

ORIGINAL ARTICLE

The MMP-2 and MMP-9 promoter polymorphisms and susceptibility to salivary gland cancer

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Summary

Purpose: Matrix metalloproteinases (MMPs) are a family of endopeptidases that may play an important role in the development of salivary gland cancer (SGC). MMP-2 and MMP-9, members of the gelatinase protein family, are capable of degrading type IV collagen of basement membranes, and their overexpression is often associated with tumor aggressiveness and poor prognosis. The aim of this study was to establish the role of single nucleotide polymorphisms (SNPs) in MMP-2 and MMP-9 genes as putative susceptibility factors for the development of SGC.

Methods: The MMP-2 -1306 C>T, MMP-2 -1575 G>A and MMP-9 -1562 C>T polymorphisms were analyzed in 93 SGC cases and 100 controls using PCR-RFLP.

Results: The T allele for the MMP-2-1306 C>T polymorphism exhibited its effect in heterozygous carriers, increasing the risk for SGC (odds ratio/OR 1.98, 95% CI 1.07-

3.65, $p=0.03$). According to the dominant model, CT+TT genotypes had a 2-fold increased risk of developing SGCs ($p=0.02$). When the dominant model was applied for the MMP2 -1575 G>A, individuals with GA+AA genotypes exhibited a 1.77-fold increase in cancer risk, but with borderline significance ($p=0.049$). Heterozygous carriers of the variant T allele for the MMP-9 -1562 C>T polymorphism had roughly a 2-fold increase in susceptibility for SGC compared to wild type homozygotes (CC) ($p=0.02$).

Conclusion: Our findings suggest MMP-2-1306 C>T and MMP-9-1562 C>T polymorphisms genotypes seem to influence the development of SGCs, whereas MMP-2 -1575 G>A seems to be of a minor importance.

Key words: matrix metalloproteinases, PCR-RFLP, polymorphism, salivary gland cancer

Introduction

SGC is an uncommon malignancy with an overall incidence in the Western world of about 2.5–3.0 per 100 000 per year [1]. SGC comprises a morphologically remarkably heterogeneous group of tumors, with more than 40 histological types, some of which are extremely rare. Besides the morphological variations between individual tumors, the morphology can vary greatly within the same tumor mass, making the diagnosis and classification of salivary gland tumors a major

challenge [2]. It is very complicated to use prognostic tools when determining the aggressiveness of a malignant neoplasm [1,3].

Some studies have showed that MMPs may play an important role in the development of SGC [4]. MMPs are a family of zinc-dependent endopeptidases that are capable of degrading the extracellular matrix (ECM). Since they can degrade essentially all components of ECM including collagens, elastin, proteoglycans, laminin and fi-

bronectin, their role is crucial in the invasion and metastasis of most malignancies [5]. It has been shown that MMPs could be involved in several steps of cancer development, such as cancer cell growth, differentiation, apoptosis, migration, invasion, and metastasis [6]. They also cleave several non-matrix proteins, including growth factors, cytokines, chemokines, and their receptors, in this manner regulating cell growth and inflammation. Elevated levels of MMPs have been associated with the invasive properties of cancer and their role as prognostic factors has been studied widely in different cancer types [7,8].

Although many MMPs are thought to have a role in carcinogenesis, most attention has focused on MMP-2 and MMP-9, members of the gelatinase protein family. MMP-2 and MMP-9 are capable of degrading type IV collagen, the most abundant component of the basement membrane, that provides structural support for cells and influences cell signaling and polarity. Therefore, the destabilization of the basement membrane is an essential step for both the local and metastatic spread of most cancers. These molecules are over-expressed in a variety of malignant tumors and their expression and activity are often associated with tumor aggressiveness and poor prognosis [9]. Elevated levels of MMP-2 and/or MMP-9 are found in many malignant tumors, such as breast, brain, ovarian, pancreas, colorectal, bladder, prostate and lung cancers and melanoma [10-12]. The deregulation of MMP-2 and MMP-9 expression can be attributed to single nucleotide polymorphisms (SNPs) in the gene promoter region. The MMP-2 -1306 C>T (rs243865) polymorphism is located in the CCACC box of the Sp1-binding site. The MMP-2 -1575G/A (rs243866) variant is located immediately to 5' to a half-palindromic potential oestrogen receptor binding site and the G allele functions as an enhancer [13]. The MMP-9 -1562 C>T (rs3918242) polymorphism has been shown to upregulate the promoter activity and the presence of the -1562T allele has also been reported to be associated with the increase in gene expression [14].

