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Glutathione S-transferase polymorphisms and traditional classification in Korean population with cerebrovascular disease

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SUMMARY

Glutathione S-transferase polymorphisms (GST) were examined in 98 cases with cerebrovascular disease (CVD) to test the hypothesis that GST polymorphisms confer a risk to an individual to develop CVD. Tobacco smoke is a major cause of both cancer and vascular disease. We therefore were stratified the subjects with CVD for smoking status, and then examined whether polymorphisms in this detoxification enzyme gene, GST, influence risk of CVD. Neither GSTM1 nor GSTT1 genotypes in the CVD group was significantly different from the control group (n=230), even in smokers. We attempted the combined analyses for GSTM1 and GSTT1 genotypes in CVD for smoking status. No significant association observed between the combined genotypes and CVD. We also classified the subjects and control group into four types according to Sasang Constitutional Medicine, Korean Traditional Oriental Medicine, and investigated the association among GST genotypes, CVD, and Sasang constitutional classification. Our observations do not confirm the effect of the GSTM1 and GSTT1 genotypes as a risk factor for CVD, even in smokers. Furthermore, we first attempted to evaluate the efficacy of Sasang Constitutional Medicine, and to find an association with CVD.

Key words: Cerebrovascular disease; Glutathione S-transferase; Polymorphism; Sasang constitution; Koreans

INTRODUCTION

Cerebrovascular disease (CVD) is a multifactorial disease caused by the interactions of several genetic and environmental factors. Strong evidence from twin and family studies shows that familial predisposition, in addition to such recognized risk factors as high blood pressure, smoking, diabetes, obesity, and advanced age, contributes to the pathogenesis of stroke, including CVD (Sharma, 1996). Especially, tobacco smoking has an important role in the etiopathogenesis of diseases such as vascular and cancer of the respiratory tract. The more than 3800 chemicals identified in tobacco smoke include at least 40 known carcinogens such as tobacco-specific nitrosamines, aromatic amines, and tars (Hoffmann and Hecht, 1990). Even though the smoking-associated cancer risk has been attributed to exposure to these hazardous compounds, the mechanistic relationship between smoking and CVD has remained unclear. One possibility is that, in a process parallel to carcinogenesis, tobacco smoke-induced DNA damage causes cell proliferation in the intima of arteries, thereby contributing to atherosclerotic plaque formation (Bridges *et al.*, 1990). The damage could result from direct chemical binding to DNA or be a consequence of inflammation and oxidative stress consistent with a response-to-injury model (Ross, 1993). The hypothesis that DNA damage plays a role in vascular disease has received support from observations in animal models and in humans

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(Bridges *et al.*, 1990; De Flora *et al.*, 1997). In experimental animals, chemicals in tobacco smoke (e.g., benzo[a]pyrene, 1,3-butadiene) and environmental tobacco smoke have been reported to induce and stimulate atherosclerotic plaque formation (Penn and Snyder, 1988, 1996).

The majority of genotoxic chemicals in tobacco smoke require metabolism in order to bind to cellular macromolecules. Enzymes, including the multigene family of glutathione S-transferases (GST), detoxify these reactive metabolites to more water-soluble and readily excretable forms. A number of common polymorphisms occur that affect enzyme activity; these include gene deletions in the GSTM1 and GSTT1 genes, which result in individuals lacking in the corresponding enzyme activity (Seidegard *et al.*, 1988). Their expression therefore modulates the amount of chemical binding to DNA, and polymorphisms in these genes have been associated with myocardial infarction, as well as the tobacco-related cancers including lung, in smokers (Taningher *et al.*, 1999; Strange *et al.*, 2000). Ischemic heart disease and cerebrovascular disease have risk factors in common, such as hypertension, hyperlipidemia, and smoking; and both types of diseases are pathologically based on atherosclerosis. However, genetic risk factors in CVD have less studied as compared with those involved in ischemic heart disease. Our hypothesis was that smoke-induced DNA damage resulted from decreased enzyme activity of GST null genotype causes smooth muscle cell proliferation in the intima of arteries, thereby contributing to atherothrombotic process and the development of CVD. We therefore examined whether polymorphisms in GST genes influence risk of CVD in tobacco smokers.

