N-Acetyltransferase2 Genotype Correlated with Isoniazid Acetylation in Japanese Tuberculous Patients

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Isoniazid (INH) is metabolized by polymorphic *N*-acetyltransferase2 (NAT2). In the present study, the relationship between the *NAT2* genotype and the INH acetylator phenotype was examined in Japanese tuberculous patients and compared with healthy subjects. Subjects were classified according to the genotyping into *NAT2*5B* (*allele4*), *NAT2*6A* (*allele3*) and *NAT2*7B* (*allele2*), using the PCR-RFLP method. Twelve healthy subjects and 7 tuberculous patients participated in the INH acetylator phenotyping study, in which each subject was administered an oral dose of INH, followed by urine sampling for 24 h. Urinary concentrations of INH and *N*-acetylisoniazid (AcINH) were measured by the HPLC method. The urinary recoveries of INH (% of dose) in healthy subjects in relation to *NAT2* genotyping were as follows: 6.4±2.2 in the homozygotes for the wild-type allele, 10.7± 2.2 in the compound heterozygotes for the mutant allele, and 38.6±6.4 in the homozygotes for the mutant allele. In the patients study, the findings in the corresponding three groups were 4.0±1.7, 8.8 and 18.3±9.3. Although no significant difference was found because of the lower systemic exposure of INH in patients compared with healthy subjects, there were differences in the disposition kinetics of INH between subjects with and without mutations in the *NAT2* gene, and these findings were observed not only in healthy subjects but also in patients who had comedicated drugs and hepatic dysfunctions. The findings indicated that the metabolism of INH by NAT2 is clearly impaired in subjects with mutations in the *NAT2* gene, and thus genotyping for three *NAT2* point mutations was adequate to predict the metabolism of INH in Japanese tuberculous patients as well as healthy subjects. This *NAT2* genotyping could become a useful alternative to TDM for INH.

Key words *N*-acetyltransferase 2; genotype; isoniazid; tuberculous patients; healthy subjects; polymorphism

In the early 1950s, isoniazid (INH) was developed as chemotherapeutic agent for the treatment of tuberculosis.[2)] However, serious side effects including peripheral neuritis and hepatic toxicity were recognized in some patients, despite their dosage being similar to that taken by others. Previous studies indicated that INH was catabolized to inactive *N*-acetylisoniazid (AcINH) by *N*-acetyltransferase2 (NAT2) (Fig. 1), and that the interindividual variation of plasma concentration and urinary recovery of INH were characterized by a bimodal or trimodal distribution.[3,4)] *N*-acetylation of INH is reported to be genetically determined in a simple Mendelian fashion, and the frequencies of rapid (and/or intermediate) and slow acetylator of INH is different among racial populations.[5)] There have been studies on the relationships between the acetylator phenotype and the efficacy or side effects of INH. The rapid acetylators should take larger doses of INH than slow acetylators,[6)] and slow acetylators are at risk of adverse reactions such as peripheral neuritis,[4)] hepatic toxicity[4,7)] and systemic lupus erythematosus-like syndromes.[8,9)] These findings strongly suggested the necessity of therapeutic drug monitoring (TDM) in blood or serum to define the most appropriate dosage regimen for each individual.[10,11)]

Recently, Deguchi and his colleagues defined four *NAT2* alleles, the wild-type allele (*NAT2*4*) and three mutant alleles (*NAT2*5B*, *NAT2*6A* and *NAT2*7B*), which could explain 93—97.5% of the trimodal INH acetylator phenotype among Japanese healthy subjects.[12,13)] An individual INH

