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Original Article

Correlation of MMP-9 gene polymorphisms with aneurysmal subarachnoid hemorrhage and its prognosis

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Abstract: *Background:* Aneurysmal subarachnoid hemorrhage (aSAH)-associated gene polymorphism is of great significance for the accurate diagnosis and individualized treatment of aSAH. This study aims to investigate the expression of matrix metalloproteinase-9 (MMP-9) gene in the peripheral blood of patients with aneurysmal subarachnoid hemorrhage (aSAH) and explore the correlations of MMP-9 polymorphisms with the onset and prognosis of the disease. *Methods:* A total of 80 aSAH patients (aSAH group) and 24 healthy (control group) people receiving physical examination were enrolled in the study. Western blotting was applied to detect the expression of MMP-9 gene in the peripheral blood in aSAH patients and healthy people. The genotyping of single nucleotide polymorphisms (rs42512, rs56212 and rs61221) in the promoter region of MMP-9 gene was analyzed by means of conformation-difference gel electrophoresis. Chi-square test was applied to examine the applicability of the distribution frequency of MMP-9 genotypes with genetic equilibrium law. The correlations of MMP-9 alleles and gene polymorphisms with the onset and prognosis of aSAH were determined. *Results:* The expression of MMP-9 protein in aSAH group was significantly higher than that in control group ($P<0.05$). The Hardy-Weinberg equilibrium analysis showed that MMP-9 gene polymorphisms were in agreement with the genetic equilibrium law. According to the results of genetic association analysis, only the polymorphism rs42512 and its alleles were significantly correlated with the onset and prognosis of aSAH ($P<0.05$). However, polymorphisms rs56212 and rs61221 and their alleles had no association with the onset and prognosis of aSAH ($P>0.05$). *Conclusion:* The polymorphism rs42512 in the promoter region of MMP-9 gene is related to the onset of aSAH, which provides further evidence for the diagnosis of aSAH.

Keywords: MMP-9, gene polymorphism, aneurysmal subarachnoid hemorrhage, correlation

Introduction

As an emergency of the nervous system, subarachnoid hemorrhage (SAH) represents a subtype of stroke with high incidence and mortality rates [1]. However, compared with ischemic stroke and intracranial hemorrhage, SAH occurs in much younger populations. It has been reported that about 85% of SAH is induced by ruptured intracranial aneurysms, which leads to aneurysmal SAH (aSAH) [2, 3]. Approximately 10% of aSAH patients die before treatment due to the sudden attack and severe conditions of the disease [4]. Meanwhile, the 3-month mortality rate of aSAH patients is as high as 47-49%, and most of the survived patients still have serious sequelae [5]. Currently, there is a lack of precise prediction criteria for the occurrence and development of aSAH in clinic. Therefore, one must determine the high-risk populations of aSAH and take preventive measures timely, so as to effectively prevent and cure aSAH.

Many studies have manifested that aSAH is a disease resulting from the combined action of multiple factors. For instance, environment and gene, and advanced age, gender, hypertension and smoking history are determined as the high-risk factors for the disease [6-8]. In addition, a large number of studies have also verified that genetic susceptibility is closely related to the occurrence and development of aSAH. Hence, the investigation of an aSAH-associated gene polymorphism is of great significance for the accurate diagnosis and individualized treatment of aSAH in the future. Studies have revealed that the activity of matrix metalloproteinase-9 (MMP-9) is directly correlated with

Table 1. Primer sequences and product sizes at different sites in the promoter region of MMP-9 gene

Site	Primer sequence (5'-3')	Product (bp)
rs42512	Forward: AGCTGGATCGTATCGGGCA Reverse: GGGCAGCACGCTACGCATCGA	203
rs56212	Forward: GGCTGATGCTAGCTGATCGTA Reverse: AGCTGTACGATGCAGTC	245
rs61221	Forward: GCATCGATGCCTCGTACACAA Reverse: ACGTAGCTGATCGTAGGTCGA	299

SAH-induced blood-brain barrier damage [9, 10], but the correlations of MMP-9 gene polymorphisms with the onset and prognosis of the aSAH patients remain to be further clarified. Therefore, in this research, the expressions of MMP-9 in aSAH patients and healthy people receiving physical examination were detected, in order to identify the genetic associations of the gene polymorphisms (rs42512, rs56212 and rs61221) in the promoter region of MMP-9 with the genetics and pathogenesis of aSAH.