Also, we investigated the relationship among CVD, GSTs gene polymorphism, and Sasang constitutional classification. The Sasang Constitutional Medicine, a major branch of Korean traditional medicine, classifies people's constitutions into four types, according to the strengths and weaknesses in functions of the internal organs. Sasang constitutional philosophy forms the basis of treatment by correcting the imbalance of the internal organs caused by the constitutional properties in each body type. Accordingly, it presents different treatments according to constitution (Lee, 1996). The different constitutions bring about different reactions to the same disease. In the previous study, our group showed the regulatory effect of cytokine production in patients with cerebral infarction by Yulda-Hanso-Tang, which is a prescription for the Taeumin cerebral infarction patients according to Sasang constitution philosophy (Shin *et al.*, 2000). The differences of disease severity to be shown in Sasang constitutional classification may be due to genetic factors. Therefore, we examined interrelationship among CVD, GSTs gene polymorphism, and Sasang constitutional classification.

MATERIALS AND METHODS

Subjects

Patients with CVD (n=98) during acute stage were identified according to well-defined criteria that included computerized tomography scanning, magnetic resonance imaging (MRI), and clinical signs (hemiparesis, hemiplegia, slurred speech, facial palsy, and so forth) from Wonkwang University Oriental Medicine Hospital in Iksan, Korea. The control group consisted of 230 individuals undergoing routine health screening. None of the controls had a history of CVD. All cases and controls (all Koreans) gave informed consent before participating in the research protocol, which was approved by the ethnics committee of each hospital. Smoking status was determined from interviews with patients and controls at the time of blood sampling. Patients were asked whether they were smokers at the time of recruitment (current smoker) had never been a regular smoker (never smoker).

Discrimination of Sasang constitution of individuals

Individuals were discriminated into four types by QSCC II program as well as clinical data (weight, height, blood pressure, etc.); Teaeumin, Taeyangin, Soyangin, and Soeumin. QSCC II is the program for "objective 4-constitutional body types" under PC, which is developed by the Department of Sasang Medical in Kyung Hee University Oriental Medicine Hospital, Seoul, Korea. It has been proved for providing its accuracy and universal logical ground with its standardized diagrams according to diagnostic clinical data.

Genotyping

The blood was stored at -20°C until it was ready to be extracted. The genomic DNA was extracted by inorganic procedure (Miller *et al.*, 1988). The concentration of DNA was estimated by absorbance at 260 nm. Analysis of the GSTT1 and GSTM1 genes was conducted using the modified multiplex PCR reaction with the ubiquitous β-globin gene as an internal control (Abdel-Rahman *et al.*, 1996). Briefly a PCR reaction was carried out in a 25 μl volume containing, 100 ng of genomic DNA, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 200 μM of each dNTP, and 1U of r*Taq* DNA polymerase (Takara), with 0.2 μM of GSTM1-A/B, 0.4 μM of GSTT1-A/B, and 0.2 μM of β-globin-A/B primers (Bioneer, Korea). The primer pairs for each gene were as follows; GSTM1-A/B:5'-GAACTCCCTGAAAAGCTAAAGC-3'/5'-GTTGGGCTCAAATATACGGTGG-3', GSTT1-A/B: 5'-TTCCTTACTGGTCCTCACATCTC-3'/5'-TCACCGGATCATGGCC-AGCA-3', β-globin-A/B:5'-CAACTTCATCCACGTTCACC-3'/5'-GAAGAGCCAA-GGACAGGTAC-3'. The PCR conditions were 3 min preincubation step at 94°C, 30 cycles of 30 sec at 94°C, 30 sec at 63°C, 45 sec at 72°C, and a final postcycling 10 min extension step at 72°C (MJ Research). Ten microliters of PCR product was analyzed electrophoretically on a 2% agarose gel stained with ethidium bromide (250 ng/ml) and the presence or absence of the GSTT1 (480bp) and GSTM1 (215bp) products was determined in the presence of the control β-globin gene (268bp) (Fig. 1).

