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Association of Isoniazid-metabolizing Enzyme Genotypes and Isoniazid-induced Hepatotoxicity in Tuberculosis Patients

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Abstract. *Background: Isoniazid (INH), a key drug of antituberculosis therapy, is metabolized by arylamine N-acetyltransferase2 (NAT2), cytochrome P450 2E1 (CYP2E1) and glutathione S-transferase (GST). We studied the possible influence of genetic polymorphisms of INH-metabolizing enzymes on serum concentrations of INH and its metabolites, as well as on the incidence of hepatotoxicity. Patients and Methods: A total of 144 tuberculosis patients who received antituberculosis treatment were followed prospectively. Their NAT, CYP2E1 and GST genotypes were determined using a polymerase chain reaction with restriction fragment length polymorphism method. Blood samples were collected from the patients and serum concentrations were determined by HPLC. The severity of hepatotoxicity was judged by the increases in either aspartate aminotransferase or alanine aminotransferase levels from the upper limit of the corresponding normal range. Results: Incidence of hepatotoxicity was highest in pulmonary tuberculosis patients with the slow acetylator (SA) phenotype and lowest in those with the rapid acetylator (RA) phenotype, although no clear relationship of genetic polymorphism of INH-metabolizing enzymes on the severity of hepatotoxicity were confirmed. Conclusion: The risk of side-effects, such as hepatic disorder, may rise in these patients with an SA phenotype, because of an increase in serum INH concentration. The evidence presented in this study, albeit based on the examination of a low number of patients, suggests that a safe INH dosage for tuberculosis patients with SA phenotype should be less than the dosage which is usually recommended for tuberculosis patients.*

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Tuberculosis is one of the most widely spread contagious diseases in the world. There are 9.4 million new cases and the disease claims 1.3 million lives worldwide every year (1). Despite Japan's status as a highly developed country, it remains in the intermediate-burden category because of the intermediate prevalence of tuberculosis (19.4 tuberculosis cases per 100,000 population) (2).

Isoniazid (INH), rifampicin (RFP), pirazinamide (PZA), ethambutol (EB) and streptomycin (SM) are used in the chemotherapy of tuberculosis, with INH in particular playing an important role in pharmacotherapy. Moreover, the management of tuberculosis is associated with serious problems, including an increase in the prevalence of disease relapse in elderly patients, multidrug-resistant *Mycobacterium tuberculosis*, occurrence in acquired immunodeficiency syndrome, and development of adverse effects of anti-tuberculosis drugs (3, 4). In particular, side-effects of INH such as hepatic disorder are viewed with suspicion in treatment for tuberculosis. It is of concern that dose reduction or discontinuation of anti-tuberculosis drugs because of hepatotoxicity may cause prolonged periods of treatment or emergence of multidrug-resistant *Mycobacterium tuberculosis*, and as a result, obstacles to tuberculosis treatment, where effective medication is limited, may rise.

The metabolic pathway of INH is shown in Figure 1; the major pathway is the metabolism of INH into acetyl isoniazid (AcINH) by *N*-acetyltransferase 2 (NAT2), which is estimated to account for 50-90% of INH metabolism. A portion of AcINH is further converted to acetyl hydrazine (AcHz) by amidase-catalyzed hydrolysis. There is another amidase-catalyzed route where INH is directly hydrolyzed to yield hydrazine (Hz). NAT2 then catalyzes the acetylation of Hz to yield AcHz and subsequently to diacetyl hydrazine before excretion. AcHz can also enter the cytochrome P450 2E1 (CYP2E1)-mediated metabolic pathway in the liver, which is linked to the glutathione-S-transferase (GST)-mediated metabolic pathway, to become an excretable