Patients and methods

Objects

A total of 80 aSAH patients treated in Xiangyang Central Hospital from January 2015 to May 2018 were selected as the research objects, including 46 males and 34 females aged of 55.71±12.34 years old. 4 mL venous blood was collected, added with sodium citrate for anticoagulation and frozen in a refrigerator at -20°C for standby use. In addition, 24 healthy people receiving physical examination in the same time period were enrolled as controls, including 13 males and 11 females aged of 57.51±12.19 years old. This research was approved by the Ethics Committee of our hospital, and all the enrolled objects signed an informed consent.

Detection by Western blotting

After the peripheral blood in Control group and aSAH group was centrifuged at 1500 g, 4°C, the supernatant was maintained into EP tubes. Later, the protein concentration was determined through BCA method and ultraviolet spectrophotometric assay, and all the sample proteins was adjusted at equal concentration. Next, the proteins were subpackaged and preserved in the refrigerator at -80°C. The total protein was extracted and subjected to SDS-PAGE. After that, the protein in the gel was transferred onto a PVDF membrane, followed by incubation with primary antibody at 4°C overnight, incubation with goat-anti-rabbit secondary antibody in the dark for 1 h. Odyssey membrane scanner was used to scanned and quantified the protein bands. The level of the targeted protein was normalized by GAPDH.

Extraction of deoxyribonucleic acid (DNA)

The genomic DNA of EDTA-anticoagulated blood was extracted according to the instructions of DNA extraction kit (Guge Bio-Technology Co., Ltd.). Then 2 μL DNA was taken to measure the mass in 1.5% agarose gel electrophoresis, along with ultraviolet spectrophotometer.

Polymerase chain reaction (PCR) amplification

Primers for rs42512, rs56212 and rs61221 in the promoter region of MMP-9 gene were designed separately for amplification, and the primer sequences at each site were shown in **Table 1**. PCR system (20 μL): DNA template (2.0 μL), 2× MIX (10.0 μL), forward primers (0.4 μL), reverse primer (0.4 μL) and ddH_2O (7.2 μL). PCR amplification conditions: at 95°C for 120 s, 94°C for 30 s, 57°C for 90 s and 72°C for 60 s, 35 cycles in total, followed by extension at 72°C for 10 min. Next, the amplification of the gene fragments was examined by agarose gel electrophoresis.

Ligase detection reaction

The upstream and downstream probes applied in the reaction were designed and synthesized by BGI. All the upstream probes were prepared into a probe mixture at a concentration of 12.5 pmol/μL modified by 5' terminal phosphorylation. Ligase detection reaction system (3.05 μL): ligases (0.05 μL), buffer solution (1 μL), PCR product (1 μL) and probe mixture (1 μL). PCR amplification conditions: at 95°C for 120 s, 94°C for 15 s and 50°C for 25 s for a total of 30 cycles. After that, the concentration was measured using the ultraviolet spectrophotometer. Subsequently, BGI was entrusted to sequence the target gene and analyze its sements. All the data were analyzed using GeneMapper (**Table 2**).

Table 2. Probe sequences of the ligase reaction and product sizes at different sites of MMP-9

Site	Probe	Primer sequence (5'-3')	Product (bp)
rs42512	rs42512	P-ACGTAGCCCGACCCTTTTTTTTTTT-FAM	97
	rs42512-C	ACCCCTTTTTTTTTTTTTACCCATTTTTTTTTAT	
	rs42512-T	TTCGCTGATCAAATTTTTGCGACGAGTTTCAGCTAGA	
rs56212	rs56212	P-AGCCATGCACCCAATTTTTTTTTTTTTTTTTTT-FAM	131
	rs56212-A	TTTTTTTTTTTTACCGTGTTTTTTCGTAGCTAAAC	
	rs56212-T	AGGGGGGCCCCCTTTTTTTTTTTTTTTTACGATCGATG	
rs61221	rs1066283	P-ACGGGATGCCATTTTTTTTTTTTTTTTTT-FAM	144
	rs1066283-A	ACCCCTGATCTTTTTTTTTTTTTTTTTTTGCGGACGAG	
	rs1066283-G	ACGATGCTAGGGTTTTTTTTTTTTGCGGCCAAA	