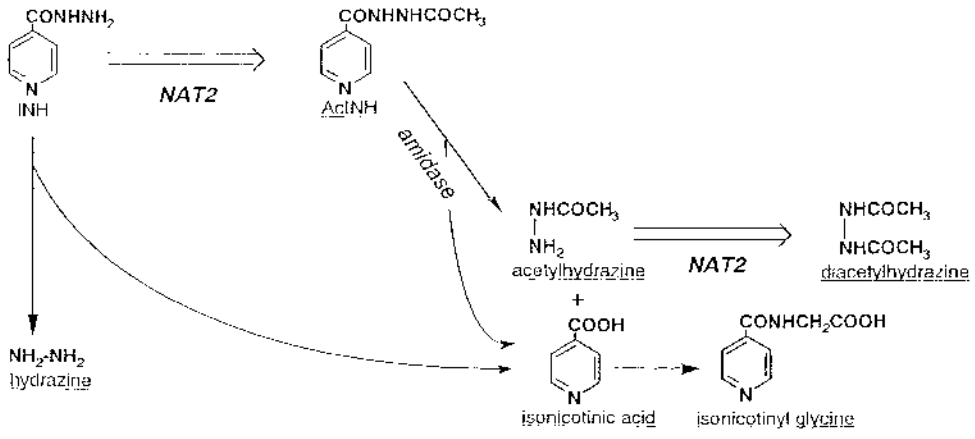

Fig. 1. Pathways for the Metabolism of INH in Humans

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acetylator for each phenotype is characterized by genotyping the *NAT2* gene polymorphism: the rapid acetylator phenotype (the homozygotes for the wild-type allele, *NAT2*4/*4*), intermediate (the heterozygotes for the mutant allele, *NAT2*4/*5B*, **4/*6A* and **4/*7B*), and slow (the homozygotes for the mutant allele, *NAT2*5B/*5B*, **5B/*6A*, **5B/*7B*, **6A/*6A*, **6A/*7B*, and **7B/*7B*). It was also suggested that the genotyping of these four alleles could predict the acetylator phenotype of procainamide in healthy Japanese subjects.[14] Phenotyping poses some drawbacks and difficulties for routine work because it includes the risk of toxicity and the interruption of drug therapy during test drugs administration. Compared with phenotyping, genotyping is simple and rapid, and subjects are free from the exposure to test drug. Thus, genotyping of the drug metabolizing enzymes which exhibit the genetic polymorphism is expected to be an alternative to TDM.[10,11] What should be clarified for the application of clinical genotyping is the correlation of the genotype and phenotype in patients, not in healthy subjects, since co-administration of other drugs and hepatic and renal dysfunction sometimes result in the quantitative and qualitative alteration of enzymes, which may change the phenotype, and subsequently make the genotyping invalid as an indicator of the phenotype.[15] In this study, we investigated the relationship between the *NAT2* genotype and the acetylator phenotype of INH in 12 healthy subjects and 7 tuberculous patients with various types of co-administered drugs and various states of renal and hepatic dysfunction.

MATERIALS AND METHODS

Chemicals INH was purchased from Wako Pure Chemical Industries, Ltd., Osaka. AcINH was synthesized by the method of Fox and Gibas.[16] Five kinds of polymerase chain reaction (PCR) primers for *NAT2* genotyping were synthesized by Rikaken Co. (Nagoya, Japan).[14] All other chemicals were of reagent grades and obtained commercially.

***NAT2* Genotyping** The *NAT2* genotype was defined based on the three point mutations, that is, the wild-type allele (*NAT2*4*) or three mutant alleles (*NAT2*5B*, *NAT2*6A* and *NAT2*7B*) according to Deguchi and colleagues,[12—14] which was diagnosed by the PCR–restriction fragment length polymorphism (RFLP) method using 0.5 ml blood samples.

The *NAT2* genotypes of the 12 healthy subjects were *NAT2*4/*4* ($n=3$), *NAT2*4/*6A* ($n=3$), *NAT2*4/*7B* ($n=3$), *NAT2*5B/*7B* ($n=1$), *NAT2*6A/*7B* ($n=1$) and *NAT2*7B/*7B* ($n=1$). Those of the 7 patients were *NAT2*4/*4* ($n=3$), *NAT2*4/*6A* ($n=2$), *NAT2*6A/*6A* ($n=1$) and *NAT2*6A/*7B* ($n=1$). The aims of the *NAT2* genotyping and acetylator phenotyping using INH were fully explained to each subject, and written informed consent was obtained.