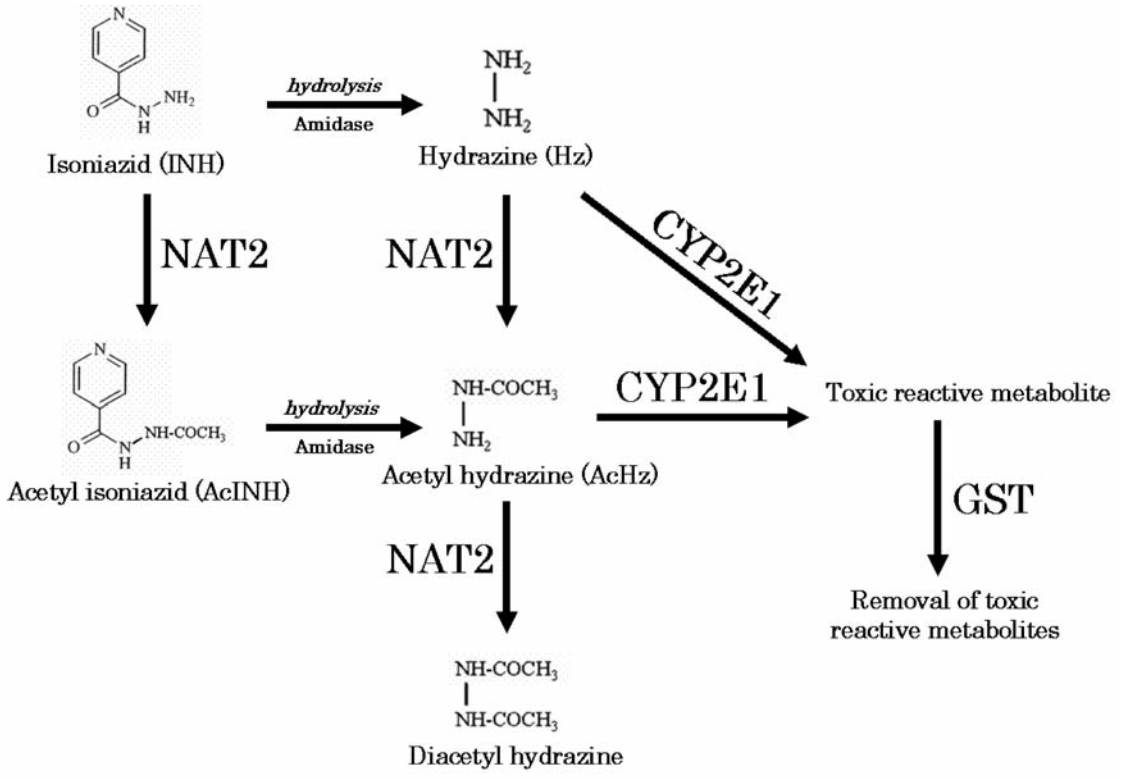

Figure 1. *Metabolic pathways of isoniazid in man. INH, Isoniazid; AcINH, acetylisoniazid; Hz, hydrazine; AcHz, acetylhydrazine; DiAcHz, diacetylhydrazine.*

metabolite. AcINH has little antibacterial effect on tubercle bacillus (about 1/100 the efficacy of INH), and therefore, for proper use, it is important to optimize the dosage and control serum INH concentrations.

Genetic polymorphism in *NAT2*, one of the causes of inter-individual variation in INH metabolism, is related to the antibacterial effect and side-effects of INH (5-7). People with low NAT2 activity have a higher risk of developing hepatic disorder than those with high NAT2 activity (8, 9). Considering that NAT2 is not the only enzyme involved in the metabolic pathway of INH, it is necessary to address the possible influence of genetic polymorphisms of the *CYP2E1* and *GST* genes, which are also involved in the metabolic pathway of INH, on the risk of hepatotoxicity.

With respect to *NAT2* genetic polymorphism, three mutant alleles (*NAT2**5B, *NAT2**6A and *NAT2**7B) have been found in Japanese populations to date, in addition to the wild-type *NAT2**4 allele (10, 11). Individuals who are homozygous for *NAT2**4 (wild-type allele) are classified as having the rapid acetylator (RA) phenotype, while those homozygous for mutant alleles, as well as heterozygous individuals (wild-type allele/mutant allele), are classified as having the slow acetylator (SA) and intermediate acetylator (IA) phenotypes, respectively (5). Regarding genetic polymorphism of *CYP2E1*, a member of the cytochrome P450 superfamily of enzymes, 12 allelic variants have been reported in addition to the wild-type allele. Among them are *CYP2E1**5B, of which mutations in the 5'-flanking region can be detected by PstI and RsaI digestion (12), and *CYP2E1**6, of which mutations in intron 6 can be detected by DraI digestion (13), and both are implicated in the altered enzymatic activity of *CYP2E1*. GSTs are enzymes that play a role in the detoxification system by conjugating cytochrome P450-activated substances with glutathione and are also involved in AcHz metabolism (14); consequently, the glutathione-S-transferase M1 (*GSTM1*) and glutathione-S-transferase T1 (*GSTT1*) genes have been studied for genetic polymorphism (15). The *GSTM1* locus has three allelic variants (M1a, M1b and M1 null), while the *GSTT1* locus has two allelic variants (T1 and T1 null), and the null genotype of both *GSTM1* and *GSTT1* are likely to result in loss of enzyme activities (16).

In this study, we examined cases of INH-induced hepatotoxicity in tuberculosis patients in order to elucidate the possible influence of genetic polymorphisms of INH-metabolizing enzymes on serum concentrations of INH and its metabolites, as well as on the prevalence of hepatotoxicity. Moreover, we investigated the relationship between pharmacokinetic (PK) parameters of INH and

NAT2 polymorphisms in tuberculosis patients undergoing INH treatment to obtain information for the control of the dosage of INH.

Patients and Methods

The present study was performed under the approval of the Ethical Review Boards of the National Hospital Organization Chiba-East Hospital and the Graduate School of Pharmaceutical Sciences, Chiba University.

Study participants. Among the inpatients with active pulmonary tuberculosis at the National Hospital Organization Chiba-East Hospital, 144 patients who were treated with the standard Japanese chemotherapy regimen (administration of INH, RFP and PZA, plus EB or SM during the first two months, followed by administration of INH and RFP plus EB or SM during the final four months), followed up for more than three months after treatment, and who consented to this study were investigated.

Anti-tuberculosis drugs were orally administered at 10 am to patients as standard treatment when deemed feasible. Blood samples were collected at 2 h after administration, before lunch, to determine concentrations of INH, AcINH, Hz and AcHz, and the genotyping of *NAT2, CYP2E1* and *GST*.

Genotyping of INH metabolic enzymes. Genomic DNA was extracted from 200 μl of patients' peripheral blood using a QIAamp® DNA Blood MiniKit (Qiagen Inc., Hilden, Germany) according to the manufacturer's instructions. Determination of genotypes was performed using polymerase chain reaction (PCR) with restriction fragment length polymorphism.

NAT2: Three restriction enzymes were individually used for genotyping. First, *KpnI* digestion distinguished the wild-type allele *NAT2**4 (yielding a 655-bp fragment) from the mutant allele *NAT2**5B (yielding a 710-bp fragment). Next, *TaqI* digestion distinguished the wild-type allele *NAT2**4 (yielding a 377-bp fragment, a 170-bp fragment and a 163-bp fragment) from the mutant allele *NAT2**6A (yielding a 377-bp fragment and a 333-bp fragment). *BamHI* digestion distinguished the wild-type allele *NAT2**4 (a 431-bp fragment and a 279-bp fragment) from the mutant allele *NAT2**7B (a 710-bp fragment). Patients who had homozygote wild-type alleles were judged as rapid acetylators (RA), those with heterozygote wild-type and mutant alleles as intermediate acetylators (IA), and those with homozygote mutant alleles as slow acetylators (SA).