The aim of this study was to establish the role of SNPs in the MMP-2 and MMP-9 genes as putative susceptibility factors for the development of SGCs in the Serbian population.

Methods

Study subjects

This retrospective study included 93 patients di-

agnosed with SGCs, surgically treated at the Clinic of Otorhinolaryngology and Maxillofacial Surgery, Clinical Center of Serbia from 2004 to 2013. The inclusion criterion was the diagnosis of any malignant head and neck salivary gland tumor and the exclusion criterion was the presence of recurrences. For the control group, buccal swabs from 100 healthy volunteers were collected, matching the study group in sex and age. All procedures were approved by the Ethics Committee of the School of Medicine, University of Belgrade, in accordance with the Declaration of Helsinki.

Genotyping

DNA was extracted from formalin-fixed and paraffin-embedded (FFPE) SGC tissue or buccal swabs (controls) using QIAamp DNA Mini Kit (Qiagen Inc, Hilden, Germany), according to the manufacturer's recommendations. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis was used to evaluate the MMP-2 and MMP-9 genotypes. PCR was carried out in a total volume of 25 µL, containing 300 ng genomic DNA, 0.4 µM of each primer and 1U Taq DNA polymerase (Thermo Fisher Scientific Inc, Waltham, MA, USA). The solution was incubated for 3 min at 95 °C, followed by 35 cycles of 1 min at 95 °C, 45 s at primer specific annealing temperature (Table 1) and 45 s at 72 °C, with a final extension of 72 °C for 7 min.

The primer sequences and annealing temperatures are shown in Table 1.

MMP-2 -1575G > A (rs243866) genotyping

The 175 bp PCR product was then digested with 3 U NlaIII (Thermo Fisher Scientific Inc) overnight at 37°C and the fragments were separated on an 8% polyacrylamide gel stained with ethidium bromide. The GG genotype showed a 175 bp product; the GA genotype had 175 bp, 112 bp, and 63 bp fragments; and AA had 112 bp and 63 bp fragments.

MMP-2 -1306C > T(rs243865) genotyping

The 193 bp product was digested with 3 U BfaI (Thermo Fisher Scientific Inc) overnight at 37°C and the fragments were separated on an 8% polyacrylamide gel stained with ethidium bromide. The CC genotype showed a 193 bp digestion product, the CT genotype had 193 bp, 167 bp, and 26 bp fragments and the TT genotype had 167 bp and 26 bp fragments.

MMP-9 -1562 C>T (rs3918242) genotyping

The 435 bp PCR product was digested with 3 U of SphI (Thermo Fisher Scientific Inc) overnight and the fragments were separated on an 8% polyacrylamide gel stained with ethidium bromide. After digestion, CC homozygotes showed 1 band of 435 bp, TT homozygotes had 2 bands (247 and 188 bp) and CT heterozygotes had

Table 1. Primer sequences and annealing temperatures for MMP-2 -1306 C>T, MMP-2 -1575 G>A and MMP-9 -1562 C>T polymorphisms