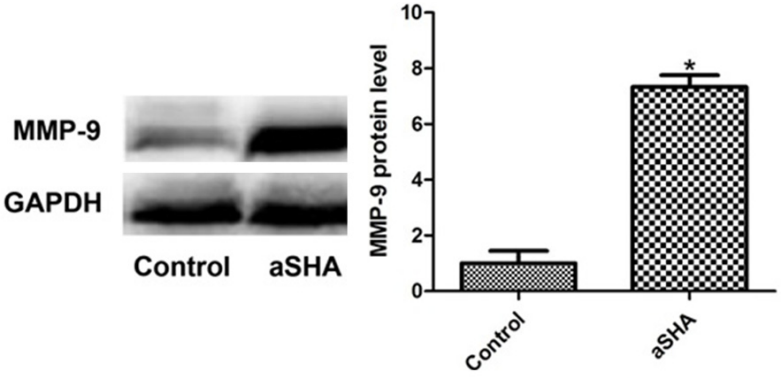

Figure 1. Expression level of MMP-9 in peripheral blood between the two groups of patients: Control: Control group, aSAH: aneurysmal subarachnoid hemorrhage group, *P<0.05 vs. control group, a significant difference.

Statistical analysis

SPSS 22.0 was adopted to analyze all the data. The enumeration data were presented as frequency and percentage, and measurement data were expressed as mean ± standard deviation. The genotype frequency in the samples was calculated and examined by Hardy-Weinberg genetic equilibrium formula. Chi-square test was used for examination and multiple comparisons of enumeration data, and t-test and analysis of variance were adopted for the measurement data. P<0.05 was considered a significant difference.

Results

Expression of MMP-9 in peripheral blood between the two groups of patients

According to the results of western blotting, the level of serum MMP-9 in aSAH group was elevated markedly, which was about 8.65 times that in the control group (P<0.05) (**Figure 1**). This suggests that MMP-9 plays a crucial role in the occurrence and development of aSAH. MMP-9 may serve as a diagnostic marker for aSAH in the peripheral blood.

MMP-9 gene polymorphisms

As shown in **Figure 2**, the DNA fragments obtained from elution at different sites were subjected to agarose gel electrophoresis, and it was indicated that the PCR purification results at the three sites were preferable. We then analyzed the results of rs42512, rs56212 and rs61221 in MMP-9 gene. In both Control group and aSAH group, rs42512, rs56212 and rs61221 were cleaved by BstU I restriction enzyme. The results manifested that rs42512 had alleles C and T and genotypes CC, AT and AT. There were two alleles (A and T) and three genotypes (AA, AT and TT) in rs56212, and rs61221 had A and G alleles as well as genotypes AA, AG and GG (**Figure 3**).

Hardy-Weinberg equilibrium test

The Hardy-Weinberg equilibrium formula was applied to examine the results of linkage disequilibrium test at different sites in MMP-9 gene. According to **Table 3**, these sites were in agreement with the equilibrium test in each group (r^2<0.33).

Correlations of MMP-9 gene polymorphisms with the onset of aSAH

The genotype frequencies of each polymorphism in the patients in Control group and

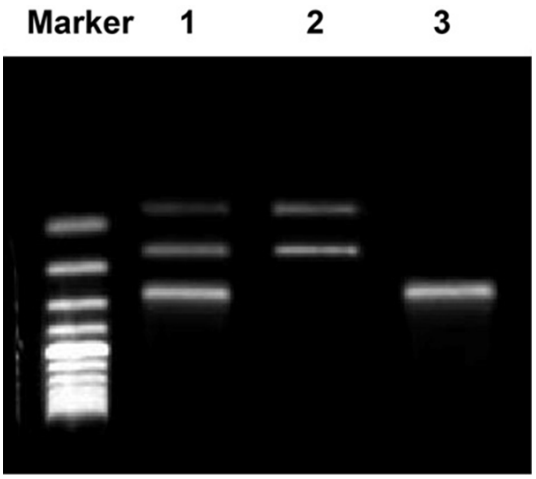

Figure 2. Purification results at different sites in MMP-9 gene. 1: rs42512, 2: rs56212, 3: rs61221.