CYP2E1: RsaI was used to distinguish the wild-type allele c1 (yielding a 352-bp fragment) from the mutant allele c2 (yielding a 413-bp fragment). On the other hand, *DraI* was used to distinguish the wild-type allele D (yielding a 251-bp fragment and a 125-bp fragment) from the mutant allele C (yielding a 376-bp fragment).

GSTM1: A 219-bp PCR product detected by gel electrophoresis indicated the wild-type genotype, while the absence of detectable PCR products indicated the null genotype.

GSTT1: A 459-bp PCR product detected by gel electrophoresis indicated the wild-type genotype, while the absence of detectable PCR products indicated the null genotype.

Analysis of INH and INH metabolites in serum. Blood samples were collected from the patients at 2 h (also, at 1, 4, 6 and 8 h post-administration for some) after administration of first-line anti-tuberculosis drugs that contained INH (approximately 5 mg/kg/day), followed by isolation of the serum. The serum concentrations of INH, AcINH, Hz and AcHz were determined by HPLC; the analytical conditions and parameters for HPLC was established by our group with reference to a previously reported method (17, 18). Concentrations lower than the lower limit of quantification were considered as 0.

Determination of serum concentration. INH: Two hundred microliters of trichloroacetic acid was added to 200 μl of serum, and the mixture was centrifuged at 13,000 × g for 5 min. The resulting supernatant (100 μl) was mixed with 10 μl of water and 20 μl of 1% *trans*-cinnamaldehyde in methanol and incubated for 10 min at room temperature. Twenty microliters of the resulting mixture was injected into the HPLC system. NANOSPACE SI-1 (SHISEIDO) equipped with CAPCELL MF Ph-1 (4.0×10 mm, SHISEIDO) as a guard cartridge was used for the HPLC system. INH was separated in a CAPCELL PAK C18 UG120 column (1.5 mm I.D.×250 mm, 5 μm, SHISEIDO) and measured at a detection wavelength of 340 nm. The column temperature was maintained at 50˚C. The composition of mobile phase used in INH detection was a mixture of 50 mM K2HPO4, acetonitrile and isopropanol (65:28:7, v/v). The flow rate for analysis was 100 μl/min.

AcINH: The mixture of 200 μl of serum and 200 μl of trichloroacetic acid was centrifuged at 13,000 × g for 5 min. The resulting supernatant (100 μl) was mixed with 10 μl of 6 M HCl and incubated for 1 h at 80˚C. Twenty microliters of 1% *trans*-cinnamaldehyde in methanol was added, and the mixture was incubated for 10 min at room temperature. Twenty microliters of the resulting mixture was injected into the HPLC system under the same conditions described above. AcINH serum concentrations were determined using calibration curves and the equation below, where *Sa, Sb* and *Sc* are the slopes of the calibration curves for measurement of INH, hydrolyzed INH and hydrolyzed AcINH, respectively, and *Aa* and *Ab* are the HPLC peak areas of INH and hydrolyzed INH of the sample, respectively. The constant, 1.3065, is the ratio of the molecular weight of AcINH to that of INH.

$$[\mathrm{AcINH}]=[Ab–Aa(Sb/Sa)](1.3065/Sc).$$

Hz: The mixture of 200 μl of serum and 200 μl of trichloroacetic acid was centrifuged at 13,000 × g for 5 min. The resulting supernatant (100 μl) was mixed with 10 μl of water and 20 μl of 1% trans-cinnamaldehyde in methanol and incubated for 10 min at room temperature. One hundred microliters of the mixture was injected into the HPLC system. NANOSPACE SI-1 (SHISEIDO) equipped with CAPCELL MF Ph-1 (4.0×10 mm, SHISEIDO) as a guard cartridge was used for the HPLC system. Hz was separated in a Discovery® HS PEG column (4.6 mm I.D.×150 mm, 5 μm, SUPELCO) and measured at a detection wavelength of 340 nm. The column temperature was maintained at 50˚C. The composition of mobile phase was 35% acetonitrile in water. The flow rate for analysis was 800 μl/min.

AcHz: Two hundred microliters of a phenacetin solution (16 μg/ml), 100 μl of 10% acetic acid and 3 ml of n-hexane were added to 500 μl of serum, and the mixture was centrifuged at 2,000 × g for 5 min. The organic layer was removed by aspiration. The remaining aqueous layer was mixed with 300 μl of 0.1% salicylaldehyde and 400 μl of 10% acetic acid, and the resulting mixture was shaken in a water bath at 60˚C for 30 minutes. After being cooled to room temperature, the mixture was further mixed with 1 ml of 1 M K_2HPO_4 and then with 6 ml of diethylether. After centrifugation at 2,000 × g for 5 min, 5 ml of the organic layer was transferred to a fresh tube and evaporated to dryness at 40˚C using

a centrifugal evaporator. The resulting pellet was mixed well with 200 μl of water, and 20 μl of the solution was injected into the HPLC system. NANOSPACE SI-1 (SHISEIDO) equipped with CAPCELL MF Ph-1 (4.0×10 mm, SHISEIDO) as a guard cartridge was used for the HPLC system. AcHz was separated in a CAPCELL PAK C18 UG120 column (1.5 mm I.D.×250 mm, 5 μm, SHISEIDO) and measured at a detection wavelength of 280 nm. The column temperature was maintained at 35˚C. The composition of mobile phases was 0.4% triethylamine in a mixture of acetonitrile/H_2O (1:3).The flow rate for analysis was 100 μl/min until 2 min and 130 μl/min after 2 min.