SNP		Primer sequence	Annealing temperature
MMP-2 -1306 C>T	Sense	CTT CCT AGG CTG GTC CTT ACT GA	63°C
	antisense	CTG AGA CCT GAA GAG CTA AAG AGC T	
MMP-2 -1575 G>A	Sense	GTC TGA AGC CCA CTG AGA CC	63°C
	antisense	CTA GGA AGG GGG CAG ATA GG	
MMP-9 -1562 C>T	Sense	GCC TGG CAC ATA GTA GGC CC	60°C
	antisense	CTT CCT AGC CAG CCG GCA TC	

Table 2. Genotype distribution and allelic frequencies (p [%] and q [%]) and logistic regression analysis data of MMP-2 -1306C>T, MMP-2 -1575G>A and MMP-9 -1562C>T polymorphisms

MMP-2 -1306 C>T	SGC ($\Sigma=93$)	Controls ($\Sigma=100$)	OR	95% CI	p value
CC	35	55	REF		
CT	44	35	1.98	1.07-3.65	0.03
TT	14	10	2.2	0.88-5.50	0.09
CT+TT	58	45	2.03	1.14-3.60	0.02
p (%)	61	73	REF		
q (%)	39	27	1.73	0.95-3.14	0.07
MMP-2 -1575 G>A	SGC($\Sigma=93$)	Controls ($\Sigma=100$)	OR	95% CI	p value
GG	38	55	REF		
GA	39	35	1.61	0.87-2.99	0.13
AA	16	10	2.3	0.95-5.65	0.06
GA+AA	55	45	1.77	1.00-3.13	0.049
p (%)	62	74	REF		
q (%)	38	26	1.74	0.96-3.19	0.07
MMP-9 -1562 C>T	SGC($\Sigma=93$)	Controls ($\Sigma=100$)	OR	95% CI	p value
CC	53	73	REF		
CT	37	25	2.04	1.10-3.78	0.02
TT	3	2	2.07	0.33-12.80	0.65
CT+TT	40	27	2.04	1.12-3.73	0.02
p (%)	77	86	REF		
q (%)	23	14	1.83	0.88-3.82	0.10

OR:odds ratio; 95% CI:95% confidence interval; p value: Pearson χ^2 or Fisher exact test two-sided value

3 bands (435, 247 and 188 bp).

Genotypes were confirmed by randomly re-genotyping 10% of the samples. There were no discrepancies between the genotypes determined in duplicate.

Statistics

Differences in allele and genotype frequencies between SGCs and controls were evaluated by Pearson χ^2 -test or Fisher exact test. The association of MMP-2 -1306 C>T, MMP-2 -1575 G>A and MMP-9 -1562 C>T variants with SGC risk was estimated using unconditional logistic regression analysis to calculate OR and their 95% CI. All statistical tests were two-sided at a significance level of 0.05. Calculations were performed

using the Statistical Package for the Social Sciences (SPSS). Hardy-Weinberg equilibrium (HWE) was tested using a goodness-of-fit χ^2 test.

Results

The genotype and allele frequencies along with the risk estimates for the 3 genotyped SNPs are shown in Table 2. Observed and expected frequencies for all 3 polymorphic sites met the HWE criteria.

The T allele for the MMP-2 -1306 C>T polymorphism exhibited its effect in heterozygous carriers, leading to increased risk of SGC develop-

ment (OR 1.98, 95% CI 1.07-3.65, $p=0.03$). In addition, according to the dominant model, individuals with CT+TT genotypes had a 2-fold increased risk of developing the SGC ($p=0.02$).

When the dominant model was applied for the MMP2 -1575 G>A, individuals with GA+AA genotypes exhibited a 1.77-fold increase in cancer risk, but only with borderline significance ($p=0.049$).

A significant difference in genotype frequencies was also found between the SGC group and controls for the MMP-9 -1562 C>T polymorphism. Heterozygous carriers of the variant allele T had roughly a 2-fold increase in susceptibility for SGC compared to wild type homozygotes (CC) ($p=0.02$). This increase in susceptibility was also confirmed using the dominant model (OR 2.04, 95% CI 1.12-3.73, $p=0.02$ for the CT+TT genotypes).

