Original article

Genotypes of glutathione S-transferase M1 and N-acetyltransferase 2 in Japanese patients with gastric cancer

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Abstract:

Background. Genetic polymorphisms of some metabolizing enzymes, such as glutathione S-transferase M1 (*GSTM1*) and N-acetyltransferase 2 (*NAT2*), have recently been shown to affect individual susceptibility to various types of cancers. However, the link between the *GSTM1* polymorphism and gastric cancer is controversial and there are few studies focusing on the relation between a combination of the two enzyme genotypes and gastric cancer risk.

Methods. Genotypes of *GSTM1* and *NAT2* were determined by polymerase chain reaction (PCR) or restriction fragment length polymorphism (RFLP) following PCR in 147 Japanese patients with gastric cancer and 112 autopsied Japanese patients without stomach, lung, urinary bladder, or colon cancer.

Results. The frequency of the *GSTM1* null genotype was significantly increased in the cancer patients (91 individuals; 61.9%) compared with the controls (55 individuals; 49.1%) ($P < 0.05$; odds ratio, 1.68). The incidence of patients with the homozygously wild genotype of *NAT2* (rapid acetylator) was slightly lower in the cancer group (59 individuals; 40.1%) than in the control group (58 individuals; 51.8%). The proportion of patients with both the *GSTM1* gene and homozygously wild genotype of *NAT2* was significantly smaller in the cancer group (19 individuals; 12.9%) than in the control group (29 individuals; 25.9%) ($P < 0.05$). In addition, in a comparison of four subgroups of gastric cancer which were classified by both the genotype of *GSTM1* and the predicted phenotype of *NAT2*, the number of subjects in the subgroup “*GSTM1*+ / rapid” was significantly smaller than the expected numbers of the other three subgroups ($P < 0.05$).

Conclusion. It is suggested that a combination of *GSTM1* and *NAT2* decreases the risk of gastric cancer in Japanese patients.

Key words: glutathione-S transferase M1, N acetyltransferase 2, gastric cancer, Japanese

Introduction

Environmental genotoxic chemical agents arising from the diet, smoking, and air pollution are responsible for carcinogenesis in humans. It is generally accepted that the majority of carcinogenic chemicals do not produce their biological effects per se, but require metabolic activation by host enzymes: phase I enzymes including cytochrome P450 enzymes and phase II enzymes including epoxide hydroxylase, N-acetyltransferase 2 (*NAT2*), and glutathione S-transferases (GSTs) [1]. Genetic polymorphisms have recently been shown in many of these enzymes, indicating that there are genetic differences among individuals in the ability to metabolize chemicals [2]. Such genetic polymorphisms may affect the individual susceptibility to chemical carcinogenesis. From this point of view, the relationship between the genetic polymorphisms and carcinogenicity has been extensively examined to determine which polymorphic enzymes are highly associated with the susceptibility to cancers (see review [3]).

GSTs, which catalyze the conjugation of glutathione to a wide range of electrophilic compounds, are a gene family consisting of four classes named alpha, mu, pi, and theta [4]. Glutathione S-transferase M1 (*GSTM1*), a member of the *GST mu* family, is one of the most studied genotypes in relation to the individual susceptibility to cancers. For example, a considerable number of investigations indicate that individuals with a homozygous deletion of the *GSTM1* gene (*GSTM1* null genotype) are susceptible to lung and urinary bladder cancers [1,3,5]. However, the link between the *GSTM1* polymorphism and gastric carcinoma is controversial [6–10].

NAT2, which catalyzes the acetylation of certain therapeutic and carcinogenic arylamine chemicals, is also involved in the effects and toxicity of various substances. In humans and other mammals, differences in *NAT2* activity are genetically determined, and the

phenotypes are classified as “rapid”, “intermediate”, and “slow” acetylators. This genetic heterogeneity in NAT2 activity has been studied in relation to susceptibility to drug intoxication and certain cancers, such as urinary bladder and colorectal cancers [3]. However, to our knowledge, no reports have been published on the relation between NAT2 genotype and susceptibility to gastric cancer.

In the present study, we determined the genotypes of *GSTM1* and *NAT2* in 147 Japanese patients with gastric cancer and 112 autopsy cases of patients without stomach, lung, urinary bladder, or colon cancer to examine whether the polymorphisms of the two enzymes are related to susceptibility to gastric cancer.