Estimation of PK parameters. The following patients were excluded: those without basic data necessary for analysis; those with pre-existing diseases (hepatic disorders, hepatic steatosis, cirrhosis, hepatitis B infection and hepatitis C infection) that may affect the clearance (CL/F) of INH; and those whose blood samples were collected four to five times (collection time-points: pre-INH administration, 1, 2, 4, 6 and 8 h after INH administration). A total of 184 points obtained from 36 patients were used for estimation of PK parameters. Serum concentration data was analyzed using the nonlinear least-squares method and MULTI software (19). We used the one-compartment model with the primary absorption process for the analyses.

Classification of hepatotoxicity in tuberculous patients. The severity of hepatotoxicity (hepatotoxicity A-D) was judged by the increase in either aspartate aminotransferase (AST) or alanine aminotransferase (ALT) levels from the upper limit of the corresponding normal range (AST, 33 U/l; ALT, 42 U/l): Hepatotoxicity A, above the upper limit and less than two-fold increase; hepatotoxicity B, two- to three-fold increase; hepatotoxicity C, three- to four-fold increase; hepatotoxicity D, greater than four-fold increase. Hepatotoxicity C and D were defined as considerable hepatotoxicity, while hepatotoxicity A and B as mild hepatotoxicity.

Statistical analysis. Numerical data are expressed as the mean±standard deviation. Statistical significance was analyzed using the Bartlett test to check the equality of variances; this was followed by one-way analysis of variance (ANOVA) if the variances were equal. If the variances were unequal, statistical significance was analyzed using the Kruskal-Wallis test. The ratios were analyzed for statistical significance using the χ^2 test. All statistical analyses were performed using SAS version 9.2 (SAS Institute Japan Inc.), and statistical significance was defined as $p<0.05$.

Results

Influence of genetic polymorphisms of INH-metabolizing enzymes on INH and INH metabolite concentration and hepatotoxicity prevalence. Characteristics of 144 tuberculosis patients are presented in Table I. The percentage of male and female patients in the subject group were 80.6% (116/144) and 19.4% (28/144), respectively. These figures were not largely different from the gender distribution in tuberculosis patients in Japan (male 62.1%, female 37.9%). Mean age was 52.1±18.0 years, while mean body weight was 55.0±10.6 kg. The mean dosage of INH, RFP and PZA used were slightly lower than the standard dosages (INH, 5 mg/kg; RPF, 10 mg/kg; PZA, 25 mg/kg; Table I).

Table II shows the characteristics of patients, dosage of anti-tuberculosis drugs per kilogram body weight and serum levels of INH and its metabolites for each of the *NAT2* phenotypes (RA, IA and SA) inferred from *NAT2* genetic polymorphisms. Sixty-five, sixty-six and thirteen patients had RA, IA and SA phenotypes, respectively. The percentage of female patients in the SA group was 30.8%, which was higher than that in the RA group (18.5%) and IA group (18.2%), albeit not significantly. There were no significant differences in age, body weight and anti-tuberculosis drug dosage among the three phenotype groups. The mean INH concentration at two hours after administration of anti-tuberculosis drugs was highest in the SA group; the INH concentration in the RA and IA groups was less than 50% that of the SA group and approximately 32% lower than that of the SA group, respectively. On the other hand, the mean AcINH concentration was highest in the RA group; the AcINH concentration in the SA and IA groups was approximately 82% and 46% lower than that in the RA group, respectively. The AcINH/INH ratio, indicative of acetylation capacity, was 2.21±0.95 in the RA group, which was the highest among the three groups. The mean Hz concentration was highest in the SA group; the Hz concentration in the RA and IA groups was approximately 35% and 19% lower than that in the SA group, respectively.

Table III shows the prevalence of each genotype of *CYP2E1, GSTM1* and *GSTT1* in the RA, IA and SA groups. The prevalence of the *CYP2E1* c1/c1 genotype was higher in the IA group than in the RA and SA groups, albeit not significantly. In the SA group, the prevalence of the *CYP2E1* D/D genotype was higher than in the other two groups, and there was no case of the *CYP2E1* C/C genotype, but differences were not significant. The prevalence of the *GSTM1* null genotype was lowest in the RA group, while highest in the SA group, but again differences were not significant. There were no significant differences in the prevalence of the GSTT1 null genotype among the three groups.

Table VI shows the prevalence of hepatotoxicity in three *NAT2* phenotype groups. The severity of hepatotoxicity was classified into four levels (graded low to high as hepatotoxicity A-D) (see Patients and Methods section). Treatment with anti-tuberculosis drugs was withdrawn for patients with hepatotoxicity D, but not from those with hepatotoxicity A-C. Levels of AST and ALT were decreased during treatment in patients with hepatotoxicity A-C without drug withdrawal. The percentage of tuberculosis patients who developed hepatotoxicity of any degree was higher in the SA group (8/13, 61.5%) than in the RA group (21/65, 32.3%) and IA group (23/66, 34.8%). However, the percentage of patients who developed a considerable degree of hepatotoxicity, which is clinically significant, in the SA group was 15.4% (2/13), which was not significantly different from the 15.4% (10/65) in the RA group and 10.6%

Table I. *Characteristics of tuberculous patients*.