Statistical analysis

The mean levels of all numerical values were tested by the Student *t* test. Comparisons of the allele frequencies of the GSTM1 and GSTT1 genotypes between the control and patients were carried out using the Pearson chi-square test. The combined GSTM1 and GSTT1 genotype distributions were tested using the Fisher's two-tailed exact test or Pearson chi-square test. Results obtained for the GSTM1 and GSTT1 genotypes were also analyzed with reference to current smoking status using the Fisher's two-tailed exact test or Pearson chi-square test. Distribution of Sasang constitutions in CVD patients and controls [15] were tested using the Pearson chi-square test. All statistical analyses were performed using SPSS v9.00 (SPSS Inc.) statistical analysis software. A *P*-value less than 0.05 were considered statistically significant.

RESULTS

Clinical characteristics of patients according to GSTs genotypes

The characteristics of the patients with CVD according to the GSTs genotypes are summarized in Table 1. A total of 98 patients were included in the analysis. The levels of total cholesterol and triglyceride were lower in GSTM1 null genotype than in GSTM1 positive genotype. In contrast, the levels in GSTT1 null genotype were higher than that in GSTT1 positive genotype, but the difference was not

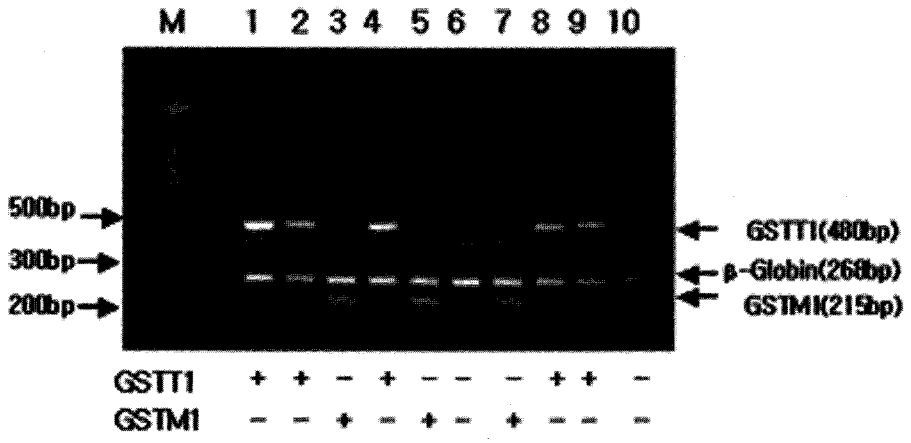

Fig. 1. Multiplex PCR for GSTM1 and GSTT1 genes. β-globin gene was used as an internal positive control. The PCR products indicate GST M1 (215bp), β-globin (268bp), and GST T1 (480bp), respectively. M represents 100bp DNA marker. The -/+ indicates the null genotype or present genotype of GST.

Table 1. Clinical characteristics of patients (n=98)

Characteristics	Mean	GSTM1		GSTT1	
		Null	Present	Null	Present
Age (year)	46.9±21.1	46.4±21.4	47.6±20.9	49.7±21.4	44.2±20.6
Sex (m:f, %)	49.5:50.5	52.9:47.1	45.2:54.8	47.1:52.9	52.4:47.6
Cholesterol (mmol/l)	198.1±51.0	193.4±50.3	204.7±52.1	204.8±51.3	189.8±50.2
Triglyceride (mmol/l)	174.3±144.6	159.5±101.9	195.4±189.5	190.2±118.8	154.9±170.8
Hypertension, %	58.5	55.8	61.9	48.1	71.4[a]
Diabetes Mellitus, %	17	15.4	19.0	13.5	21.4
Smoker, %	36	41.7	28.9	38.3	33.3
Alcohol, %	43.5	47.9	37.8	48.9	36.8

[a]$P<0.05$ compared with null genotype with the present genotype by χ-square test (2-sided).