Patients and methods

Study subjects and DNA preparation

One hundred and forty-seven patients with gastric cancer and 112 autopsy controls were examined in this study. All were Japanese. The patients with gastric cancer underwent surgical resection at the Department of Surgery of the Cancer Research Institute Hospital of Kanazawa University and the Komatsu City General Hospital. The age range of the patients with gastric cancer was 30–84 years (mean age, 62.5 ± 11.3 years; there were 98 men and 49 women). The control study with age-matched autopsy subjects (age at death, 20–95 years; mean age, 64.0 ± 17.5 years; 74 men and 38 women) excluded patients with stomach, lung, urinary bladder, or colon cancer, because several studies have indicated that *GSTM1* deficiency is a risk factor for the development of these cancers [1,3,5]. Smoking histories were not available in this study, because the information was incomplete in a considerable number of the cases and controls. Genomic DNA was isolated from carcinoma tissue and the adjacent normal mucosa as described [11]. As a control, genomic DNA was prepared from the autopsy liver in the same way.

Detection of *GSTM1* gene

Identification of the *GSTM1* genotype was performed according to the method of Kempkes et al. [12]. Two sets of oligonucleotide primers were used to amplify simultaneously the polymorphic *GSTM1* (5'-GAACT-CCCTGAAAAGCTAAAGC-3' and 5'-GTTGGG-CTCAAATATACGGTGG-3') and a segment of β -globin (5'-CAACTTCATCCACGTTTACC-3' and 5'-GAAGAGCCAAGGACAGGTAC-3'). A polymerase chain reaction (PCR) was performed in 100 μ l of the final reaction volume containing 1 μ g of DNA extracted from the tissue; 10 mM Tris-HCl, pH 8.3; 50 mM

KCl; 1.5 mM $MgCl_2$; 0.01% (w/v) gelatin; 200 mM each of dATP, dCTP, dGTP, and dTTP; 0.2 mM of each primer; and 2.5 units of a thermal stable DNA polymerase (Amplitaq; Perkin-Elmer Cetus, Norwalk, CT, USA). Forty cycles of amplification were done in a DNA Thermal Cycler (Perkin-Elmer Cetus) with a 94°C denaturation step (1 min), 55°C annealing step (1 min), and 72°C extension step (1 min). After the last cycle, the temperature was held at 72°C for 7 min. A 9- μ l volume of the PCR reaction mixture was electrophoresed on a 2% agarose gel containing ethidium bromide to verify the successful multiplication of the β -globin gene (268 bp) and to identify whether a 215-bp PCR product derived from the *GSTM1* gene was present or not (Fig. 1). To avoid false-positives, we carefully handled all specimens and reagents according to a strict recommended set of protocols [13,14].

Genotyping of NAT2

Determination of the NAT2 genotype was performed according to the method of Abe et al. [15], with some modifications. A set of oligonucleotide primers was used to amplify the 559-bp NAT2 genomic sequence (5'-TGACGGCAGGAATTACATTGTC-3' and 5'-ACACAAGGGTTTATTTTGTTC-3'). Forty cycles of amplification were done with a 94°C denaturation step (50 s), 55°C annealing step (50 s), and 65°C extension step (50 s). A 100- μ l PCR reaction mixture was electrophoresed on a 2% agarose gel containing ethidium bromide, and the PCR product of 559 bp was recovered from the gel with a diethylaminoethyl (DEAE) membrane. Four hundred ng of the 559-bp product was digested by incubation in a 10- μ l reaction mixture containing 10 units of *Bam* HI and *Asp* 718 at 37°C for 3 h, then digested by adding 10 units of *Taq* I

Fig. 1. Representative electrophoresis of polymerase chain reaction (PCR) products for *GSTM1* in cancer patients. A positive case is shown in lane 1 (adjacent normal mucosa) and lane 2 (cancer tissue); a negative case is shown in lane 3 (adjacent normal mucosa) and lane 4 (cancer tissue). The β -globin gene was simultaneously amplified to verify sufficient extraction of DNA from tissue samples. M, DNA size marker (ϕ X174 DNA digested with *Hae*III)

Fig. 2. Restriction enzyme sites of PCR products for four types of NAT2 alleles. Numbering is cited from [34]. Hatched boxes indicate the specific fragments for alleles 2, 3, and 4, which are generated by restriction enzyme digestion

and incubating at 65°C for an additional 3 h. The digested PCR products were electrophoresed on an agarose gel (3% agarose and 1.5% Nusieve GTG agarose [FMC Bioproducts, Rockland, ME, USA]) containing ethidium bromide.