INH Acetylator Phenotyping Study with Healthy Subjects Twelve non-smoking healthy subjects (age: 21—51 years, body weight: 45—81 kg, 8 male, 4 female) participated in the INH acetylator phenotyping study. They were prohibited from taking any drugs for one week prior to INH administration until the final urine sampling. After overnight fasting, each subject took a single oral dose of INH (5.0—6.3 mg/kg, 225—450 mg) as a Sumifon® tablet (Sumitomo Pharmaceuticals Co., Ltd., Osaka, Japan). They were also prohibited from taking any food and drink except for water for 3 h after INH dosing. Urine was collected every 1 h up to 6 h after administration, then collected at appropriate intervals up to 24 h. Generally, the "urinary recovery of INH (% of dose)," the "urinary recovery of AcINH (% of dose)" and the "urinary recovery ratio of AcINH to INH (AcINH/INH)" at 0—2, 3—4 h or 6—8 h after INH administration were used for the acetylator phenotyping method of INH.[17—19] Since 75—95% of a dose of INH is excreted in urine within 24 h after INH administration, the urine recovered during the 24 h after administration is necessary for the exact definition of the acetylator phenotype.[20]

Blank urine as a reference for the drug assay had been obtained the day before INH administration. All urine samples were weighed and an aliquot of each sample was stored at −20 °C until assayed.

INH Acetylator Phenotyping Study with Tuberculous Patients Seven inpatients with pulmonary tuberculosis (age: 47—68 years, 3 male, 4 female) also participated in this study. Clinical characteristics, INH dose and concomitant drugs are summarized in Table 1. All patients were co-administered pyridoxal phosphate to prevent the occurrence of pyridoxine-deficiency anemia. Two (patients No. 6 and No. 7) were also diagnosed with hepatic dysfunction,

Table 1. Characteristics of Hospitalized Patients with Tuberculosis

Patient No.	*NAT2* genotype[a]	Age	Gender	GOT	GPT	Serum creatinine	INH dose	Co-administered drugs
1	*NAT2*4/*4*	60	Male	16	16	0.7	300 mg/d	Pyridoxal phosphate, Teprenone, Prednisolone, Famotidine
2	*NAT2*4/*4*	52	Female	30	69	0.9	300 mg/d	Pyridoxal phosphate, Phenobarbital, Famotidine, Atenolol
3	*NAT2*4/*4*	47	Female	20	27	0.4	100 mg/d	Pyridoxal phosphate, Minocycline HCl, Mizoribine, Alfacalcidol
4	*NAT2*4/*6A*	82	Female	24	11	1.0	200 mg/d	Rifampicin, Pyridoxal phosphate, Ranitidine HCl, Isosorbic mononitrate, Lisinopril
5	*NAT2*4/*6A*	67	Male	39	55	0.5	300 mg/d	Rifampicin, Pyridoxal phosphate
6	*NAT2*6A/*6A*	60	Male	107	219	0.7	400 mg/d	Pyridoxal phosphate, Manidipine HCl, Allopurinol
7(1)[b]	*NAT2*6A/*7B*	68	Female	699	265	0.6	200 mg/d	Rifampicin, Valproic acid, Pyridoxal phosphate, Ticlopidine HCl
7(2)[b]	*NAT2*6A/*7B*	68	Female	44	24	0.6	200 mg/d	Pyridoxal phosphate

a) Based on the three point mutations defined by Deguchi and colleagues.[12,13] *b*) Patient No. 7 was included twice in this study, with hepatic dysfunction (1) and normal hepatic function (2).

whereas the others showed normal renal and hepatic function. All patients had taken Sumifon® tablets orally for more than 2 weeks and the urine samples were collected after INH administration in the morning for 24 h. For patient No. 7, urine samples were also collected after a dramatic recovery from hepatic dysfunction possibly caused by sodium valproate.

Determination of INH and AcINH The stocked urine samples were thawed at room temperature, then centrifuged at 3000 rpm (950×***g***) for 10 min. The supernatant was passed through a microporous membrane filter (0.45 μm, MILLEX-HA®, Nihon MILLIPORE, Tokyo), then filtered again through a 0.2 μm filter (LCR4-LG® Nihon MILLIPORE). A total of 350 μl of the filtered samples was diluted with the same volume of acetonitrile and centrifuged at 13000 rpm (16000×***g***) for 10 min. The supernatant was diluted 50-fold with distilled water and 20 μl of this solution was subjected to HPLC analysis.[21)]