	Tuberculous patients (n=144)
Gender (male/female)	116/28
Age (years)*	52.1±18.0
Height (meters)*	1.65±0.08
Body weight (kg)*	55.0±10.6
Heavy alcoholism (+/-)**	29/115
Hepatitis virus B or C infection (+/-)	12/132
INH dosage (mg/kg/day)*	4.83±0.62
RFP dosage (mg/kg/day)*	8.59±1.46
PZA dosage (mg/kg/day)*	22.25±4.06
NAT2 (RA/IA/SA)	65/66/13
CYP2E1 (c1c1/c1c2/ c2c2)	89/43/12
CYP2E1 (DD/DC/ CC)	68/62/14
GSTM1 (wild/null)	67/77
GSTT1 (wild/null)	88/56

*Data are expressed as the mean±standard deviation. **Heavy alcoholism means more than 540 ml in terms of rice wine.

Table II. *Characteristics of patients, dosage of anti-tuberculosis drugs and serum levels of INH and its metabolites according to NAT2 phenotype group*.

Characteristic	NAT2 phenotype		
	RA	IA	SA
Number of patients	65	66	13
Gender (male:female)	53:12	54:12	9:4
Age (years)*	55.1±17.5	49.6±18.0	49.4±19.2
Body weight (kg)*	55.6±10.5	55.2±11.0	50.7±8.3
HBV-positive patients	0	1	1
HCV-positive patients	5	5	0
Heavy alcoholism (+/-)**	12	14	3
INH dose (g/body/day)*	0.27±0.04	0.26±0.04	0.25±0.03
RFP dose (g/body/day)*	0.46±0.08	0.46±0.06	0.47±0.06
PZA dose (g/body/day)*	1.21±0.20	1.21±0.16	1.24±0.15
INH dose (mg/kg/day)*	4.84±0.68	4.77±0.58	5.05±0.49
RFP dose (mg/kg/day)*	8.44±1.54	8.60±1.42	9.34±0.89
PZA dose (mg/kg/day)*	22.01±4.04	22.47±3.14	24.51±2.59
INH concentration (μg/ml)*	2.22±0.90	3.37±1.47	4.94±0.81
AcINH concentration (μg/ml)*	4.66±2.08	2.51±1.06	0.86±0.59
AcINH/INH*	2.21±0.95	0.83±0.43	0.19±0.16
Hz concentration (ng/ml)*	19.84±8.76	24.84±12.25	30.68±12.47
AcHz concentration (ng/ml)*	2.11±1.53	2.02±1.37	0.96±0.63

*Data are expressed as the mean±standard deviation. **Heavy alcoholism means more than 540 ml in terms of rice wine.

Table III. *Prevalence of CYP2E1, GSTM1 and GSTT1 genetic polymorphisms according to NAT2 phenotype group*.

Gene	Genotype	*NAT2* phenotype			Total
		RA	IA	SA	
		65	66	13	144
CYP2E1 (*RsaI* detection)	c1/c1	37 (56.9%)	45 (68.2%)	7 (53.8%)	89 (61.8%)
	c1/c2	22 (33.8%)	17 (25.8%)	4 (30.8%)	43 (29.9%)
	c2/c2	6 (9.2%)	4 (6.1%)	2 (15.4%)	12 (8.3%)
CYP2E1 (*DraI* detection)	D/D	30 (46.2%)	31 (47.0%)	7 (53.8%)	68 (47.2%)
	D/C	26 (40.0%)	30 (45.5%)	6 (46.2%)	62 (43.1%)
	C/C	9 (13.8%)	5 (7.6%)	0	14 (9.7%)
GSTM1	Wild-type	36 (55.4%)	28 (42.4%)	3 (23.1%)	67 (46.5%)
	Null	29 (44.6%)	38 (57.6%)	10 (76.9%)	77 (53.5%)
GSTT1	Wild-type	45 (69.2%)	34 (51.5%)	9 (69.2%)	88 (61.1%)
	Null	20 (30.8%)	32 (48.5%)	4 (30.8%)	56 (38.9%)

The percentage of genotypes for the corresponding genes in each NAT2 phenotype group is shown in brackets.

Table IV. *Prevalence of hepatotoxicity by NAT2 phenotype group. The percentage of hepatotoxicity with different levels of severity according to NAT2 phenotype group is shown in brackets*.

	NAT2 phenotype			Total
	RA	IA	SA	
	65	66	13	144
Hepatotoxicity A	9	10	5	24
Hepatotoxicity B	2	5	1	8
Hepatotoxicity C	4	0	1	5
Hepatotoxicity D	6	8	1	15
Total	21 (32.3%)	23 (34.8%)	8 (61.5%)	52 (36.1%)
A+B	11 (16.9%)	14 (21.2%)	6 (46.2%)	32 (22.2%)
C+D	10 (15.4%)	7 (10.6%)	2 (15.4%)	21 (14.6%)

The percentage of hepatotoxicity with different levels of severity by NAT2 phenotype group is shown in brackets.

(7/66) in the IA group. In particular, the percentage of patients with hepatotoxicity D, who did not complete therapy with anti-tuberculosis drugs, was 9.2% (6/65), 12.1% (8/66) and 7.7% (1/13) in the RA, IA and SA groups, respectively.