Over 15 alleles of NAT2 have been reported in humans to date [16]. By the methods as described above, major alleles of human NAT2 (alleles 1, 2, 3, and 4, which were named by a Japanese investigator [17]) could be identified (Fig. 2). Alleles 1, 2, 3, and 4 corresponded to NAT2*4 (wild type), NAT2*7A/*7B (mutant), NAT2*6A/*6B (mutant), and NAT2*5A/*5B (mutant), respectively, according to the international nomenclature for NAT [16]. Most of the present patients with NAT2 genotype I (a homozygous combination of wild type) could be classified as rapid acetylators, the patients with NAT2 genotypes II/III/IV (a heterozygous combination of wild type and mutant) as intermediate acetylators, and the patients with NAT2 genotypes V/VI/VII/VIII/IX/X (a homozygous or heterozygous combination of mutant) as slow acetylators (Table 1) [17,18].

Statistical analysis

Statistical analyses using the χ^2 and proportion tests were performed on the results of GSTM1 and NAT2 polymorphisms. Odds ratios with 95% confidence intervals were also calculated.

Results

β -Globin and NAT2 genes were amplified in all 112 autopsy controls. In the group of 147 gastric cancer patients, the amplification of these genes succeeded in

all cases when we used DNA isolated from normal gastric mucosa. When we used DNA isolated from tumor tissue, the DNA amplification succeeded in all but one case because of the marked degradation of DNA in the tumor. All 146 of the cancer tissues with successful amplification of the β -globin gene showed the same genotypes of GSTM1 and NAT2 in the adjacent normal mucosa. Figure 3 shows the electrophoretic patterns of NAT2 genotypes that were identified in this study. Table 1 summarizes the genotypes of GSTM1 and NAT2 determined in the normal mucosa of the 147 cancer cases and the liver of the 112 controls. The frequency of the GSTM1 null genotype was significantly higher in the cancer patients (91 individuals; 61.9%) than in the controls (55 individuals; 49.1%). The odds ratio (OR) was 1.68 (95% confidence interval [CI], 1.03–2.75). The incidence of patients with homozygously wild genotype of NAT2 (rapid acetylator genotype) was lower in the cancer group (59 individuals; 40.1%) than in the control group (58 individuals; 51.8%), although the difference was not significant. However, the proportion of individuals with both the GSTM1 gene and the rapid acetylator genotype in the cancer group (19 cases; 12.9%) was significantly smaller than that in the control group (29 cases; 25.9%) ($P < 0.05$). In addition, among the four subgroups of gastric cancer cases classified by both the genotype of GSTM1 and the predicted phenotype of NAT2 (GSTM1+/rapid, GSTM1+/non-rapid, GSTM1 null/rapid, and GSTM1 null/non-rapid), the number of patients in the subgroup “GSTM1+/rapid” was significantly smaller than the expected numbers of the other three subgroups ($P < 0.05$) which were corrected according to each ratio of the corresponding control subgroups. We found that 34.7% of the gastric cancer patients had both the GSTM1 null and non-rapid acetylator genotypes, which

Table 1. Distribution of genotypes of *GSTM1* and *NAT2* in gastric cancer patients and controls

Genotype of NAT2	Combination of NAT2 alleles		Predicted NAT2 phenotype	Patients with cancer		Controls	
	Japanese nomenclature ^a	International nomenclature ^b		GSTM1(+)	GSTM1(-)	GSTM1(+)	GSTM1(-)
I	1 + 1	NAT2*4 + NAT2*4	Rapid	19	40	29	29
II	1 + 2	NAT2*4 + NAT2*7A/*7B	Intermediate	10	12	9	7
III	1 + 3	NAT2*4 + NAT2*6A/*6B	Intermediate	16	26	14	11
IV	1 + 4	NAT2*4 + NAT2*5A/*5B	Intermediate	2	2	1	3
V	2 + 2	NAT2*7A/*7B + NAT2*7A/*7B	Slow	2	0	0	0
VI	2 + 3	NAT2*7A/*7B + NAT2*6A/*6B	Slow	3	3	2	2
VII	2 + 4	NAT2*7A/*7B + NAT2*5A/*5B	Slow	0	0	0	1
VIII	3 + 3	NAT2*6A/*6B + NAT2*6A/*6B	Slow	4	8	2	2
IX	3 + 4	NAT2*6A/*6B + NAT2*5A/*5B	Slow	0	0	0	0
X	4 + 4	NAT2*5A/*5B + NAT2*5A/*5B	Slow	0	0	0	0
Total				56	91	57	55

^aReference [17]^bReference [16]

was non-significantly higher than the proportion in the controls (23.2%) (Table 2).

