is expressed in low levels, its expression being highly stimulated in many tumours, including oral squamous cell carcinoma (39). This SNP is a functional polymorphism as C to T replacement at -1562 position in the promoter affects gene transcription. Allele T confer an increased gene promoter activity.

MMP-9 is overexpressed and plays an important role in head and neck cancers (40), but a recent meta-analysis (41) showed no significant association between the *MMP-9* -1562 C/T polymorphism and HNSCC risk. High *MMP-9* expression could determine HNSCC progression via mechanisms other than regulation by the

MMP-9 -1562 C/T polymorphism (41). Still, this meta-analysis has several important limitations ranging from the number of studies included (5 studies), studied populations with different potential risk factors (Asian, African and only one Caucasian population), tumour locations to control group selection. Another recent meta-analysis showed that *MMP-9* rs3918242 may be a risk factor for breast cancer (42).

We found a significant association between the presence of T allele in both matrix metalloproteinases *MMP-2* and *MMP-9* polymorphisms and head and neck cancer occurrence. Both genotypes showed a significant risk for head and neck cancer in case of presence of the T allele. The calculated OR showed a 2-fold increased risk for head and neck cancer compared to control group. The stratification of our studied groups showed that the age and gender of participants could be confounding factors (age for *MMP-2* -735 C/T, but not for *MMP-9* -1562 C/T polymorphism and gender for *MMP-9* -1562 C/T, but not for *MMP-2* -735 C/T polymorphism).

In conclusion, *MMP-2* -735 C/T (rs2285053) and *MMP-9* -1562 C/T (rs3918242) polymorphisms can be important genetic markers for head and neck cancer susceptibility. This finding is important in terms of genetic screening. Considering our strata results, further prospective studies on larger cohorts are needed to validate these results.

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Authors' contributions

SS and DCG have equal contribution to the article; conceived and designed the experiments: SS, DM, DCG; performed the experiments: SS, CP; analysed the data: DM, SS, AZ, CP, DCG; contributed with patients/sample collection/ materials: AZ, DM, DCG; wrote the paper: SS, DCG, DM; discussed and commented on results and final manuscript approval: SS, DCG, AZ, CP, DM.

Conflict of interest

None to declare.

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