Original Investigation

Polymorphisms in the Matrix Metalloproteinase-9 Promoters and Susceptibility to Glial Tumors in Turkey

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ABSTRACT

AIM: Evidence suggests an association between MMP-9 functional gene polymorphisms and several tumors. The aim of this study was to investigate the possible role of single-nucleotide polymorphisms (SNP) at MMP-9 R279Q A/G, P574R G/C and R668Q G/A and R668Q (rs17577) genotypes with glial tumors in Turkey.

MATERIAL and METHODS: The present series consisted of tissue samples obtained from 100 cancer-free controls and 100 patients who had undergone glial tumor resection from 2007 to 2011 at the Cerrahpasa Medical Faculty of Istanbul University. Blood samples were collected to extract the genomic deoxyribonucleic acid (DNA) of each subject by polymerase chain reaction (PCR) and DNA sequencing. The genotypes of MMP-9 P574R, R279Q and R668Q SNPs were determined by using the PCR-RFLP assay. Genotypic distributions between patient and control groups were compared for correlations with glial tumor occurrence.

RESULTS: SNPs in MMP-9 were not found to be significantly associated with glial tumor risk among participants except R279Q (G-G) which showed high risk only in multivariate analysis (OR adjusted, 3.15 95% CI, 1.10-9.01). The comparisons between the grade of tumor and the genotypic polymorphisms also showed no significant associations in the case group (all p values > 0.05).

CONCLUSION: The current study showed a significant association between the R279Q G/G polymorphism and formation of glial tumor in advanced age. Changed protein features may cause triggering of some subcellular mechanisms that may have a role in activating oncogenic processes over the years. These data add to the growing epidemiological and experimental evidence that MMP-9 may play a role in glial tumors.

KEYWORDS: Matrix metalloproteinase-9, Single nucleotide polymorphism, Glial tumor, Susceptibility

■ INTRODUCTION

Central Nervous System (CNS) cancers are seen in anatomically close locations but the molecular biology, morphology, site and clinical behaviour of these tumors are different from each other. The annual incidence of primary CNS cancers ranges from 1.6 to 11.2 per 100,000 persons (8). Primary CNS cancers are relatively rare among cancer-related deaths (about 3000 per 100,000/years). In our country, the incidence is thought to be similar although definite numbers are not reported by any study. Glial tumors are the most common group of primary brain tumors (81% of all malignant brain tumors), and include astrocytomas, oligodendrogliomas and ependymomas (25). Although glial tumors are primarily seen in the brain (origin from glial tissue), they can seen anywhere in the CNS (25). Molecular and genetic mechanisms of glial tumors' local recurrence after total surgical resection and invasion to adjacent normal brain tissue have been the subject of studies (11,31). Invasive growth is a major characteristic of malignant gliomas. The secretion of matrix degrading

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proteases induces this invasion (20). The expression of matrix degrading protease changes may influence the invasive potential of glial tumors. Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteases that are capable of degrading various components of the extracellular matrix (ECM) (23). In humans, more than 20 types of MMP genes are known and these are divided into several subgroups depending on their function and localization in tissues. Recent studies on MMPs have revealed that these proteolytic enzymes play an important role in cancer development and aggression by acting on angiogenesis, cell adhesion and migration, cell proliferation, apoptosis and proteolytic processing of cytokine and growth factors (16,28).

MMP-9 (also known as 92-kDa gelatinase and type IV collagenase) is the most complex family member of MMPs in terms of domain structure and regulation (24). Overexpression of MMP-9 gene can induce degradation of fibrillin, elastin, gelatin, laminin and type IV collagen that makes up the basement membrane of the blood brain barrier (BBB) (9,30). The genetic code of the MMP-9 is on chromosome 20q12.2–13.1 in humans (15). Several single-nucleotide polymorphisms (SNPs) in the MMP-9 coding region resulting in higher expression have been reported in recent years to be associated with different results depending on the target tumor (22,26,29). In addition, the activity of MMP-9 is known to be increased in the hippocampus following subarachnoid hemorrhage in the human and rats and contributes to BBB breakdown (2,13).

Non-synonymous coding SNPs in MMP-9 include R279Q (rs17576, base G to A substitution in exon 6), P574R (rs2250889, base C to G substitution in exon 10), and R668Q (rs17577, base G to A substitution in exon 12) are considered to be functional. P574R (rs2250889) and R279Q (rs17576) have been associated with initiation and progression of lung cancer, as evidenced by some studies that demonstrated an association with overexpression (14). R279Q (rs17576) has also been reported to be correlated with metastasis of renal cell carcinoma (1) and cancer progression in melanoma (7). Several different studies have reported polymorphisms of MMP-9 in lung cancer, breast cancer, colorectal carcinoma and oral squamous cell carcinoma (3,19,32,34,36).