HPLC (LC-6A series, Shimadzu, Kyoto, Japan) consisted of a system controller (SCL-6B, Shimadzu), a variable wavelength ultraviolet detector (SPD-6AV, Shimadzu) adjusted to 266 nm, and a data processor (Chromatopac C-R4A, Shimadzu). The stationary phase was a reverse phase Chemcobond 5-ODS-H column (4.6 mm i.d.×250 mm, particle size 5 μm, Chemco, Osaka, Japan). The mobile phase consisted of 10 mM NaH_2PO_4 solution, 1 mM SDS and 20% acetonitrile. The flow rate was 1.5 ml/min and the column temperature was maintained at 35 °C. The retention time of INH and AcINH was 10.5 min and 5.5 min, respectively. The calibration curves were linear over a concentration range of 0.125 to 4.0 μg/ml (r^2>0.999) for INH and 0.125 to 8.0 μg/ml (r^2>0.999) for AcINH with a day-to-day variance and within-a-day variance being neglected.

Pharmacokinetic Analysis Urinary recovery profiles of INH and AcINH were compared among the different genotypes in healthy subjects and patients. The urinary recovery (% of dose) of INH, AcINH and INH+AcINH, and the urinary recovery ratio AcINH/INH were calculated. For healthy subjects, the mean residence time in the body (*MRT*) was also calculated from the time-profiles of urinary recovery of INH according to the following equation,

$$MRT=\frac{1}{Ae^{\infty}}\int_0^{\infty}\left[Ae^{\infty}-Ae(t)\right]dt$$

where Ae^{∞} is the total amount of urine recovered and $Ae(t)$ is the cumulative amount excreted in urine up to time t. The amount excreted up to 24 h was used as Ae^{∞} because the excretion after that period was negligible. The numerical integration was carried out by the linear trapezoidal method without any extrapolation to infinity.

Statistical analyses were performed using unpaired t-test. Statistical significance was set at $p<0.05$.

RESULTS

INH Acetylator Phenotyping Study with Healthy Subjects Figure 2 shows the cumulative urinary recovery *versus* time curves for INH and AcINH following a single oral administration of INH to 12 healthy subjects. The recovery of INH in *NAT2*4/*4*, *NAT2*4/*6A* and *NAT2*4/*7B* were much lower than AcINH. In contrast, urinary recoveries of both INH and AcINH showed similar profiles in *NAT2*5B/*7B*, *NAT2*6A/*7B* and *NAT2*7B/*7B.*

Table 2 lists the 24 h urinary recovery of INH, AcINH and INH+AcINH, and the ratio AcINH/INH and the MRT of INH. Urinary recovery (% of dose) of INH in the homozygotes for the wild-type allele (*NAT2*4/*4*, 6.4±2.2) and the heterozygotes for the mutant allele (*NAT2*4/*6A* and *NAT2*4/*7B*, 10.7±2.2) were significantly lower than those in the homozygotes for the mutant allele (*NAT2*5B/*7B*,