Table V shows the characteristics of patients, dosage of anti-tuberculosis drugs, and serum concentrations of INH and its metabolites at 2 h after anti-tuberculosis drug administration in the different hepatotoxicity groups. There were no significant differences in age, body weight and dosage of anti-tuberculosis agents per kilogram bodyweight among the three groups. The percentage of patients with the RA, IA and SA phenotypes was 50.0%, 40.0% and 10.0%,

Table V. *Comparison of patient characteristics among the three hepatotoxicity groups.*

Characteristic	Hepatotoxicity					
	A+B (mean±sd)	Number of patients	C+D (mean±sd)	Number of patients	None (mean±sd)	Number of patients
Gender (male:female)	30:2	32	18:2	20	68:24	92
Age (years)	51.8±17.5	32	59.9±20.2	20	50.4±17.3	92
NAT2 (RA:IA:SA)	11:15:6	32	10:8:2	20	44:43:5	92
Body weight (kg)	57.1±12.0	32	56.2±10.2	20	53.9±10.1	92
INH dose (g/body/day)	0.27±0.04	32	0.26±0.05	20	0.26±0.04	92
RFP dose (g/body/day)	0.48±0.07	32	0.48±0.06	18	0.45±0.07	91
PZA dose (g/body/day)	1.26±0.19	30	1.23±0.20	16	1.19±0.17	87
INH dose (mg/kg/day)	4.78±0.64	32	4.65±0.48	20	4.88±0.63	92
RFP dose (mg/kg/day)	8.63±1.4	32	8.45±1.27	18	8.61±1.52	91
PZA dose (mg/kg/day)	22.28±3.29	30	22.2±4.79	16	22.51±3.46	87
INH concentration (μg/ml)	3.24±1.31	32	3.02±1.39	20	2.91±1.50	92
AcINH concentration (μg/ml)	2.75±1.61	32	3.61±2.35	20	3.11±3.72	92
Hz concentration (μg/ml)	25.23±11.13	32	22.58±12.31	20	22.49±11.21	92
AcHz concentration (μg/ml)	2.01±1.41	32	1.65±1.48	20	2.02±1.43	92

respectively, in the hepatotoxicity C and D group; 34.4%, 46.9% and 18.8%, respectively, in the mild (A+B) hepatotoxicity group; and 47.8%, 46.7% and 5.4%, respectively, in the no hepatotoxicity group. The percentage of patients with the SA phenotype was lower in the no hepatotoxicity group than in the other groups. Serum levels of INH, AcINH, Hz and AcHz were not significantly different among the three groups.

Table VI shows the prevalence of genetic polymorphisms of *CYP2E1, GSTM1* and *GSTT1* in the three hepatotoxicity groups. None of the genotypes of these genes showed clear association with the degree of hepatic disorder.

Changes in serum concentrations of INH and estimation of PK parameters for each NAT2 phenotype. Of the 36 patients, blood samples were collected five times at 0, 1, 2, 4, 6 h or 0, 2, 4, 6, 8 h after administration of INH; 21 patients were RA, 13 were IA, and 2 were SA. Changes of mean serum INH concentration in each NAT2 phenotype are shown in Figure 2. There was little difference between findings in RA and IA patients, although serum INH concentrations were higher in SA than RA and IA patients. PK parameters estimated by MULTI are shown in Table VII. The mean values of CL/F were 0.52 l/h/kg in RA and 0.42 l/h/kg in IA patients, suggesting a decrease of about 19% in IA patients. In SA patients, the mean value of 2 cases was 0.14 l/h/kg, which showed about a 73% decrease compared with RA. The mean values of the area under the concentration curve (AUC) were 9.37 mg h/l in RA, 12.62 mg h/l in IA, and 39.16 mg h/l in SA. The AUC value of IA was larger (135%) than that of RA, and that of SA was 4.2 times larger than that of RA and 3.1 times larger than that of IA.

Discussion

The major metabolic route of INH involves NAT2-catalyzed acetylation in the liver, where conversion of INH to AcINH is followed by hydrolysis of AcINH, to yield AcHz and NAT2-mediated acetylation of AcHz to yield diacetylhydrazine, which will eventually be excreted. *NAT2* genetic polymorphisms and the resulting variation in acetylation capacity of NAT2 are implicated in the risk of INH-induced hepatotoxicity (20). In this study, 45.1%, 45.8% and 9.0% of patients had RA, IA and SA phenotypes, respectively (Table I). The proportion of *NAT2* phenotypes in the Japanese population is thought to be about 10% SA, with RA and IA representing about 40-50%, respectively. Several research studies in Japan have also indicated this proportion (18, 21, 22). With regard to the *CYP2E1* genetic polymorphism examined in this study, 61.8%, 29.9% and 8.3% patients had c1/c1, c1/c2 and c2/c2 genotype, respectively; and 47.2%, 43.1% and 9.7% had D/D, D/C and C/C genotype, respectively (Table I). Our results are in agreement with the *CYP2E1* genotype distribution by *RsaI* restriction enzyme in healthy Asian individuals (c1/c1, 59.5%; c1/c2, 35.9%; c2/c2, 4.6%) reported by Bolt *et al*. (23) and with the *CYP2E1* genotype distribution by *DraI* restriction enzyme (D/D, 48.3%; D/C, 42.3%; C/C, 9.4%) reported previously (23). We found that 46.5% and 53.5% of patients had *GSTM1* wild-type and null genotypes, respectively, while 61.1% and 38.9% had *GSTT1* wild-type and null genotypes, respectively (Table I). These results are roughly similar to the *GSTM1* genotype distribution in a healthy Japanese population (wild-type, 45.5%; null genotype, 54.5%) reported by Komiya *et al*. (24) and to the

Table VI. *Prevalence of CYP2E1, GSTM1 and GSTT1 genetic polymorphism by hepatotoxicity group.*

Gene	Genotype	Hepatotoxicity None 92	A+B 32	C+D 20
CYP2E1 (*RsaI* detection)	c1/c1	60 (65.2%)	18 (56.3%)	11 (55%)
	c1/c2	23 (25%)	13 (40.6%)	7 (35%)
	c2/c2	9 (9.8%)	1 (3.1%)	2 (10%)
CYP2E1 (*DraI* detection)	D/D	45 (48.9%)	14 (43.8%)	9 (45%)
	D/C	37 (40.2%)	15 (46.9%)	10 (50%)
	C/C	10 (10.9%)	3 (9.4%)	1 (5%)
GSTM1	Wild-type	42 (45.7%)	7 (21.9%)	8 (40%)
	Null	50 (54.3%)	25 (78.1%)	12 (60%)
GSTT1	Wild-type	52 (56.5%)	23 (71.9%)	13 (65%)
	Null	40 (43.5%)	9 (28.1%)	7 (35%)

The percentage of each genotype of INH-metabolizing enzymes according to hepatotoxicity group is shown in brackets.