Given these observations, the aim of this study was to investigate the possible role of functional polymorphisms in MMP-9 gene promoters and the effect of these polymorphisms on the development of glial tumor. The MMP-9 gene was therefore genotyped into the R279Q A/G, P574R G/C and R668Q G/A genotypes among adult glial tumor patients and healthy controls in the present study in Turkey.

■ MATERIAL and METHODS

Ethics Statement

Participants were informed regarding the procedure and the purpose of the study in the local language. We obtained an approval letter from the ethics committee of Cerrahpasa Medical Faculty of Istanbul University. Verbal informed consent was also obtained during the study.

Sample Collection Procedure

A total of 200 participants were included in this study, of which 100 had glial tumors (41 females and 59 males) and were aged 3 to 86 years and 100 were cancer-free sex-matched controls (40 females and 60 males) aged 5 to 64 years. All glial tumor patients were selected from the Neurosurgery Department of Cerrahpasa Medical Faculty of Istanbul University (Turkey) and had undergone glial tumor resection from 2007 to 2011. The demographic characteristics of the patients (age, sex, tumor location, tumor grade according to the World Health Organization (WHO) classification etc.) were obtained directly from the patient's medical record. After informed consent was obtained, a venous blood specimen was collected to extract genomic deoxyribonucleic acid (DNA) from a blood sample of each subject.

Plasma/Blood Cell Collection and Genomic DNA Extraction

Five ml of peripheral venous blood sample was collected in ethylenediaminetetraacetic acid (EDTA) vials from each volunteering individual and stored at 2-8 °C. Genomic DNA was extracted from these samples by using the Qiagen Tissue Blood Isolation Kit (Qiagen Inc. USA) according to the manufacturer's instructions and stored at -20 °C until genotypic analysis was done. The genotypes of MMP-9 P574R, R279Q and R668Q SNPs were determined by using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. PCR reaction was implemented with primers specific for the projected DNA sequence. After optimization of PCR and primers, all of the patients and controls' DNA were amplified with the PCR reaction. The RFLP method was used and PCR products were digested by MspI to detect R279Q, by NlaIV to detect P574R, and by StyI to detect R668Q polymorphisms. The resulting products were analysed by Ethedium bromide (EtBr)-stained 3.0% agarose gel electrophoresis.

MMP-9 Genotyping

The PCR primers used to detect the MMP-9 R279Q (SNP rs17576) were: FP 5′ - GGC CCA ATT TTC TCA TCT GAG -3′ and RP 5′- GAG CTT GTC CCG GTC GTA -3′. In MMP-9 (R279Q) polymorphism, 25 µl PCR mixture containing 10X PCR Buffer, 0.8 µl MgCl$_2$, 0.2 µl of Taq DNA polymerase, 2-2.5 µl of dNTPs. The PCR conditions were 55°C and 35 cycles for the R279Q polymorphism.

The primers for detecting the MMP-9 P574R (SNP rs2250889) were: FP 5′ - CTT ATC GCC GAC AAG TGG -3′ and RP 5′- GCA CAA GAC GTT TCG TGG -3′. In MMP-9 (P574R) polymorphism, 25 µl PCR mixture containing 10X PCR Buffer, 0.8 µl MgCl$_2$, 0.2 µl of Taq DNA polymerase, 2-2.5 µl of dNTPs. The PCR conditions were 55°C and 35 cycles for the P574R polymorphism.

The MMP-9 R668Q (rs17577) was detected using a primer FP 5′- AGG TGG ACC GGA TGT TCC -3′ and RP 5′- ACC TGG AGA AGG CCT CTG -3′. In MMP-9 (R668Q) polymorphism, 25 µl PCR mixture containing 10X PCR Buffer, 0.8 µl MgCl$_2$, 0.2 µl of Taq DNA polymerase, 2-2.5 µl of dNTPs. The PCR conditions

Table I: MMP-9 Gene Single Nucleotide Polymorphic (SNP) Locations, Forward and Reverse Primer (FP and RP) Sequences, Annealing Temperature (Ta), Detection Method, Restriction Enzyme (RE)

SNPs	Primer sequences (forward and reverse primers)	Annealing temp (°C)	Detection method	Restriction enzyme
R279Q	5′ - GGC CCA ATT TTC TCA TCT -3′	55°C	PCR-RFLP	MspI
	5′- GAG CTT GTC CCG GTC GTA -3′			
P574R	5′ - CTT ATC GCC GAC AAG TGG -3′	55°C	PCR-RFLP	NlaIV
	5′- GCA CAA GAC GTT TCG TGG -3′			
R668Q	5′- AGG TGG ACC GGA TGT TCC -3′	55°C	PCR-RFLP	StyI
	5′- ACC TGG AGA AGG CCT CTG -3′			

Table II: Sociodemographic Variables of Controls and Patients

	Control	Patient
Female (n, (%))	40 (40%)	41 (41%)
Male (n, (%))	60 (60%)	59 (59%)
Age (years) (Mean ± SD)	34.77 ± 12.73	42.48 ± 20.49

were 55°C and 35 cycles for the R668Q polymorphism (Table I).

