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Original Articles

Study on the Relationship between Polymorphisms in Glutathione S-transferase and Ischemic Cerebrovascular Disease

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Objective : Glutathione S-transferase polymorphism (GST) were examined in 120 cases with ischemic cerebrovascular disease (ICVD) to test the hyperthesis that GST polymorphisms confer a risk to an individual to develop ICVD. Tobacco smoking is a major cause of both cancer and vascular disease.

Methods : therefore We were stratified the subjects with ICVD for smoking status, and then examined whether polymorphisms in this detoxification enzyme gene, GST, influence risk of ICVD

Results : Neither GSTM1 nor GSTT1 genotypes in the ICVD group was significantly different from the control group (n=207), even in smokers. We attempted the combined analyses for GSTM1 and GSTT1 genotypes in ICVD for smoking status. No significant association observed between the combined genotypes and ICVD

Conclusion : Our observation do not confirm the effect of the GSTM1 and GSTT1 genotypes as a risk factor for ICVD, even in smokers.

Key Words: Glutathione S-transferase polymorphism (GST), schemic cerebrovascular disease (ICVD), tobacco smoking.

Introduction

Ischemic cerebrovascular disease (ICVD) 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 ICVD[1]. 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 smoking include at least 40 known carcinogens such as tobacco-specific nitrosamines, aromatic amines, and tars[2]. Even though the smoking-associated cancer risk has been attributed to exposure to these hazardous compounds, the mechanistic relation-

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ship between smoking and ICVD has remained unclear. One possibility is that, in a process parallel to carcinogenesis, tobacco smoking-induced DNA damage causes cell proliferation in the intima of arteries, thereby contributing to atherosclerotic plaque formation[3]. 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[4]. The hypothesis that DNA damage plays a role in vascular disease has received support from observations in animal models and in humans[3,5]. In experimental animals, chemicals in tobacco smoking (e.g., benzo[a]pyrene, 1,3-butadiene) and environmental tobacco smoking have been reported to induce and stimulate atherosclerotic plaque formation[6,7].

The majority of genotoxic chemicals in tobacco smoking 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. The GST super gene family of enzymes catalyze the detoxification of electrophillic compounds such as carcinogens and cytotoxic drugs by glutathione conjugation[8,9]. The cytosolic GST enzyme family consists of six gene classes, classified according to their primary structure, termed alpha, mu, pi, sigma, theta and zeta[10]. Several studies have indicated that GST enzymes may play a role in cancer predisposition with GSTM1 and GSTT1 null phenotypes specifically implicated in lung and colorectal cancers[11,12]. 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. Therefore their expression 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[13,14]. 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 ICVD have less studied as compared with those involved in ischemic heart disease.

My hypothesis was that smoke-induced DNA damage resulted from decreased enzyme activity of GST null genotype causes smooth muscle cellproliferation in the intima of arteries, thereby contributing to atherothrombotic process and the development of ICVD. Therefore I examined whether polymorphisms in GST genes influence risk of ICVD in tobacco smokers.

Materoals and Methods

1. Subjects

Patients with Ischemic cerebrovascular disease (ICVD) (n=120) during the 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 207 individuals undergoing routine health screening. None of the controls had a history of ICVD. All cases and controls (all Koreans) gave informed consent before participating in the research protocol, which was approved by the ethics 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') or had never been a regular smoker ('never smoker').

2. Genotyping

The blood was stored at -20C until it was ready to be extracted. The genomic DNA was extracted by inorganic procedure[15]. 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[16]. 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 $MgCl_2$, 200μM of each dNTP, and 1U of rTaq 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'-TTGGGCTCAAATATACGGTGG-3', GSTT1-A/B:
5'-TTCCTTACTGGTCCTCACATCTC-3'/5'-TCACCGGATCATGGC-CAGCA-3', β
-globin-A/B:
5'-CAACTTCATCCACGTTCACC-3'/5'-GAAGAGCC-AAGGACAGGTAC-3'.

The PCR conditions were 3 min preincubation step at 94℃, 30 cycle of 30 sec at 94℃, 30 sec at 63℃, 45 sec at 72℃, and a final postcycling 10 min extension step at 72℃ (MJResearch). 10 μl of PCR product were analyzed electrophoretically on a 2% agarose gel stained with ethidium bromide (250 ng/ml) and the presence or absence of the GSTT1 (480 bp) and GSTM1 (215bp) products were determined in the presence of the control β-globin gene (268 bp).