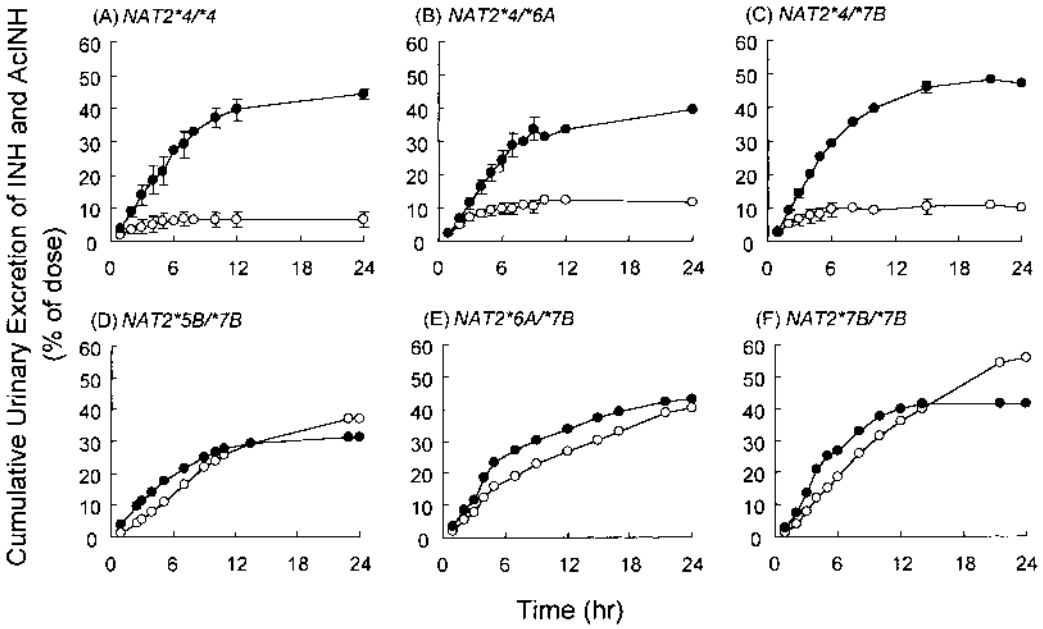

Fig. 2. Cumulative Urinary Recovery *versus* Time Curves for INH (○) and AcINH (●) Following a Single Oral Administration of INH to 12 Healthy Subjects

(A) *NAT2*4/*4*; (B) *NAT2*4/*6A*; (C) *NAT2*4/*7B*; (D) *NAT2*5B/*7B*; (E) *NAT2*6A/NAT2*7B*; (F) *NAT2*7B/*7B.* Each value represents the mean±S.D. (n=3) for panels (A), (B) and (C). Single-subject findings are presented in panels (D), (E) and (F).

Table 2. Urinary Recovery and the Mean Residence Time for INH and AcINH Following Oral Administration of INH in 12 Healthy Subjects and 7 Patients (Mean±S.D.)

NAT2 Genotype[a]		24 h Urinary recovery				*MRT* INH (h)
		INH (% dose)	AcINH (% dose)	INH+AcINH (% dose)	AcINH/INH	
Healthy subjects						
*NAT2*4/*4*	(*n*=3)	6.4±2.2**	44.4±1.6	50.8±3.2*	7.39±1.99**	2.90±0.51**
*NAT2*4/*6A*	(*n*=3)	11.4±1.7	39.6±4.6	51.0±2.9	3.57±0.99	3.38±0.30
*NAT2*4/*7B*	(*n*=3)	10.0±2.8	47.2±3.0	57.2±4.3	4.95±1.25	3.08±0.49
Subtotal[b]	(*n*=6)	10.7±2.2**	43.4±5.4	54.1±4.7**	4.26±1.26**	3.23±0.40**
*NA.T2*5B/*7B*	(*n*=1)	31.3	37.1	68.4	1.19	5.75
*NAT2*6A/*7B*	(*n*=1)	43.1	40.1	83.2	0.93	7.07
*NAT2*7B/*7B*	(*n*=1)	41.3	55.9	97.2	1.35	5.03
Subtotal[c]	(*n*=3)	38.6±6.4	44.4±10.1	82.9±14.4	1.16±0.21	5.95±1.04
Patients						Patients No.
*NAT2*4/*4*	(*n*=3)	4.0±1.7	37.3±14.8	41.3±16.5	9.53±0.50**	No. 1—3
*NAT2*4/*5B*	(*n*=1)	7.0	29.6	36.5	4.25	No. 4
*NAT2*4/*5B*	(*n*=1)	10.7	37.8	48.5	3.53	No. 5
Subtotal[b]	(*n*=2)	8.8	33.7	42.5	3.89	
*NAT2*6A/*6A*	(*n*=1)	21.5	22.8	44.3	1.06	No. 6
*NAT2*6A/*7B*[d,e]	(*n*=1)	7.8	15.7	23.6	2.01	No. 7(1)
*NAT2*6A/*7B*[d]	(*n*=1)	25.7	21.0	46.6	0.82	No. 7(2)
Subtotal[c]	(*n*=3)	18.3±9.3#	19.8±3.7#	38.2±12.7#	1.30±0.63	

The recovery for AcINH was presented as equivalent to INH. *a*) Based on the three point mutations defined by Deguchi and colleagues.[12,13] *b*) Mean±S.D. of the heterozygotes for the mutant allele (*NAT2*5B*, *NAT2*6A* and *NAT2*7B*). *c*) Mean±S.D. of the homozygotes for the mutant allele (*NAT2*5B*, *NAT2*6A* and *NAT2*7B*). *d*) Patient No. 7 was included twice in this study; with hepatic dysfunction (1) and normal hepatic function (2). *e*) Urine samples in patient No. 7(1) were taken until 10 h after INH administration in the morning. **; $p<0.01$, *; $p<0.05$: Significantly different from the homozygotes for the mutant allele, in healthy subjects or patients. #; $p<0.05$: Significantly different from healthy subjects with three genotype groups.

