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Original Article

Association of *MMP-2* polymorphisms with occurrence risk of stroke

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Abstract: Aims: This study was designed to discuss the association between matrix metalloproteinase-2 (*MMP-2*) gene C-735T, C-1306T polymorphisms and stroke, meanwhile, to detect the interaction of *MMP-2* polymorphisms in stroke. Methods: In 95 patients with stroke and 120 healthy controls, who were matched in age and gender, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to determine the genotypes of C-735T and C-1306T polymorphisms. The distribution differences of genotypes and alleles between the case and control groups were tested by chi-square. Odds ratio (OR) and 95% confidence interval (CI) were applied to express the relative risk of stroke. Crossover analysis was conducted to check the interaction of *MMP-2* polymorphisms. Results: In *MMP-2* C-735T polymorphism, both of TT genotype and T allele showed the significant frequency difference between the case and control groups ($P<0.05$), which indicated the carriers easily suffered from stroke (OR=3.26, 95% CI=1.07-9.90; OR=1.68, 95% CI=1.06-2.68). Similarly, the CT genotype and T allele in C-1306T polymorphism had statistically significant difference between two groups ($P<0.05$) and they might be associated with the onset risk of stroke (OR=2.83, 95% CI=1.20-6.70; OR=3.89, 95% CI=1.77-8.55). In addition, these two polymorphisms of *MMP-2* gene had the strong interaction. Conclusion: *MMP-2* C-735T and C-1306T polymorphisms are related to the occurrence risk of stroke and both of two play a role in stroke synergistically.

Keywords: *MMP-2*, polymorphism, stroke, interaction

Introduction

Stroke is the second leading cause of death in the world with the characteristics of high morbidity, high disability rate, high mortality, high recurrence rate and various complications [1, 2]. About 1.5-2.0 million people are attacked by stroke every year in China [3]. Stroke is usually divided into hemorrhagic and ischemic stroke and the lifetime prevalence of the latter is higher than that of the former [4]. The symptoms and signs of stroke are sudden weakness, numbness of arms and legs, confusion, language disorder and loss of vision to one side [5]. So stroke brings on heavy economic and life burden for patients themselves and their family, but the effective methods to prevent and treat stroke are not found nowadays. In previous studies, hypertension, diabetes, hyperlipidemia, cigarette consumption were proved to have influences on stroke [6]. What's more, the publications have reported that stroke is caused by the interaction of genetic and environmental factors [7]. Therefore, researches on stroke pathogenesis, treatment and prognosis have always been the focuses of medical field for years.

Matrix metalloproteinases (MMPs) is a type of zinc-dependent endoproteinases and can degrade extracellular matrix (ECM) and transmembrane protein of basement membrane under the physiological conditions [8]. MMPs involve in many physiological processes, such as cell growth, proliferation, differentiation, migration, apoptosis and even the interaction of cells [9]. MMPs are secreted in the form of inactive proenzymes and obtain activity when they are cracked by extracellular proteinases [10]. MMP-2 is an important member in MMPs family encoded by *MMP-2* gene and plays a leading role in the lesion of blood-brain barriers [11]. Plenty of studies indicate that *MMP-2* participates in the formation, migration, fracture

Table 1. Primer sequences of *MMP-2* gene C-735T, C-1306T polymorphisms

Locus	Primer sequence	
C-735T	For.	5'-GGATTCTTGGCTTGGCGCAGGA-3'
	Rev.	5'-GGGGGCTGGGTAAAATGAGGCTG-3'
C-1306T	For.	5'-CTTCCTAGGCTGGTCCTTACTG-3'
	Rev.	5'-CTGAGACCTGAAGACCTAAAGAGCT-3'

collapse of atheromatous plaque, and cerebral ischemia reperfusion, hemorrhagic transformation, and neuron apoptosis through degrading ECM [12-14], which may be closely connected with the occurrence and development of stroke. However, so far, the research on the association of *MMP-2* polymorphisms with stroke is rare.

Therefore, in this study, *MMP-2* C-735T, C-1306T polymorphisms were selected to reveal the association with stroke susceptibility and explain the etiology of stroke. Meanwhile, the interaction between the two polymorphisms of *MMP-2* was analyzed based on stroke to ensure the role of *MMP-2* polymorphisms in stroke.

Materials and methods

Research objects

The case-control study was conducted and a total of 215 subjects were enrolled. All research objects in this study were Han population from northern China without blood relationship each other. This study was reviewed and approved by the Research Ethics Committee of Liaocheng People's Hospital. The process of sample collection was conducted in accordance with the national ethics criteria of human genome research. What's more, all subjects were informed the study process and the written consents were also signed by patients and their family.