3. *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. 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

1. *Clinical characteristics of ICVD patients*

Table 1 shows the clinical characteristics of the present subjects by smoking. A total of 120 patients were included in the analysis; 53 patients (55.8%) were female. For sex, there was significant difference; in never smokers, 49 patients (75.4%) were female, but 4 patients (13.3%) in current smokers ($p<0.05$). The levels of total cholesterol, HDL cholesterol, and triglyceride were lower in current smokers than in never smokers but the difference was not statistically significant ($p>0.05$).

2. *Relationship among GST genotype, ICVD and smoking status*

The frequencies of GSTM1 and GSTT1 null genotypes did not differ between ICVD and control group (p>0.05) (Table 2). When subjects were stratified for smoking status, the association between GSTs null genotypes and ICVD were examined. The frequency of GSTM1 null genotypes in current smokers with ICVD was higher than that in controls of smokers (Controls vs. ICVD; 56.5% vs. 65.4%). In contrast, the frequency of GSTT1 null genotypes in current smokers with ICVD was lower than that in controls of smokers

Table 1. Clinical Characteristics of ICVD Patients Characteristics Total (Mean±S.D.) Never Smokers (n=65) Current Smokers (n=30)*p* Value

Age (year)*	48.1±22.2	68.6±10.6	61.2±12.3	.003
Total cholesterol (mg/dl)	42:53	16:49	26:4	.001
Triglyceride (mg/dl)	187.5±47.0	190.7±50.4	180.6±38.8	.342
Obesity, n (%)	46.8±12.6	48.6±13.2	43.2±10.2	.056
	134.9±83.8	139.7±92.6	124.7±61.5	.429
	25(26.3)	20(30.8)	5(16.7)	.147
	13(16.9)	10(18.2)	3(13.6)	.631
	27(28.4)	18(27.7)	9(30)	.817

Statistical tests by Student ttest (2-tailed) or chi-square test.

*p<0.05.

Table 2. Distribution of GSTM1 and GSTT1 Genotypes in Controls and ICVD

	Controls (%)	ICVD	2	*p* value
GSTM1	n=207	n=120	.113	.736
Null	122(58.9)	73(60.8)		
Present	85(41.1)	47(39.2)		
GSTT1	n=206	n=120	.475	.491
Null	106(51.5)	57(47.5)		
Present	100(48.5)	63(52.5)		

Statistical tests by 2-test (2-tailed).

Table 3. Individual Analysis of GSTM1 and GSTT1 Genotypes by Smoking Status

GST M1		Never smokers			Current smokers		
Null		Controls (%) (n=57)	ICVD (%) (n=55)	*p* value	Controls (%) (n=23)	ICVD (%) (n=26)	*p* value
	Null	36(62.1)	34(61.8)	.978	13(56.5)	17(65.4)	.525
		26(45.6)	27(49.1)	.713	12(52.2)	10(38.5)	.396

Statistical tests by 2-test or Fisher's exact test (2-tailed).

Table 4. Combined Analysis of GSTM1 and GSTT1 Genotypes in Controls and ICVD

GSTM1	GSTT1	Controls (%) (n=206)	ICVD (%) (n=120)
Null	Null	55(26.7)	31(25.8)
Null	Present	66(32.0)	42(35)
Present	Null	51(24.8)	26(21.7)
Present	Present	34(16.5)	21(17.5)

Statistical tests by 2-test (2-tailed).

Table 5. Combined Analysis of GSTM1 and GSTT1 Genotypes by Smoking Status

GST M1	GST T1	Never smokers			Current smokers		
		Controls (%) (n=57)	ICVD (%) (n=55)	*p* value	Controls (%) (n=23)	ICVD (%) (n=26)	*p* value
Null7	Null	14(24.6) (30.4)	14(25.5)	1.0	7(30.4)	7(26.9)	1.0
Null Present21 (36.8)20(36.4)1.06(26.1)10(38.5).382							
Present	Null	12(21.1)	13(23.6)	.822	5(21.7)	3(11.5)	.448
Present	Present	10(17.5)	8(14.5)	.798	5(21.7)	6(23.1)	1.0

Statistical tests by 2-test or Fisher's exact test (2-tailed).

(Controls vs. ICVD; 52.2% vs. 38.5%). However, none of these differenced was statistically significant (p>0.05) (Table 3).