*NAT2*6A/*7B* and *NAT2*7B/*7B*, 38.6±6.4), although those of AcINH were comparable among these three genotype groups. Those of INH+AcINH in *NAT2*4/*4*, *NAT2*4/*6A* and *NAT2*4/*7B* were also much lower than *NAT2*5B/*7B*, *NAT2*6A/*7B* and *NAT2*7B/*7B*. AcINH/INH was significantly higher in *NAT2*4/*4*, *NAT2*4/*6A* and *NAT2*4/*7B* than in the other *NAT2* genotypes. MRT of INH was 3—4 h shorter in *NAT2*4/*4*, *NAT2*4/*6A* and *NAT2*4/*7B* than in *NAT2*5B/*7B*, *NAT2*6A/*7B* and *NAT2*7B/*7B.*

INH Acetylator Phenotyping Study with Tuberculous Patients The findings on the tuberculous patients are also presented in Table 2. As with healthy subjects, urinary recovery of INH showed a tendency to depend on the *NAT2* genotype, however, those of AcINH and INH+AcINH showed wide values of 15.7—50.7% and 23.5—56.0%, respectively, and were not correlated with their *NAT2* genotype. However, the urinary recovery ratio AcINH/INH could be classified into three groups according to the *NAT2* genotypes, *NAT2*4/*4* (9.53±0.54%), *NAT2*4/*6A* (4.25 and 3.53), and *NAT2*6A/*6A* (1.06) and *NAT2*6A/*7B* (2.01 or 0.82). Among the various pharmacokinetic parameters, AcINH/INH was the most useful index to describe individual acetylator phenotypes with respect to *NAT2* genotyping in the present patient study.

Comparison between Healthy Subjects and Tuberculous Patients Figure 3 shows the urinary recovery of INH and AcINH/INH in the 12 healthy subjects and 7 patients after oral INH administration. Although the urinary recovery of INH in the patients was lower than in the healthy subjects, there was a good correlation between the *NAT2* genotype and the trimodal distribution of the two pharmacokinetic parameters in both healthy groups.

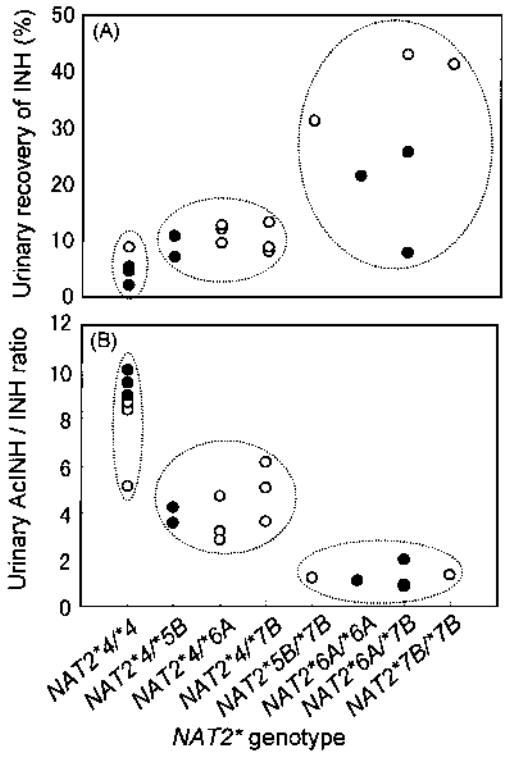

Fig. 3. The Urinary Recovery of INH (A) and the Urinary Recovery Ratio AcINH/INH (B) in All Subjects

○=healthy subjects; ●=tubercular patients. Each symbol indicates one subject. Subjects were classified into three groups based on their *NAT2* genotypes.