Discussion

The frequency of the *GSTM1* null genotype and the distribution of the *NAT2* genotype in the controls in the present study were in agreement with values reported previously in Japan [9,19–23]. The frequency of the *GSTM1* null genotype in Japan ranges from 42.6% [22] to 50.8% [9]. As shown in the control group in the present study, the predicted rapid and non-rapid acetylators have almost the same ratio in Japan [17,18,23]. The controls in the present study showed that the proportions of the four subgroups classified by both the genotype of *GSTM1* and the predicted phenotype of NAT2 (*GSTM1*+/rapid, *GSTM1*+/non-rapid, *GSTM1* null/rapid, and *GSTM1* null/non-rapid) were very similar, indicating that these two genes were inherited independently. In fact, *GSTM1* and *NAT2* genes have been identified on different chromosomes; chromosomes 1p13 [24] and 8p22 [25], respectively. In this sense, it can be concluded that the controls in the present study contained no biases that could result in errors in the statistical analysis.

Controversial case-control studies have been reported as to the association between the *GSTM1* null genotype and susceptibility to gastric cancer. Two research groups [7,10] have reported that the *GSTM1* null genotype is associated with an increased risk of gastric cancer in Japan. Katoh et al. [10] examined the *GSTM1* genotype of 139 patients with gastric adenocarcinoma and 126 controls and observed that the frequency of the *GSTM1* null genotype was significantly higher in the gastric cancer group (56.8%) than in the control group (43.6%) (OR, 1.7; 95% CI, 1.05–2.76). Harada et al. [7] also showed a close association between deficiency of the *GSTM1* gene and increased risk of gastric adenocarcinoma, in a small-scale study; 14 of 19 patients with gastric cancer (73.7%) and 40 of 84 controls (47.6%) lacked the *GSTM1* gene. However, another genetic study in Japan [9] denied the association, demonstrating that the *GSTM1* null genotype was identified in 30 of 64 patients with gastric adenocarcinoma (46.9%) and 61 of 120 controls (50.8%). A relatively large-scale, genetic study of English Caucasians [8] also demonstrated no association between deficiency of the *GSTM1* gene and increased risk of gastric adenocarcinoma; 52.9% (72/136) of the cancer cases and 54.8% (316/577) of the controls lacked the *GSTM1* gene. However, a biochemical study of the *GSTM1* phenotypes in Northern European subjects showed a significantly greater proportion of *GSTM1*-deficient subjects among patients with gastric adenocarcinoma compared with controls

Fig. 3. Electrophoresis of *NAT2* genotypes identified by restriction fragment length polymorphism following PCR in this study. A homozygous combination of allele 2 can be differentiated from a heterozygous combination of alleles 1 and 2 by the absence of a 99-bp band generated with *TaqI* and *BamHI*

Table 2. Numbers of subjects in subgroups classified by predicted phenotype of *NAT2* and genotype of *GSTM1* in gastric cancer patients and controls

Predicted <i>NAT2</i> phenotype	Patients with cancer		Controls	
	<i>GSTM1</i> (+) (%)	<i>GSTM1</i> (-) (%)	<i>GSTM1</i> (+) (%)	<i>GSTM1</i> (-) (%)
Rapid	19 (12.9%)	40 (27.2%)	29 (25.9%)	29 (25.9%)
Non-rapid	37 (25.1%)	51 (34.7%)	28 (25.0%)	26 (23.2%)
Total	56 (38.1%)	91 (61.9%)	57 (50.9%)	55 (49.1%)