Table VII. *Pharmacokinetic parameters of INH estimated with the MULTI program. Data are expressed as mean±standard deviation for RA and IA, and as the average of 2 cases for SA.*

NAT2 phenotype	Number of patients	CL Mean (l/h/kg)	CL CV (%)	AUC Mean (mg h/l)	AUC CV (%)
RA	21	0.525	19.2	9.4	23.7
IA	13	0.42	25.9	12.6	29.7
SA	2	0.145 (0.117, 0.172)	---	39.1 (44.4, 33.9)	---

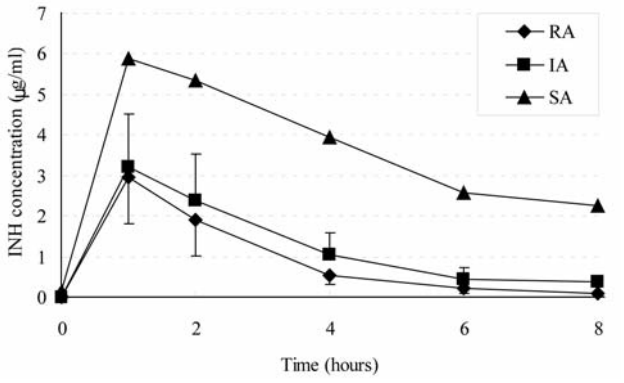

Figure 2. *Serum concentrations of INH after oral administration according to NAT2 phenotype group. Data are expressed as the mean±standard deviation in RA and IA. Averages of two cases are plotted for SA.*

GSTT1 genotype distribution (wild-type, 51.7%; null genotype, 48.3%), reported previously (24).

High levels of AcHz attributed to rapid acetylation of INH were implicated in the high incidence of INH-induced hepatotoxicity in the RA group (20, 25). On the other hand, there is contradicting evidence that AcHz levels are high in the SA group but low in the RA group because of rapid conversion of AcHz to diacetylhydrazine, an excretable metabolite of INH (26). It was also reported that the prevalence of elevated AST and/or ALT levels that were at least three times the upper limit of the normal range was significantly higher in the SA group (26%) than in the RA group (11%) (9). Furthermore, it was found that the SA group in a Japanese population was susceptible to INH-induced hepatotoxicity (8). However, the association between *NAT2* genetic polymorphism and hepatotoxicity was not always supported (27, 28), thus it remains unclear. Recently, experimental results showed that Hz, a product of INH hydrolysis, can induce hepatotoxicity (29, 30) and is more potent than AcHz (30), suggesting the involvement of CYP2E1 and GST in the mediation of Hz and AcHz metabolism in the development of hepatotoxicity (14, 31, 32). CYP2E1 catalyzes the oxidation of drugs in phase I of drug metabolism and is associated with hepatotoxicity induced by various drugs. On the other hand, GST catalyzes glucuronic acid conjugation in phase II of drug metabolism, thereby facilitating depletion of pharmacologically active compounds. Various studies have demonstrated the implication of *CYP2E1* and *GST* genetic polymorphisms in anti-tuberculosis drug-induced hepatotoxicity. For example, the *CYP2E1* c1/c1 genotype has been linked to the high incidence of INH-induced hepatotoxicity (31). Among patients with latent tuberculosis, a significantly high prevalence of INH-induced hepatotoxicity was observed with *CYP2E1* c1/c1, but not with any of the *NAT2* genotypes (32). For GST genotypes, it was shown that the risk of anti-tuberculosis drug-induced hepatotoxicity was high with the *GSTM1* null genotype (14, 33), but not significantly different among any of the *NAT2* or *GSTT1* genotypes (33). Converse results were reported in a different study where a significantly high prevalence of drug-induced hepatotoxicity was found with the *GSTT1* null genotype, but not with the *GSTM1* null genotype (34).

In the present study, we examined genetic polymorphisms of *NAT2, CYP2E1* and *GST*, serum concentrations of INH and its metabolites and levels of hepatotoxicity markers during treatment with anti-tuberculosis agents. The severity of hepatotoxicity was first grouped into four levels based on the increments of AST or ALT above the upper limit of the corresponding normal range (≤2-fold, 2- to 3-fold, 3- to 4-fold and >4-fold increments) then further classified into the mild hepatotoxicity group (above upper limit of normal with elevation of ≤3-fold) and the considerable hepatotoxicity group (elevation greater than 3-fold). Elevated levels of AST or ALT during treatment with anti-tuberculosis drugs often revert to normal ranges without withdrawal of drugs when increments are mild (35). In the present study, we found differences in the concentrations of INH and its metabolites (AcINH, Hz and AcHz) in serum among the *NAT2* phenotypes (RA, IA and SA) inferred from *NAT2* genetic polymorphism. The INH concentrations were highest in the SA group and lowest in the RA group (SA>IA>RA). Reverse trends were observed when comparing the concentrations of AcINH, the principal metabolite of INH, and AcHz, a product of AcINH hydrolysis (RA>IA>SA). These results suggest the strong influence of the *NAT2* phenotype on INH metabolism, leading to increased INH accumulation and consequent lowering of the blood levels of INH metabolites in individuals with lower acetylation capacity. We also found that serum levels of Hz were highest in patients with the SA phenotype and lowest in those with the RA phenotype (SA>IA>RA). Two lines of evidence suggest possible mechanisms for this trend. The first, found in this study, is that the Hz/AcINH ratio, an indicator of the balance between a hydrolysis route and an acetylation route in the INH metabolism pathway, was highest in the SA group and lowest in the RA group (SA>IA>RA). This indicates a skew toward the hydrolysis route as acetylation activity declines. In other words, a higher concentration of INH in patients with the slower acetylation phenotypes (SA and IA) caused the acceleration of hydrolysis, resulting in high Hz concentrations. The second line of evidence is that, considering the involvement of NAT2 in the metabolism of Hz, as well as that of INH, the AcINH/INH ratios are indicative of the status of metabolic conversion of Hz to AcHz (7), and the slower acetylation process leads to the accumulation of Hz in the SA and IA phenotypes. Taken together, elevation of serum Hz levels in the SA phenotype can possibly be attributed to increases in Hz production and decreases in metabolic conversion of Hz.