Table 2. Frequencies of GSTM1 and GSTT1 null genotypes

	Controls, n (%) (n=230)	CVD, n (%) (n=98)	P[a]
GSTM1 null genotype	134(58.3)	54(55.1)	0.597
GSTT1 null genotype	121(52.8)	54(55.1)	0.707
Never smokers			
GSTM1 null genotype	36(62.1)	28(50.9)	0.232
GSTT1 null genotype	26(45.6)	29(52.7)	0.452
Current smokers			
GSTM1 null genotype	13(56.5)	20(64.5)	0.551
GSTT1 null genotype	12(52.2)	18(58.1)	0.667

[a]P compared with controls with the CVDs by χ-square test (2-sided).

statistically significant. The frequency of hypertension was significantly higher in GSTT1 positive genotype than in null genotype (71.4 vs 48.1%, χ=5.219, P=0.025) (Table 1).

Frequencies of GST genotypes

The frequencies of GSTM1 and GSTT1 null genotypes did not differ between CVD and control group. When subjects were stratified for smoking status, the association between GSTs null genotypes and CVD were examined. The frequencies of GSTM1 and GSTT1 null genotypes in current smokers with CVD were higher than that in controls of smokers (GSTM1; 56.5 vs 64.5%, GSTT1; 52.2 vs 58.1%). However, none of these differenced was statistically significant (Table 2).

We also analyzed the genotypes of GSTM1 and GSTT1 in combination to evaluate whether combination of these genotypes is associated with CVD. The frequency of the null genotype of both GSTM1 and GSTT1 in patients with CVD (n=25, 25.5%) was significantly not different from that of the control group (n=62, 27.1%), even in current smokers (38.7% vs 30.4%)(Table 3).

Distribution of four types of Sasang constitution

Sasang constitutional classification was done by QSCC II program, and identified as Taeyangin, Taeumin, Soyangin, and Soeumin, respectively. The distribution of each constitution in 98 patients with CVD was as follows: Taeyangin, 0 (0%); Taeumin, 56 (57.1%); Soyangin, 31 (31.6%); and Soeumin, 11 (11.2%). It was significantly different from the distribution in 1589 control subjects: Taeyangin, 0 (0%); Taeumin, 553 (34.8%); Soyangin, 485 (30.5%); and Soeumin, 551 (34.7%) (Park *et al.*, 1999). The frequency of Taeumins with CVD (n=56, 57.1%) was higher than that of Taeumins without CVD (n=553, 34.8%), and the frequency of Soeumins with CVD (n=11, 11.2%) was significantly lower than that of Soeumins without CVD (n=551, 34.7%) (χ^2=15.425, $P<0.001$) (Table 4). These results indicate that the susceptibility of CVD in Taeumins is higher than the other constitutions, but on the other hand, Soeumins seem to have a protective property against CVD.

The distribution of GSTM1 and GSTT1 null genotype according to Sasang constitution was also investigated in

Table 3. Combined analysis of GSTM1 and GSTT1 genotypes by smoking

GSTM1	GSTT1	Total			Never smokers			Current smokers		
		Control, n (%) (n=229)	CVD, n (%) (n=98)	P^a	Control, n (%) (n=57)	CVD, n (%) (n=55)	P^a	Control, n (%) (n=23)	CVD, n (%) (n=31)	P^a
Null	Null	62(27.1)	25(25.5)	0.769	14(24.6)	9(16.4)	0.283	7(30.4)	12(38.7)	0.577
Null	Present	71(31.0)	29(29.6)	0.800	21(36.8)	19(34.5)	0.800	6(26.1)	8(25.8)	1.0
Present	Null	59(25.8)	29(29.6)	0.475	12(21.1)	20(36.4)	0.073	5(21.7)	6(19.4)	1.0
Present	Present	37(16.1)	15(15.3)	0.847	10(17.5)	7(12.7)	0.478	5(21.7)	5(16.1)	0.728

[a]*P* compared with controls with the CVDs by χ-square test or Fisher's exact test (2-sided) in total, never smokers, and current smokers, respectively.

Table 4. Distribution of Sasang constitutions in CVD patients and controls

Sasang Constitution	Control, n (%) (n=1589[a])	CVD, n (%) (n=98)	p^b
Taeumin	553(34.8)	56(57.1)	0.000
Soyangin	485(30.5)	31(31.6)	0.817
Soeumin	551(34.7)	11(11.2)	0.000
Taeyangin	0(0)	0	-

The [a]was quoted from Park *et al.*, 1999.
[b]*P* compared with controls with the CVDs by χ-square test (2-sided).