Discussion

SGCs are relatively rare malignancies. Several studies have indicated the role of genetic factors in the pathogenesis of SGCs. Being a multifactorial and multistep disease, there might be complex interactions between multiple risk alleles. In this case-control study, we assessed the individual role of 3 functional SNPs in 2 genes (MMP-2 and MMP-9) implicated as potential modifiers for the predisposition to SGCs. Our results showed that MMP-2 -1306 CT, MMP-2 -1306 CT+TT genotypes and MMP-9 -1562 CT and MMP-9 -1562 CT+TT genotypes were significantly associated with an increased risk of SGCs. However, there was little association of MMP-2 -1575 G>A with SGC risk.

The MMP-2 -1306 C>T transition in a core recognition sequence of Sp1 (CCACC box), abolishes the Sp1-binding site and also diminishes promoter activity. Transient transfection experiments showed that reporter gene expression driven by the C allele was significantly greater than reporter gene expression driven by the T allele, both in epithelial cells and macrophages [15]. This SNP has an interactive effect on MMP-2 transcription. To date, several studies have evaluated this genetic variation in MMP-2 in relation to cancer susceptibility but the results are conflicting. A large study showed that the MMP-2 -1306 C>T polymorphism is associated with increased risk of breast cancer but the effect was not significant [16]. However, a meta-analysis from Asian population suggested that CC genotype of MMP-2 -1306 C>T polymorphism may contribute to head and neck cancer susceptibility [17] and another meta-analysis again from Asian population revealed that -1306T allele acts as a protective factor in

digestive tract cancers [18]. On the other hand, a recent study from India showed that the T allele increased the risk of gallbladder cancer [19]. Also, another study showed that genotypes MMP-2 -1306 CT and MMP-2 -1306 TT increased the risk of prostate cancer [20]. Up until now there is no report on MMP-2 polymorphisms in SGCs, and the present study showed increased risk for SGCs owing to variant containing MMP-2 -1306 CT and CT+TT genotypes of these SNPs. This could be explained by the possibility that MMP-2 polymorphism influences the inflammatory response by acting on alternate substrates as MMP-2 was shown to contribute to inflammation by being an alternative activator of pro-interleukin 1- β in the absence of the cytokines' favored activator caspase-1 [21].

The MMP-2 -1575G>A variant was located immediately to 5' to a half-palindromic potential estrogen receptor binding site and -1575G allele functioned as an enhancer, whereas the -1575A allele lost its transcription activation. This polymorphism has been found to play an important role in the development of certain diseases such as various metabolic disorders or cardiovascular diseases [22-24]. The significance of this polymorphism in carcinogenesis is yet to be determined. The results of our study did not implicate this polymorphism as a risk factor for SGCs, since the increase in susceptibility, with borderline significance, was only found when the dominant model was applied (GA + AA vs GG).

MMP-9 has also been implicated in tumor invasion and metastasis. However, only scarce work has been carried out on MMP-9 and SGC. Our study indicates that the heterozygous carriers of the variant allele have a 2-fold increase in risk of developing SGCs. The variant T allele is associated with higher promoter activity compared to the more common C allele [25]. In line with this, a previous study on Serbian population reported a 4-fold increase in the risk for pleomorphic salivary gland adenoma in heterozygous carriers of the variant allele T [26]. Several studies showed the association between the -1562T allele and vascular disease, gastric cancer, and head and neck squamous cell carcinoma [27-29]. Contrary to those reports, Park et al. [30] found that the MMP-9 -1562CC genotype was more common in patients with colorectal cancer. However, the risk of lymph node metastasis of colorectal cancer was higher in patients with -1562T allele.

In conclusion, our findings suggest MMP-2 -1306 C>T and MMP-9 -1562 C>T polymorphisms

seem to influence the development of SGCs, whereas MMP-2 -1575 G>A polymorphism seems to be of a minor importance.

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Conflict of interests

The authors declare no conflict of interests.

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