DISCUSSION

We observed a good relationship between the *NAT2* genotype and the acetylator phenotype in the 12 healthy subjects and 7 tuberculous patients in this study (Fig. 3). To examine the applicability of genotyping instead of TDM, urinary recovery was used as a phenotypic index, and the correlations with the urinary recovery of INH and the urinary recovery ratio AcINH/INH were investigated. INH urinary recovery was dependent on the *NAT2* genotype for both healthy subjects and patients, but a significant difference was found only for healthy subjects (Table 2). This could be explained by the enhancement of the INH metabolic pathway other than NAT2 or the decreased oral absorption efficiency in the patients.[20] That is, systemic exposure of INH was lower in the patients, resulting in an ambiguous correlation between the *NAT2* genotype and the urinary recovery of INH. To compensate for the insufficient exposure of INH, the urinary recovery ratio AcINH/INH was calculated for each healthy subject and patient. As shown in Fig. 3, there was a good correlation between the *NAT2* genotype and the urinary recovery ratio AcINH/INH in both healthy subjects and patients, and they could be superimposed.

There was no correlation between the *NAT2* genotype and the urinary recovery of AcINH or AcINH+INH especially for patients, which was because AcINH metabolism was easily affected by the concomitant drugs and hepatic dysfunction. All patients with tuberculosis were taking many concomitant drugs with INH, including phenobarbital (patient No. 2), rifampicin (patients No. 4, 5 and 7(1)) and sodium valproate (patient No. 7(1)). Cytochrome P450 and uridine diphosphate glucronosyltransferase enzymes were reported to be induced by phenobarbital and rifampicin, while sodium valproate inhibits them.[22,23] Rifampicin also induces hepatic amidase, which catabolizes AcINH into acetylhydrazine (Fig. 1).[23] Co-administration of drugs sometimes results in quantitative and qualitative alteration of the drug metabolizing system, and hepatic dysfunction could also alter this system.

However, when urinary recovery of INH and/or AcINH/INH was used as an index, the NAT2 activity in healthy subjects and patients with various types of co-administered drugs and various states of hepatic dysfunction showed trimodal distributions, and most were well-correlated with the *NAT2* genotype. These findings indicated that the *NAT2* genotyping has great potential as a complement to TDM for several drugs metabolized by NAT2 (*e.g.* INH, procainamide and some polycyclic amines).[14,24—27]

To date, a total of 17 mutant alleles have been found in the human *NAT2* gene.[28,29] Parkin *et al.* suggested the concordance between the *NAT2* genotypes composed of the recently defined 14 mutant alleles and the acetylator phenotype of INH in patients with tuberculosis.[30] These findings could provide sophisticated determinations of dosage regimens optimized for individuals. In addition, further continuous sequence analysis with an expanded population should find numerous additional mutations of the *NAT2* gene. The main purpose of the present study was to clarify the applicability of genotyping as an alternative to TDM in the clinical setting. Extensive genotyping is expensive and requires a very long time to analyze. The pharmacokinetic investigations conducted necessitate explanations for the trimodal distribution, and, we therefore, conducted a simple and rapid mutant analysis based on the three point mutations defined by Deguchi *et al.*,[12,13] *NAT2*5B* (*allele4*), *NAT2*6A* (*allele3*) and *NAT2*7B* (*allele2*), to explain it. This genotyping method was highly predictive of the trimodal INH acetylator phenotype with 93—97.5% accuracy, and also in this study, 95% (18/19) of acetylator phenotypes were also predicted by this genotyping with the exception of one healthy subject with *NAT2*4/*4*, whose phenotype was close to that of the compound heterozygotes for the mutant allele, but still far from the homozygote for that allele (Fig. 3(B)). Therefore, this simple and rapid genotyping method was adequate to predict the acetylator phenotype in Japanese, and was consistent with previous studies.

In conclusion, the definition of the three point mutations of the *NAT2* gene (*NAT2*5B*, *NAT2*6A* and *NAT2*7B*) predicted the INH acetylator phenotype in healthy Japanese subjects and patients. Concomitant drugs and hepatic dysfunction did not appear to influence the genotyping as an indicator, when the AcINH/INH ratio was used as a phenotypic index for *NAT2*. This *NAT2* genotyping method is simple and rapid, and, it is thus suggested that the genotyping of the drug metabolizing enzymes could be a useful alternative to TDM in clinical diagnoses.

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