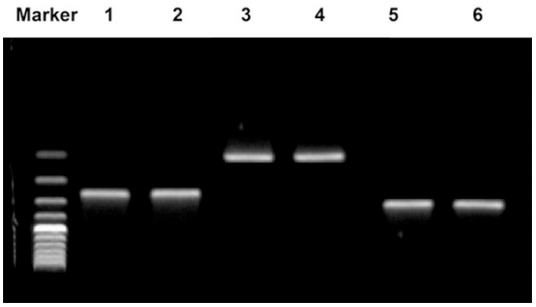

Figure 3. Analysis results of rs42512, rs56212 and rs61221 in MMP-9 gene.

Table 3. Results of linkage disequilibrium test at different sites in MMP-9 gene in each group

Site	r^2		
	rs42512	rs56212	rs61221
rs42512	-	0.013	0.121
rs56212	0.013	-	0.249
rs61221	0.121	0.249	-

aSAH group were shown in **Table 4**. It could be seen that the gene polymorphism rs42512 had significant association with the onset of aSAH (P<0.05), while rs56212 and rs61221 were not statistically correlated with the onset of aSAH (P>0.05).

Correlations of MMP-9 allele types with the onset of aSAH

According to the frequencies of the allele types at 3 sites in the aSAH group and control group shown in **Table 5**, the allele types, C, T of rs-42512 were strongly related to the onset of aSAH (P<0.05), while correlations of the allele types of rs56212 and rs61221 with the onset of aSAH were not discovered (P>0.05).

Correlations of rs42512, rs56212 and rs61221 in MMP-9 gene with survival analysis of patients

The allele types at different sites in aSAH patients were grouped and their correlations with the patients' prognosis were analyzed using Kaplan-Meier method (**Figure 4**). The results indicated that the prognosis of aSAH patients with T allele of rs42512 in MMP-9 was superior to that of patients with C allele (P<0.05), while the allele types at the other two sites were not associated with the prognosis of aSAH patients (P>0.05).

Discussion

Aneurysmal subarachnoid hemorrhage (aSAH) is a severe clinical disease, and it is very difficult for the patients to recover completely and even survive although they have received surgical and medical treatments. A majority of survived patients also present varying degrees of aphasia, hemiplegia and deafness, which seriously affect life quality [11, 12]. Therefore, the determination of gene polymorphisms of aSAH can provide valuable references for the individualized treatment by physicians in the future.

The MMP family plays crucial roles in ischemic and hemorrhagic strokes. The study of Solveig et al revealed that in SAH patients, there was a much higher MMP-9 level but a notably lower MMP-2 level in the peripheral blood than those in healthy people [13]. In another study with rat models of SAH, the level and activity of MMP-9 started rising at 12 h and reached the top at 24 h after SAH in the rats. Meanwhile, the apoptosis of the vascular endothelial cells was triggered at 12 h after SAH, and reached a peak at 24 h after SAH. Moreover, the laminin declined to the lowest level, and Caspase-3 reached the highest level at 24 h after SAH. The study demonstrates that the impacts of MMP-9 on SAH are possibly mediated by the apoptosis of vascular endothelial cells [14]. The activation of nuclear factor-kappa B (NF-κB)/MMP-9 signaling pathway also represents a leading cause of white matter fiber injury and blood-brain barrier

Table 4. Distribution of different genotypes and aSAH at different sites in MMP-9 gene

Group	rs42512			rs56212			rs61221		
	CC	CT	TT	AA	AT	TT	AA	AG	GG
aSAH	10.2%	60.0%	39.8%	23.1%	50.9%	26.0%	20.1%	50.9%	29.0%
Control	24.5%	58.4%	17.1%	24.6%	51.2%	24.2%	19.3%	51.2%	29.5%
C^2		8.678			0.864			0.532	
P		0.002			0.173			0.812	

Table 5. Distribution of different allele types and aSAH at different sites in MMP-9 gene

Group	rs42512		rs56212		rs61221	
	C	T	A	T	A	G
aSAH	74.45%	25.55%	45.00%	55.00%	45.23%	54.77%
Control	30.22%	69.78%	51.21%	48.79%	42.08%	57.92%
C^2	12.511		3.432		0.644	
P	0.007		0.341		0.412	