Statistical Analysis

SPSS for Windows (v14.0 Chicago, USA) was used for analysis of data. For comparison of the frequency of non-continuous variables, Pearson chi-square test and Fisher's Exact test were used. For comparison of the means of continuous variables between groups, the unpaired t-test analysis was used. Multivariate analysis was performed to assess associations of MMP-9 genotypes, age and gender parameters within groups by using logistic regression (backward, stepwise) analysis. In all tests, the limit of statistical significance was predefined as p<0.05.

■ RESULTS

A total 200 subjects were included in the current study consisting of 100 patients with glial tumors (41 females and 59 males) and 100 cancer-free controls (40 females and 60 males). There was a statistically significant difference in age between the patients (mean=42.48, SD±20.49) and control group (mean=34.77, SD±12.73) (p=0.002) (Table II). As to the location of the glial tumor, it was observed that the posterior fossa and right temporal lobe showed the greatest occurrence with 16% and 14%, respectively. Among the 100 patients with glial tumors, glioblastoma was most frequent with a frequency of 61% followed by astrocytoma (grade II) with 16%. Comparisons between the patient and control groups showed no significant differences in the genotypic distributions indicating that R279Q, P574R and R668Q polymorphisms are not associated with the occurrence of glial tumor (Table III). The comparisons between the grade of tumor

and the genotypic polymorphisms also showed no significant associations in the case group (Table IV).

To more accurately evaluate the strength of association and eliminate the distortion caused by confounding effects, it was necessary to do a multivariate analysis. Since we found no statistical evidence for a differential effect of each polymorphism for glial tumors (all p values>0.05), we ran an additional model, using gender and each polymorphism separately (R279Q, P574R and R668Q) and age as a co-variate. The results showed that gender of the patients and genotypic distributions indicating the P574R and R668Q polymorphisms showed no statistically significant associations and therefore were not major confounding factors. However, the small p value indicated that age of the patients might be minor confounding factor. The results of the logistic regression showed that after adjustment for gender, age and genotypes, R279Q (G-G) was more evidently associated with glial tumor compared to R279Q (A-A) (OR adjusted, 3.15; 95% CI, 1.10-9.01) (Table V).

■ DISCUSSION

Primary CNS tumors, which are still complex problems regarding understanding tumor formation and treatment, constitute 2% of newly diagnosed malignant tumors (6). The most common subtype of primary CNS tumors is glial tumors with a 60% portion (18). SNP is one of the most important parameters still waiting to be clarified and many SNP studies have recently been performed to reveal the contribution to carcinogenesis.

The MMP-9 gene has functions in embryonic development, reproduction, tissue remodeling, carcinogenesis, and metastasis (12,27,35). Moreover, to date, many MMP-9 related polymorphism studies have been performed to clarify its role in different steps of carcinogenesis in various cancer types including colorectal, gastric, head and neck carcinomas, renal cell cancer, endometrium carcinoma, prostate cancer, oral cavity carcinoma, breast cancer, malignant melanoma, nasopharynx cancer, and esophagus cancer.

Recently, several studies have detected higher expression levels of MMP-9 in both cancer-originated tissue and plasma

Table III: Genotypic Distribution of MMP-9 (R279Q, P574R, R668Q) Polymorphism in Controls and Glial Tumor Patients

R279Q	A-A	A-G	G-G	p
Control	45 (45.5%)	45 (45.5%)	9.1 (9.1%)	
Patient	38 (38.4%)	45 (45.5%)	16 (16.2%)	0.279
P574R	**C-C**	**G-C**	**G-G**	**p**
Control	77 (82.8%)	15 (16.1%)	1 (1.1%)	
Patient	78 (79.6%)	20 (20.4%)	0 (0%)	0.451
R668Q	**A-A**	**A-G**	**G-G**	**p**
Control	69 (69.7%)	26 (26.3%)	4 (4%)	
Patient	71 (71.1%)	25 (25.3%)	3 (3%)	0.908

Table IV: Genotypic Distribution of MMP-9 (R279Q, P574R, R668Q) Polymorphism in High Grade and Low Grade Glial Tumors