The case group consisted of 95 patients with stroke from outpatients and inpatients in neurology department of Liaocheng People's Hospital, including 55 males and 40 females at the age of 32-83 with the mean age of 63.56±9.83. Certainly, the cases were diagnosed by pathobiology and patients with undiagnosed stroke or others relative diseases, such as tumors were excluded. 120 healthy people as the control group had been conducted the physical examination in the same hospital during the same period with the case group, including 68 males and 52 females at the age of 36-78 with the mean age of 64.13±10.22. The healthy physical examinees were frequency-matched with the cases by age and gender and they also had no the history of stroke or tumors. Clinical information of research objects, such as gender, age, and symptom, sign and so on were collected and recorded, and then were made into an Excel form. People who drunk more than once every week were as a drinker, and cigarette was consumed two or more for every day as a smoker. The others indexed was detected according to the conventional criteria.

DNA extraction

The research objects were informed the study purposes and processes in detail. 2 ml fasting venous blood of the objects was collected and stored in the anticoagulative tube with ethylenediamine tetraacetic acid (EDTA). According to manufacturer's instructions, peripheral blood leucocyte genome DNA was extracted by Beijing TIANGEN biochemical blood genome DNA extraction kit, and then put in -20°C refrigerator for standby application.

Genotyping of MMP-2 polymorphisms

In present study, the method of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to determine the genotypes of *MMP-2* polymorphisms. Firstly, primeval design: we referred to the Genebank database on NCBI website for the published sequence of *MMP-2* C-735T, C-1306T polymorphisms. Primer Premier 5.0 software was adopted to design primer following general primer design principles and the primer sequences were showed in **Table 1**. Shanghai Sangon Biotech Co., Ltd took charge of synthesizing the primers.

And then, PCR reaction system: The total reaction system was a volume of 25 μl mixture, including 2.0 μl DNA template, 12.5 μl PCR Mix solution, each 1.0 μl of forward and reverse primers and 8.5 μl sterile ddH_2O. PCR amplification conditions: the amplification process started with 94°C initial denaturation for 5 min; followed by 30 cycles of 94°C degeneration for 30 s, 58°C annealing for 45 s, 72°C extension

Table 2. Detailed characteristics of subjects in case and control groups

Index		Case, n=95 (%)	Control, n=120 (%)	*P*
Age	Mean age/ $\overline{x}$ ±s	63.56±9.83	64.13±10.22	>0.05
Gender	Males	55 (57.89)	68 (56.67)	>0.05
	Females	40 (42.11)	52 (43.33)	
Obesity (BMI>28.0)	Yes	41 (43.16)	33 (27.50)	<0.05
	No	54 (56.84)	87 (72.50)	
Hypertension	Yes	59 (62.11)	38 (31.67)	<0.01
	No	36 (37.89)	82 (68.33)	
Drinking	Yes	47 (49.47)	53 (44.17)	>0.05
	No	48 (50.53)	67 (55.83)	
Diabetes	Yes	23 (24.21)	9 (7.50)	<0.01
	No	72 (75.79)	111 (92.50)	
Hyperlipidemia	Yes	33 (34.74)	14 (11.67)	<0.01
	No	62 (65.26)	106 (88.33)	
Smoking	Yes	44 (46.32)	38 (31.67)	<0.05
	No	51 (53.68)	82 (68.33)	

Note: BMI: body mass index.

for 45 s; and finally 72°C extension for 10 min. The PCR products were checked and preserved at 4°C for standby application.

Finally, enzyme digestion reaction: the enzyme digestion was finished in 20 μl reaction system, including 2 μl restriction enzyme (*Hinf*I for C-735T, *Xsp*I for C-1306T), 10 μl PCR products, 2 μl 10× Loading Buffer Solution and 6 μl sterile ddH_2O. The reaction mixture was incubated in a water bath at 37°C for the overnight. 2% agarose gel electrophoresis (AGE) was performed to separate and determine the genotypes of *MMP-2* polymorphisms.

Statistical analysis

PASW Statistics 18.0 was used to count data and analyze the association between *MMP-2* polymorphisms and stroke. χ^2 test was applied to estimate if the genotype distributions of *MMP-2* polymorphisms in the control group conformed to Hardy-Weinberg equilibrium (HWE). The distribution differences of genotypes, alleles and the others indexes between the case and control groups were also tested by χ^2, and the influences of gene polymorphisms on stroke were evaluated by odds ratio (OR) and 95% confidence interval (95% CI) and $P<0.05$ had statistical significance. Furthermore, crossover analysis was used to analyze the interaction intensity of *MMP-2* C-735T, C-1306T polymorphisms in stroke. The interaction intensity was represented by the synergy index (S), attributable proportion of in-interaction (AP) and relative excess risk of interaction (RERI).