I also analyzed the genotypes of GSTM1 and GSTT1 in combination to evaluate whether combination of these genotypes is associated with ICVD. The frequency of the null genotype of both GSTM1 and GSTT1 in patients with ICVD (n=31, 25.8%) was significantly not different from that of the control group (n=55, 26.7%) (Table 4), even in current smokers (Control vs. ICVD; 30.4% vs. 26.9%) (p>0.05) (Table 5).

Discussion

ICVD 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. I expected that GST is implicated in the detoxification of carcinogens present in tobacco smoking and, consequently, polymorphisms in these genes may confer susceptibility to ICVD, if DNA damage is important in disease process. Therefore, I examined the relation between tobacco smoking, genetic polymorphisms of GST and ICVD. However, no significant association between the GST genotype, smoking and ICVD observed in combined analysis, as well as in individual analysis of GSTM1 and GSTT1 genotype. In contrast, Wilson et al.[14] suggested that the expression of GST modulates the amount of chemical binding to DNA and GSTM1 null genotype were associated with myocardial infarction. Though ICVD and myocardial infarction are pathologically based on atherosclerosis, there are almost no data with respect to GST genotypes and CVD. Further studies are required 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 my control group was high (52.8%), compared with that in Caucasians (20.4%)[17], African-American (21.8%)[18] and Mexican-American (9.7%)[17]. In contrast, it was similar to Japanese (52.0%)[19] and Chinese (64.6%)[17]. For GSTM1 null genotype, the frequency of my control group (58.3%) was a little higher than that in Japanese (42.5%)[19] and Caucasian (48%)[18]. The frequencies of the GSTM1 and GSTT1 null genotype in my control group was a little

lower than that reported by Yim et al. (M1 null type: 65%, T1 null type: 62%)[20]. However, these intra-ethnic differences have been reported among African-Americans (from 20 to 24% in GSTT1 null genotype)[17,21,22], and among Caucasians (from 40 to 58% in GSTM1 null genotype)[23].

In conclusion, ICVD is a complex disease caused by the interactions of several genetic and environmental factors and GST is implicated in the detoxification of carcinogens present in tobacco smoking. Therefore, I examined the relationships between ICVD, GST polymorphism, and smoking status on the basis the hypothesis, which is that GST polymorphism is implicated with susceptibility of ICVD. However, I did not find any association among GST polymorphism, ICVD, and smoking status. 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.

References

1. Sharma, P. Genes for ischaemic stroke: strategies for their detection. *J. Hypertens.* 1996;14:277-285.
2. Hoffmann, D., and S. S. Hecht. Advances in tobacco carcinogenesis. In Chemical Carcinogenesis and Mutagenesis (C. S. Cooper, and P. L. Grover, Eds.), New York:ven Press. ;63-102.
3. Bridges, B. A., D. E. Bowyer, E. S. Hansen, A. Penn, and K. Wakabayashi. The possible involvement of somatic mutations in the development of atherosclerotic plaques. Report of ICPEMC Subcommittee 7/1 Conclusions and recommendations. *Mutat. Res.* 1990;239:143-148.
4. Ross, R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature (London)* 1993;362: 801-809.
5. De Flora, S., A. Izzotti, D. Walsh, P. Degan, G. L. Petrilli, and J. Lewtas. Molecular epidemiology of atherosclerosis. *FASEB. J.* 1997;11:1021-1031.
6. Penn, A., and C. A. Snyder. Arteriosclerotic plaque development is 'promoted' by polynuclear aromatic hydrocarbons. *Carcinogenesis* 1988;9:2185-2189.
7. Penn, A., and C. A. Snyder. 1,3 Butadiene,a vapor phase component of environmental tobacco smoke, accelerates arteriosclerotic plaque. *Circulation* 1996;93: 552-557.
8. Zhong, S., A. H. Wyllie, D. Barnes, C. R. and Wolf, N. K. Spurr. Relationship between the GSTM1 genetic polymorphism and susceptibility to bladder, breast and colon cancer. *Carcinogenesis* 1994;14:1821-1824.
9. Zhang, P., S. Liu, S. Shan, X. Ji, G. L. Gilliland, and R. N. Armstrong. Modular mutagenesis of exons 1, 2, and 8 of a glutathione S-transferase from the mu class. Mechanistic and structural consequences for chimeras of isoenzyme 3-3. *Biochemistry* 1992;31:10185-10193.
10. Board, P. G., R. T. Baker, G. Chelvanayagam, and L. S. Jermiin. Zeta, A novel class of glutathione transferases in a range of species from plants to humans. *Biochem.* J. 1997;328:929-935.
11. Ryberg, D., V. Skaug, A. Hewer, D. H. Phillips, L. W. Harries, C. R. Wolf, D. Ogreid, A. Ulvik, P. Vu, and A. Haugen. Genotypes of glutathione transferases M1 and P1 and their significance for lung DNA adduct levels and cancer risk. *Carcinogenesis* 1997;18:1285-1289.
12. Deakin, M., J. Elder, C. Hendrickse, D. Peckham, D. Baldwin, C. Pantin, N. Wild, P. Leopard, D. A. Bell, P. Jones, H. Duncan, K. Brannigan, J. Alldersea, A. A. Fryer, and R. C. Strange. Glutathione S-transferase GSTT1genotypes and susceptibility to cancer: studies of interactions with GSTM1 in lung, oral, gastric and colorectal cancers. *Carcinogenesis* 1996;17:881-884.
13. Taningher, M., D. Malacarne, A. Izzotti, D. Ugolini, and S. Parodi. Drug metabolism polymorphisms as modulators of cancer susceptibility. *Mutat. Res.*1999; 436:227-261.
14. Wilson, M. H., P. J. Grant, L. J. Hardie, and C. P. Wild. Glutathione S-transferase M1 null genotype is associated with a decreased risk of myocardial infarction. *FASEB.* J. 2000;14(5):791-796.
15. Miller, S. A., D. D. Dykes, and H. F. Polesky. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic. Acids Res.* 1988;16:1215.
16. Abdel-Rahman, S. Z., R. A. el-zein, W. A. Anwar, and W. W. Au. A multiplex PCR procedure for