Table 5. Distribution of GSTs genotypes and Sasang constitution in patients with CVD

GSTM1	GSTT1	Sasang Constitution		
		Taeumin, n (%) (n=66)	Soyangin, n (%) (n=39)	Soeumin, n (%) (n=14)
Null		40(60.6)	19(48.7)	6(42.9)
	Null	37(56.1)	22(56.4)	9(64.3)
Null	Null	20(30.3)	10(25.6)	2(14.3)
Null	Present	20(30.3)	9(23.1)	4(28.6)
Present	Null	17(25.8)	12(30.8)	7(50)
Present	Present	9(13.6)	8(20.5)	1(7.1)

CVD patients (Table 5). The frequency of both GSTM1 and GSTT1 null type in Taeumin (30.3%) was a little higher than that of the other types (25.6% in Soyangin, 14.3% in Soeumin). When these frequencies were represented as odds ratio, an interesting trend was observed. The frequency of Taeumin was higher compared with either the Soyangin or the Soeumin in those with the null genotype in GSTM1 or GSTT1 (odd ratio [OR]=1.619, *P*=0.237, Taeumin vs soyangin; [OR]=2.051, *P*=0.228, Taeumin vs Soeumin). In addition, the odd ratio was significantly increased in Taeumin with both GST M1 and GST T1 null genotype ([OR]=2.609, *P*=0.228, Taeumin vs Soeumin) (Fig. 2). These trends suppose that Taeumin with both GST M1/T1 null genotypes have higher risk on CVD than either Soyangin or Soeumin. However, further studies were required due to the small number of subjects and low statistical power.

DISSCUSSION

CVD is a multifactorial disease caused by the interactions of several genetic and environmental factors, including such recognized risk factors as high blood pressure, smoking, diabetes, obesity, and advanced age. We expected that GST is implicated in the detoxification of carcinogens present in tobacco smoke and consequently polymorphisms in these genes may confer susceptibility to CVD, if DNA damage is important in disease process. Therefore, we examined the relation among tobacco smoke, genetic polymorphisms of GST and CVD. However, no significant association among the GST genotype, smoking, and CVD observed in combined analysis, as well as in

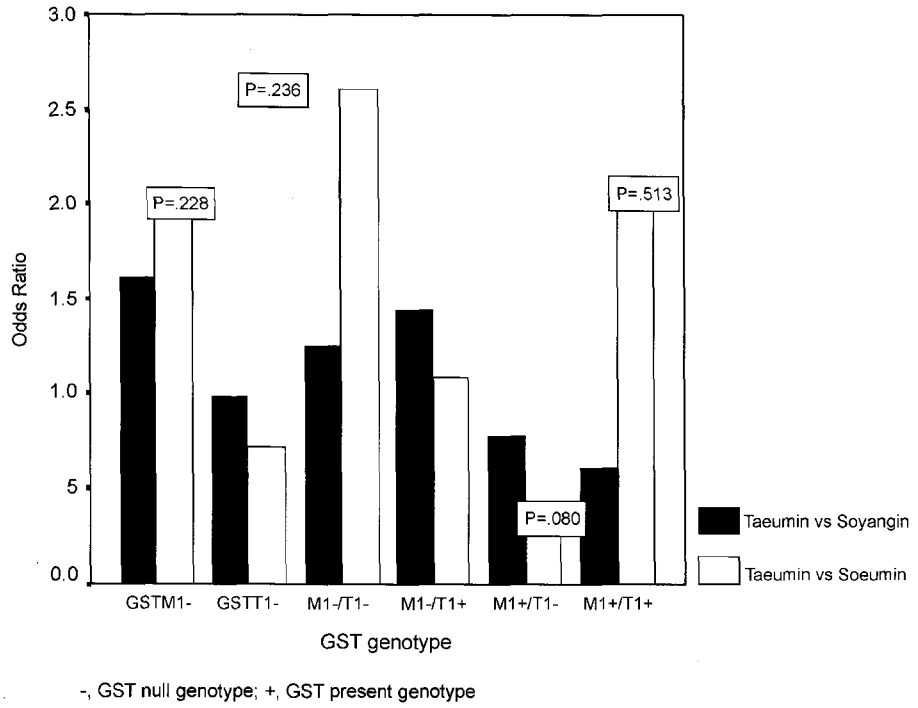

Fig. 2. Association of GST genotypes and Sasang constitutions. Bars indicate the ORs between the different combinations of genotypes for GST M1 and GST T1. The Soyangin or Soeumin of constitutions was used as the reference OR for Taeumin. The -/+ indicates the null genotype or present genotype of GST.