Results

General situations of research objects

This study consisted of 215 objects, with 95 cases and 120 controls. The case group included 55 males and 40 females with the sex ratio of 1.38:1 and the mean age was 63.56±9.83. The control group included 68 males and 52 females, in which the sex ratio was 1.31:1 and the mean age was 64.13±10.22. The age and gender between two groups had no statistically significant difference ($P>0.05$). As was the results in **Table 2**, the distributions of obesity, smoking, hypertension, diabetes, hyperlipidemia in case and control groups were significant differences ($P<0.05$). The hypertension patients even accounted for 62.11% in cases and hyperlipidemia patients also held 34.74%. What's more, the differences of hypertension, diabetes, hyperlipidemia between two groups reached to the significant level of 0.01. But drinking was not an independent influence factor for stroke ($P>0.05$).

Distributions of MMP-2 polymorphisms in case and control groups

In **Table 3**, the genotype and allele distributions of *MMP-2* C-735T, C-1306T polymorphisms were showed. Tested by χ^2, the genotype distri-

Table 3. Genotype and allele distribution comparison of *MMP-2* gene C-735T, C-1306T between case and control groups

Genotype/Allele	Case n=95 (%)	Control n=120 (%)	X^2	*P*	OR (95% CI)	P_{HWE}
C-735T						0.40
CC	56 (58.95)	83 (69.17)	-	-	1.00	
CT	28 (29.47)	32 (26.67)	0.70	0.40	1.30 (0.71-2.39)	
TT	11 (11.58)	5 (4.16)	4.74	0.03	3.26 (1.07-9.90)	
C	140 (73.68)	198 (82.50)	-	-	1.00	
T	50 (26.32)	42 (17.50)	4.90	0.03	1.68 (1.06-2.68)	
C-1306T						0.67
CC	74 (77.89)	111 (92.50)	-	-	1.00	
CT	17 (17.89)	9 (7.50)	5.99	0.01	2.83 (1.20-6.70)	
TT	4 (4.22)	0 (0)	-	-	-	
C	165 (86.84)	231 (96.25)	-	-	1.00	
T	25 (13.16)	9 (3.75)	12.89	0	3.89 (1.77-8.55)	

Table 4. Interaction of *MMP-2* C-735T, C-1306T polymorphisms in stroke

SNP1	SNP2	Case (%)	Control (%)	OR	S	AP	RERI
+	+	14 (14.74)	4 (3.33)	5.57	4.48	0.68	3.13
+	-	25 (26.31)	33 (27.50)	1.21			
-	+	7 (7.37)	5 (4.17)	2.23			
-	-	49 (51.58)	78 (65.00)	1.00			

Note: SNP: single nucleotide polymorphism; SNP1: *MMP-2* C-735T; SNP2: MMP-2 C-1306T; "-" represented the genotypes of minor allele; "+" represented the common genotype.

butions of *MMP-2* two polymorphisms in the control group conformed to HWE (*P*>0.05), which demonstrated that our study population was on behalf of a general Mendel population.

At the same time, we can see that the genotype frequencies of CC, CT, TT in C-735T polymorphism were 58.95%, 29.47%, 11.58% in case group and 69.17%, 26.67%, 4.16% in control group, respectively and The C, T allele frequencies were 47.37%, 52.63% in cases and 60.83%, 39.17% in controls. The distributions had a significant difference between two groups by TT genotype and T allele (P<0.05). Therefore, the carriage of TT genotype and T allele at C-735T polymorphism increased the probability suffering from stroke (TT vs. CC: OR=3.26, 95% CI=1.07-9.90; T vs. C: OR=1.68, 95% CI=1.06-2.68).

We only detected CC, CT genotypes of *MMP-2* C-1306T polymorphism in the control group and the frequencies were respectively 92.50%, 7.50%, but CC, CT, TT genotype frequencies in cases were 77.89%, 17.89%, 4.22%, respectively. CT genotype frequency was significantly higher in cases than that of in controls, compared with CC genotype, so was T allele (*P*<0.05). So CT genotype and T allele were correlated to the increased the risk of stroke in our study population (TT vs. CC: OR=2.83, 95% CI=1.20-6.70; T vs. C: OR=3.89, 95% CI=1.77-8.55).