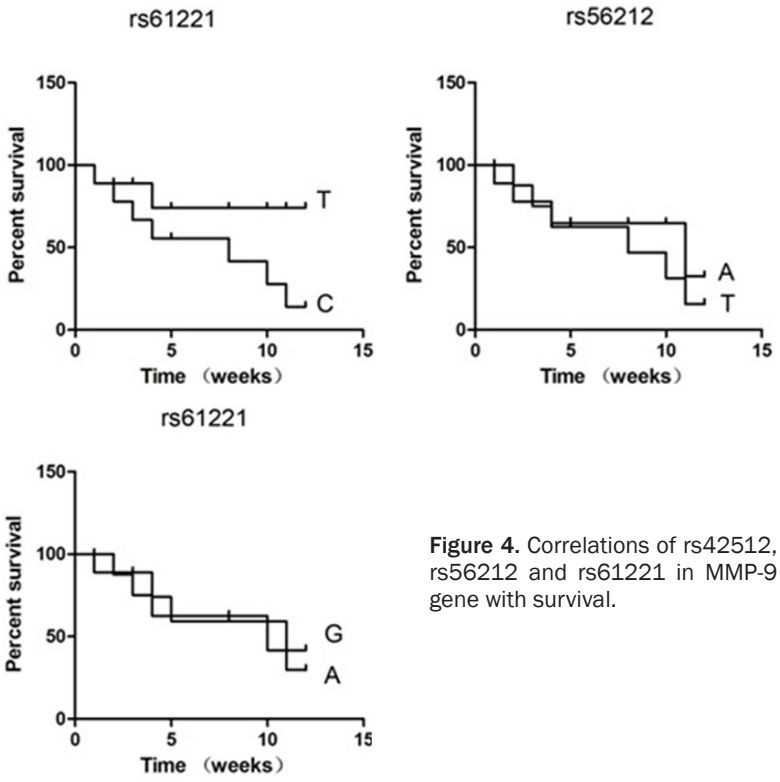

Figure 4. Correlations of rs42512, rs56212 and rs61221 in MMP-9 gene with survival.

damage in SAH. In this regard, many genes, proteins or exogenous medicines capable of repressing MMP-9 can be utilized to treat SAH. For example, it has been illustrated that the activation of NF-κB/MMP-9 signaling pathway during the inhibition of SAH can be decreased by repressing MST1 level in the hippocampus, ultimately relieving SAH [15]. Furthermore, resveratrol was found to alleviate early brain injury after SAH by inactivation of the NF-κB-dependent MMP-9 signaling pathway [16].

On the other hand, MMP-9 gene polymorphisms have close correlations with multiple diseases. Matsumura et al analyzed the -1562 C/T polymorphism in MMP-9 gene of 224 healthy people and 177 gastric cancer patients, and found no statistical difference in the genotype frequency in MMP-9 between the two groups. However, the polymorphism was significantly associated with the degree of invasion, clinical stage, and lymph node metastasis of breast cancer [17]. In terms of primary Sjögren's syndrome, the expression of MMP-9 in the patient's peripheral blood is increased evidently. Similarly, research indicated that there was no difference in the distribution of genotype frequency in MMP-9 between healthy controls and patients with Sjögren's syndrome, while the frequency of T allele in patients without Raynaud's phenomenon was remarkably higher than that in normal controls [18]. Fiotti et al analyzed the relationship between microsatellite polymorphism (MP) of MMP-9 and carotid plaque results of the patients by detecting the MP of MMP-9 in patients with carotid atherosclerosis and healthy people receiving physical examination. The results indicated that the number of

repeats (≥22 CA) in the microsatellite of MMP-9 promoter has a significant statistical correlation with carotid atherosclerosis, particularly in plaques with a thin fibrous cap [19].

In this research, the correlations of polymorphisms rs42512, rs56212 and rs61221 in MMP-9 promoter region with the incidence of aSAH among Chinese Han population were analyzed. Similar to the previous data, our study revealed that the expression of serum MMP-9 was higher in aSAH group [13]. Subsequently, the target genes were classified to record the distribution of genotype and allele frequencies at different sites in different groups. It was manifested that the gene and genotype polymorphisms of rs42512 in MMP-9 were prominently related to the incidence of aSAH ($P< 0.05$), and people with the genotype CC were more vulnerable to aSAH than those with genotype TT, which is in line with the previous finding that the MMP-9 gene polymorphism iss significantly associated with the pathogenesis of certain diseases [17]. Nevertheless, sufficient limitations still exist in this research that a large group of clinical samples, along with detailed clinical information, should be enrolled in the future while the potential bias between the European, American populations and the Chinese Han populations ought to be considered.

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Disclosure of conflict of interest

None.

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