The overall incidence of hepatotoxicity was highest in pulmonary tuberculosis patients with the SA phenotype and lowest in those with the RA phenotype. On the other hand, the incidence of intermediate or more severe hepatotoxicity was comparable among the three phenotypes. Furthermore, hepatotoxicity was not severe enough to withdraw anti-tuberculosis agents, and levels of the hepatotoxicity markers (AST and ALT) reverted to which the normal ranges during therapy in the SA phenotype group. Thus, the *NAT2* phenotype influenced the serum concentrations of INH and its metabolites at two hours after drug administration, but not the degree of hepatotoxicity in this study. While considering the possible influence of the low occurrence of the SA phenotype population (13 out of 144 patients), serum levels of INH and its metabolites were compared among the three hepatotoxicity groups (no hepatotoxicity, mild hepatotoxicity and considerable hepatotoxicity groups), but differences were negligible. Similarly, there were no clear differences in the prevalence of *CYP2E1* and *GST* genetic polymorphism among the three *NAT2* phenotypes or among the three hepatotoxicity groups. Thus, it is difficult to show that genetic polymorphisms of the INH-metabolizing enzymes can account for the development of INH-induced hepatotoxicity in this study.

We envisaged the AUC of serum concentration-time, an indicator of total drug exposure, to be better suited than serum levels of INH and INH metabolites at two hours after drug administration for determining the influence on the degree of hepatotoxicity. Thus, the AUC values were examined in 36 patients with a profile of serum INH concentrations. The mean AUC values were 15.83, 12.57 and 11.25 mg h/l in the groups with considerable hepatotoxicity, mild hepatotoxicity and no hepatotoxicity, respectively. The higher AUC values in the hepatotoxicity group, albeit not significant, suggest a possible association between INH exposure and hepatotoxicity.

The mean values of INH clearance (CL/F) estimated with MULTI were 0.52 l/h/kg in RA, 0.42 l/h/kg in IA, and 0.14 l/h/kg in SA, which are similar to those already reported (36). We thought that the number of data points in the absorption process and around the C_{max} point is not enough to estimate *Vd* and *Ka*, and this may cause considerable variation in INH CL/F. Thus, fixed *Vd* and *Ka* values were used for estimation of parameters. The AUC value of IA was larger (135%) than that of RA, and that of SA was 4.2 times larger than that of RA and 3.1 times larger than that of IA. Therefore, the risk of side-effects such as hepatic disorder may rise in SA because of an increase in serum INH concentration. As described above, the overall incidence of hepatotoxicity was high in the patients with SA phenotype. In addition, INH concentrations at two hours after INH administration were above the target range (3-5 μg/ml) in patients with the SA phenotypes (37, 38), suggesting the necessity for adjustment of INH dosage in patients with the SA phenotype. Thus, we actually estimated serum levels of INH upon altered dosage of INH using the PK parameters specific for the SA phenotype (Figure 3). It was demonstrated that reducing INH dosage to approximately 3 to 4 mg/kg is necessary to maintain serum INH concentrations within the target range.

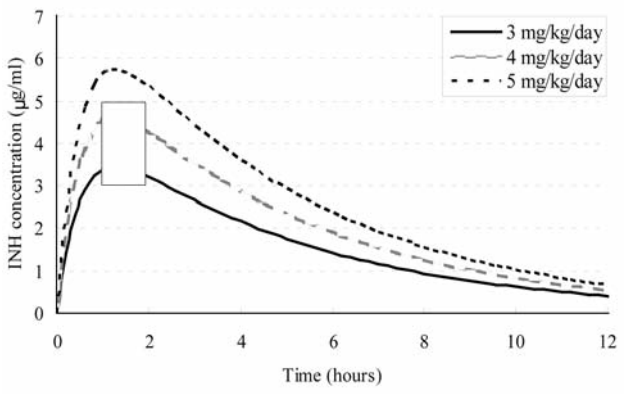

Figure 3. *Predicted serum concentration curve after oral administration of 3, 4 and 5 mg/kg INH. Serum concentration curves were estimated upon altered dosage of INH using the PK parameters specific for the SA phenotype.*

In conclusion, this study showed that both serum INH levels and the prevalence of above normal AST and ALT levels, which represent impaired liver function, were high in tuberculosis patients with the SA phenotype of *NAT2*, although no clear relationship of genetic polymorphism of INH-metabolizing enzymes on the severity of hepatotoxicity were confirmed. The evidence presented in this study, albeit based on the examination of a low number of patients, suggests that a safe INH dosage for tuberculosis patients with SA phenotypes should be less than the dosage which is usually recommended. Elucidation of the factors involved in INH-induced hepatotoxicity and the subsequent establishment of an appropriate INH regimen, which examines more patients, for example, by using a meta-analysis approach, are necessary for further progress in this area.

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