Interaction analysis of MMP-2 C-735T, C-1306T polymorphisms in stroke

The results of the interaction between *MMP-2* C-735T and C-1306T polymorphisms were listed in **Table 4**. There was the positively additive effect between two polymorphisms. When both of two polymorphisms showed the genotypes of the minor allele, the risk of strike significantly increased (S=4.48) and 68% of the patients with stroke attributed to the interaction of *MMP-2* C-735T and C-1306T polymorphisms (AP=0.68). Meanwhile, the interaction effect undertook 3.13 times risk in stroke, compared with the other risk factors (RERI=3.13).

Discussion

Stroke is not only a cerebral blood circulation disorder with sudden onset, but also one of the main diseases harming human health [15]. Among the people died of stroke, over two thirds concentrate in developing countries [16]. The onset of stroke derives from various possible reasons, in which blood pressure, blood glucose, cholesterol level and smoking are the main risk factors. Besides, other reasons including advanced age, drinking, high-salt

diet, obesity are considered as the influence factors of stroke. Cerebral ischemic stroke is the main type and accounts for 80% of total stroke [17]. It has been widely considered that stroke is a complex polygenic disease affected by the interaction of environmental and genetic factors, but the detailed pathogenesis of stroke is still not confirmed up to now.

Currently, some studies reveal that stroke is related to inflammatory response [18, 19]. Ramallal et al. assess the role of the dietary inflammatory index (DII) which is used to determine the inflammatory potential of the diet in stroke, the final results show that the risk of stroke gradually increase with each improving quartile of DII [20]. Rajan et al. ensure that low cognitive function significantly increases the onset risk of stroke and the association between the two has racial difference, that is, negro with cognitive impairment are easier to suffer from stroke that that of Caucasian [21]. The effects gene and its genetic variant on stroke susceptibility have been researched and gained several positive results. In the study of Hanscombe et al., the increased risk of ischemic stroke is found in people with genetic biomarkers influencing the factor XIII subunit B (FXIIIB), a coagulation factor [22]. What's more, genetic polymorphisms of various genes involve in the process of stroke development, including *MTHFR*, *AopE*, angiotensin converting enzyme gene (*ACE*), *eNOS*, angiotensinogen gene (*AGT*), angiotensin II type 1 receptors gene (*AT1R*) [23-25].

MMPs, a zinc dependent proteolytic enzyme family, are the important media of degrading and reconstituting ECM. They can dissolve collagen ingredients, make the proportion of plaque elements obviously alter and relatively increase lipid contents, and further add instability to plaque [26]. Gross and Lapiere earliest found MMPs when they studied metamorphosis of tadpole tail in 1962 and at least 25 members in MMPs have been found out until now. They participate in the processes of wound healing, bone resorption, pregnancy and delivery, mammary gland instauration [27]. Lots of studies also discover that *MMP*s involves in multiple pathological processes, including revascularization, tumor metastasis, inflammatory response, etc. In recent years, people find that *MMP*s widely affect nervous system, too. Among of the members in *MMP*s, *MMP-2* and *MMP-9* are studied widely in various diseases, such as breast cancer, community-acquired pneumonia [28, 29].

In present study, the association between the genetic variants of *MMP-2* and stroke susceptibility was discussed. Firstly, in the analysis of clinical information, patients with hypertension, diabetes, hyperlipidemia accounted for large proportion in stroke patients than healthy persons, which ensured the conclusion that these three index all had the important influence on stroke development. In addition, permanent cigarette consumption was also a risk factor for the onset of stroke, but the direct roles of obesity and drinking in stroke were not found in our study population. Secondly, the single polymorphism in *MMP-2* was explored the association with stroke. Both of *MMP-2* C-735T, C-1306T polymorphisms were reveal to be associated with the onset risk of stroke, not only genotype but allele. In the past, Nie et al. and Buraczynska et al. obtained the similar results [30, 31]. Finally, we analyzed the interaction of *MMP-2* C-735T, C-1306T polymorphisms because of the complicacy of stroke which was regulated by various factors. These two polymorphisms presented significantly positive correlation under the additive effect model. It could explain near 70% of stroke occurrence when they showed the genotypes of minor allele.

Generally speaking, this study supported the viewpoint that there existed association between *MMP-2* polymorphisms and stroke in the Han population from northern China. Nevertheless, in order to guarantee and improve the accuracy of results, more studies with larger and different sample populations should be performed for obtaining more supportive evidences and attaining the purpose of early discovery, diagnosis and treatment.

Disclosure of conflict of interest

None.

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