individual analysis of GSTM1 and GSTT1 genotype. In contrast, Wilson *et al.* (2000) suggested that the expression of GST modulates the amount of chemical binding to DNA, and GSTM1 null genotype were associated with myocardial infarction. This discrepancy may be derived from the difference of two tissues since the regulation of genes may be tissue or cell specific, although CVD and myocardial infarction are pathologically based on atherosclerosis. Further studies are require to clarify whether variation of the risk of GSTM1 and GSTT1 between CVD and myocardial infarction might be explained by organ specificity in the function of GST.

Another interpretation is that these conflict results may be due to ethnic differences. Indeed, the frequency of GSTT1 null genotype in our control group was high (52.8%), compared with that in Caucasians (20.4%) (Nelson *et al.*, 1995), African-American (21.8%) (Bell *et al.*, 1993), and Maxican-American (9.7%) (Nelson *et al.*, 1995). In contrast, it was similar to Japanese (52.0%) (Murata *et al.*, 2001) and Chinese (64.6%) (Nelson *et al.*, 1995). For GSTM1 null genotype, the frequency of our group (58.3%) was a little higher than that in Japanese (42.5%) (Murata *et al.*, 2001) and Caucasian (48%) (Bell *et al.*, 1993). The frequencies of the GSTM1 and GSTT1 null genotype in our control group was a little lower than that reported by Yim *et al.* (2000) (M1 null type: 65%, T1 null type: 62%). However, these intra-ethnic differences have been reported among African-Americans (from 20 to 24% in GSTT1 null genotype) (Hallier *et al.*, 1993; Nelson *et al.*, 1995; Chen *et al.*, 1996), and among Caucasians (from 40 to 58% in GSTM1 null genotype) (Strange *et al.*, 2000).

The Sasang Constitutional Medicine we examined was established by Je-Ma Lee of Korea in 1894. Since then it has been developed as a major branch of Korean traditional Oriental medicine, which occupies an important position along with Western medicine in Korea. It classifies people's constitutions into four types, such as Taeyangin, Taeumin, Soyangin and Soumin, according to the functions of the internal organs, behavioral characteristics, the symptoms of a disease, and quantitative variations including body size etc. This classification was determined by QSCC II program as well as clinical measurements (weight, height, blood pressure etc.).

Sasang Constitution philosophy was supported the report demonstrating that continuous characters governed by a large number of independent mendelian factors (polygenic characters) would display precisely the quantitative variation and family correlations described by the biometrics (Fisher, 1918). Also, Falconer extended the polygenic theory to discontinuous nonmendelian characters by postulating an underlying continuously variable susceptibility (Falconer, 1981). We suggested that Sasang constitutional medicine was similar to the approach to complex disease through the polygenic factor and nonmendelian characters by Fisher and Falconer. So, Sasang Constitutional Medicine could be applicable to whole world people as well as Korean and Asian. Also, Taeumin, who resembled the typical abdominal type of obesity in Western populations, is thought to have a higher rate of stroke, hypertension and hyperlipidemia than the other types because he or she has a large liver and small lungs. Here our data showed a consistent result with the viewpoint of Sasang Constitutional Medicine. Furthermore, the study of an association between Sasang Constitution and CVD has not been reported. We also examined the association between the Sasang constitution and GST genotypes in smokers. However, we did not find the significance due to the small number of subjects and low statistical power (data not shown). So, further studies are necessary to clarify the association with genetic factors.

In conclusion, the present results suggest that GST polymorphism is less effective than those of the other susceptible genes and the environmental factors in the development of CVD in Koreans, but that there can be a novel possibility between CVD and Sasang Constitutional Medicine.

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