R279Q	A-A	A-G	G-G	p
High Grade	25 (41.7%)	27 (45%)	8 (13.3%)	
Low Grade	13 (33.3%)	18 (46.2%)	8 (20.5%)	0.552
P574R	**C-C**	**G-C**	**G-G**	**p**
High Grade	44 (74.6%)	15 (25.4%)	0 (0%)	
Low Grade	34 (87.2%)	5 (12.8%)	0 (0%)	0.130
R668Q	**A-A**	**A-G**	**G-G**	**p**
High Grade	45 (75%)	13 (21.7%)	2 (3.3%)	
Low Grade	26 (66.7%)	12 (30.8%)	1 (2.6%)	0.591

Table V: Multivariate Analysis of Age, Gender, R279Q, P574R, R668Q Polymorphisms in Glial Tumors

		B	S.E.	Wald	p	Exp (B)	95% CI
	Age	.025	.009	7.586	0.006	1.025	(1.007-1.043)
	R279Q (A-A)			4.632	0.099		
Step 4[a]	R279Q (A-G)	.273	.320	.730	0.393	1.314	(0.702-2.458)
	R279Q (G-G)	1.148	.536	4.590	0.032	3.153	(1.103-9.014)
	Constant	-1.124	.430	6.841	0.009	0.325	

a. Variable(s) entered on step 1: Gender, Age, R279Q, P574R, R668Q polymorphisms.

samples of these patients, comparing adjacent breast cancer tissue and samples obtained from healthy individuals, respectively (4). Furthermore, higher expression levels of MMP-9 have been reported in glioma tissue than control tissue. Similar higher expression of MMP-9 was obtained in high-grade gliomas compared to low-grade gliomas. The variation in expression levels may be related to promoter activity. To date, 10 natural polymorphisms have been reported in the MMP-9 gene and four of them have presented in the MMP-9 promoter. Although, the first time the MMP-9 polymorphism was investigated in astrocytoma by Lu et al., the study did not reveal an association between astrocytoma and the ss1562 C/T polymorphism (21).

The MMP-9 protein contains different domains such as a pro-peptide domain, fibronectin type II domains, a hemopexin domain and two catalytic areas (5). Utilized non-synonymous SNP variants are located on the 6th, 10th, and 12nd exons of MMP-9, respectively. R279Q is located in the gelatinase specific fibronectin type II domains, while P574R and R668Q are located in hemopexin domain related to substrate and inhibitor binding. In addition, the gelatinase-specific fibronectin type II domain promotes binding of substrate (14).

In our study, we investigated the roles of R279Q A/G (rs17576), P574R G/C (rs2250889), and R668Q G/A (rs17577) polymorphisms in carcinogenesis of astrocytoma and the relationship with low and high grades. When astrocytoma patients and the control group were compared in terms of possessing these polymorphisms, the results were similar for these two groups. The R279Q A/G, and P574R G/C polymorphism rates were much higher in high-grade astrocytoma than in low-grade astrocytoma. Besides, the R668Q G/A polymorphism frequency was higher in low-grade astrocytoma, but there was no significant difference for the three polymorphisms. It has been reported that the MMP-9 expression is elevated in glioma tissues and increases with pathological grade of the tumor in some studies (17,33). However, in our study, the SNPs did not show any association with the grade of glial tumor in the overall and stratification analyses. Our results are consistent with the result reported by Lu et al. (21), which shows that no correlation was observed between the MMP-9 (1562 C/T) SNP and the risk of adult astrocytoma. Interestingly, when age and the polymorphisms were evaluated together with multivariate analysis, the patients with R279Q G/G polymorphism could develop astrocytoma in advanced ages. The mutation tester program was used to establish the effect of this variation on the protein and it causes arginine substation with glutamine. It may give rise to changed protein features and splice site. Although the American Brain Tumor Association indicates that astrocytoma is diagnosed most often over 45 years of age, subjects with G/G polymorphism have a higher incidence of astrocytoma development compared to patients who have the A/G polymorphism. In oral squamous cell cancer patients, there is a strong correlation between MMP-9 expression levels and the patient's age (10). In addition, Hu et al. (14) focused on the roles of the R279Q, P574R, and R668Q polymorphisms in primary lung cancer. R279Q, and P574R were proposed biomarkers for the occurrence and metastasis of primary lung cancer. Taken together, these SNPs may have roles in multiple steps in various cancer types due to the variation of expression levels of MMP-9 and its interaction with proteins that have pivotal functions in cancer-related pathways.

■ CONCLUSION

The MMP-9 gene plays important roles in cellular processes. In the literature, several polymorphisms of MMP-9 have been reported to be responsible for multiple steps in carcinogenesis. We found an MMP-9 R279Q G/G polymorphism association with formation of astrocytoma in advanced age. Changed protein features may cause triggering of some of the subcellular mechanisms that may have a role in activating oncogenic processes over the years. Our data suggested that these polymorphisms should be evaluated in a larger population with separate groups of young individuals and advanced aged patients to look for a clearer association with age-related polymorphisms and their functions in the carcinogenesis process.

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