*Significant difference, $P < 0.05$

[6]. In the present study, the *GSTM1* null genotype was significantly more frequent in the gastric cancer group than in the control group, which confirms the findings of two of the genetic studies in Japan [7,10]. The contradictory findings may be due to several factors, including the sample sizes, the choice of controls, the region, and the ethnicity of the subjects examined. For example, even a not very large difference in the incidence of *GSTM1* null genotype in same-ethnicity controls has a great influence on the conclusions of the statistical analysis; 43.6% (55/126 individuals) in the control group of the study by Katoh et al. [10] and 50.8% (61/120 individuals) in the study of Kato et al. [9]. In the present study, patients with stomach, lung, urinary bladder, or colon cancer were omitted from the control group, because it has been reported that *GSTM1* deficiency is frequently associated with these tumors [1,3,5]. A meta-analysis of the previous three studies in Japan [7,9,10] and the present study shows that the frequency of the *GSTM1* null genotype is significantly increased in the gastric cancer cases ($P < 0.01$; overall OD, 1.50; 95% CI, 1.14–1.99), suggesting that the *GSTM1* null genotype is a

small risk factor in the development of gastric cancer in the Japanese population.

In the present study, the *NAT2* genotype alone was not closely related to susceptibility to gastric cancer. However, the incidence of cancer patients with both the *GSTM1*-positive genotype and the rapid acetylator genotype was significantly lower than that in the controls. In addition, the proportion of cancer patients with these genotypes was significantly lower than that of cancer patients with the other genotypes (*GSTM1* +/non-rapid *NAT2*, *GSTM1* null/rapid *NAT2*, and *GSTM1* null/non-rapid *NAT2*). Furthermore, the incidence of individuals with both the *GSTM1* null genotype and the non-rapid acetylator genotype tended to be higher in the gastric cancer group than in the control group. These results suggest that a combination of *GSTM1* and *NAT2* decreases the risk of gastric cancer in Japanese patients and that *GSTM1* has more protective effects against cancer development than *NAT2*. Our findings are similar to those of a study on urinary bladder cancer: Brockmöller et al. [26] examined the genotypes of inherited polymorphic enzymes, *NAT2*, *GSTM1*, *GSTT1*,

microsomal epoxide hydrolase, and cytochrome P450 (*CYP1A1*, *2C19*, *2D6*, and *2E1*) in individuals with and without urinary bladder cancer and confirmed an increased bladder cancer risk in individuals with a *GSTM1* deficiency. They also found that individuals with two mutant alleles of *NAT2* were at increased bladder cancer risk if they were smokers or if they had high-risk occupations. Moreover, their study demonstrated that individuals deficient in both *NAT2* and *GSTM1* were at higher risk (crude OR, 1.48) compared with those deficient in only *GSTM1* (crude OR, 1.32) or *NAT2* (crude OR, 1.21). Katoh et al. [27] also reported that *GSTM1* null genotype and slow acetylation genotype of *NAT2* were risk factors in the development of bladder, renal, and ureter cancers, especially for smokers. The exact mechanisms explaining our results are unknown. However, *GSTM1* and *NAT2* are expressed in the human stomach [28–30], as well as the liver [2,17]. The former enzyme catalyzes the conjugation of many electrophilic compounds, including carcinogenic derivatives of polycyclic aromatic hydrocarbons [31], and the latter detoxifies aromatic amines, some of which are carcinogenic. The interaction of reactive chemical metabolites such as polycyclic aromatic hydrocarbons with DNA and protein is believed to be one of the crucial biochemical processes that damage DNA, causing cancer [1]. Hou et al. [32] studied the aromatic DNA adduct level and the genotypes of *GSTM1* and *NAT2* in non-smoking bus maintenance workers exposed to diesel exhaust, and found significantly higher levels of DNA adducts in slow acetylators without the *GSTM1* gene than in slow acetylators with the *GSTM1* gene. Taking these studies into consideration, it is possible that, in Japan, the combination of these two enzymes detoxifies carcinogenic compounds from the diet, smoking, air pollution, and so on, in the liver and gastric mucosa in situ to prevent their reaction with DNA and protein and reduce the risk of gastric cancer.

The present analysis of 146 cases with available DNA from cancer tissues demonstrated that the *GSTM1* and *NAT2* genes were well preserved. No changes of the genotypes were detected in the gastric cancer tissues by the PCR methods applied. Our previous study of colorectal cancer, using the method of restriction fragment length polymorphism with genomic DNA did not detect new mutations after the development of cancer [23]. Hubbard et al. [33] showed that the *NAT2* gene locus was not commonly deleted in colorectal cancer. Therefore, as suggested in colorectal cancer [23], the activity of *GSTM1* or *NAT2* in gastric cancer tissue and in the adjacent normal mucosa is probably at a similar level.

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