polymorphic analysis of GSTM1 and GSTT1 genes in population studies. *Cancer Lett.* 1996;107:229-233.

17. Nelson, H. H., J. K. Wiencke, D. C. Christiani, T. J. Cheng, Z. F. Zuo, B. S. Schwartz, B. K. Lee, M. R. Spitz, M. Wang, X. Wu, and K. T. Kelsey. Ethnic differences in the prevalence of the homozygous deleted genotype of glutathione S-transferase theta. *Carciogenesis* 1995;16:1243-1245.
18. Bell, D. A., J. A. Taylor, D. F. Paulson, C. N. Robertson, J. L. Mohler, and G. W. Lucier. Genetic risk and carcinogen exposure: a common inherited defect of the carcinogen-metabolism gene glutathione S-transferase M1 (GSTM1) that increases susceptibility to bladder cancer. *J. Nat.l* Cancer Inst. 1993;85:1159-1164.
19. Murata, M., M. Watanabe, M. Yamanaka, Y. Kubota, H. Ito, M. Nagao,T. Katoh, T. Kamataki, J. Kawamura, R. Yatani, and T. Shiraishi. Genetic polymorphisms in cytochrome P450 (CYP) 1A1, CYP1A2, CYP2E1, glutathione S-transferase (GST) M1 and GSTT1 and susceptibility to prostate cancer in the Japanese population. *Cancer Lett.* 2001;165(2):171-177.
20. Yim, J. J., G. Y. Park, C. T. Lee, Y. W. Kim, S. K. Han, Y. S. Shim, andC. G. Yoo. Genetic susceptibility to chronic obstructive pulmonary disease in Koreans: combined analysis of polymorphic genotypes for microsomal epoxide hydrolase and glutathione S-transferase M1 and T1. *Thorax* 2000;55(2):121-125.
21. Hallier, E., T. Langhof, M. Dannappel, M. Leutbecher, K. Schroder, H. W. Goergens, A. Muller, and H. M. Bolt. Polymorphism of glutathione conjugation of methyl bromide, ethylene oxide and dichloromethane in human blood: influence on the induction of sister chromatid exchanges (SCE) in lymphocytes. *Arch. Toxicol.* 1993;67:173178.
22. Chen C. L., Q. Liu, and M. V. Relling. Simultaneous characterization of glutathione S-transferase M1 and T1polymorphisms by polymerase chain reaction in American whites and blacks. *Pharmacogenetics* 1996;6: 187191.
23. Strange, R. C., P. W. Jones, and A. A. Fryer. Glutathione S-transferase: genetics and role in toxicology. *Toxicol. Lett.* 2000;112-113:357-363.