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CLINICAL RESEARCH

*NAT2*6A*, a haplotype of the *N*-acetyltransferase 2 gene, is an important biomarker for risk of anti-tuberculosis drug-induced hepatotoxicity in Japanese patients with tuberculosis

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Abstract

AIM: To investigate an association between *N*-acetyltransferase 2 (*NAT2*)-haplotypes/diplotypes and adverse effects in Japanese pulmonary tuberculosis patients.

METHODS: We studied 100 patients with pulmonary TB treated with anti-TB drugs including INH. The frequencies and distributions of single nucleotide polymorphisms, haplotypes, and diplotypes of *NAT2* were determined by the PCR-restriction fragment length polymorphism method, and the results were compared between TB patients with and without adverse effect, using multivariate logistic regression analysis.

RESULTS: Statistical analysis revealed that the frequency of a variant haplotype, *NAT2*6A*, was significantly increased in TB patients with hepatotoxicity, compared with those without hepatotoxicity [P = 0.001, odds ratio (OR) = 3.535]. By contrast, the frequency of a wild-type (major) haplotype, "*NAT2*4*", was significantly lower in TB patients with hepatotoxicity than those without hepatotoxicity (P < 0.001, OR = 0.265). There was no association between *NAT2*-haplotypes and skin rash or eosinophilia.

CONCLUSION: The present study shows that *NAT2* is one of the determinants of anti-TB drug-induced hepatotoxicity. Moreover, the haplotypes, *NAT2*4* and *NAT2*6A*, are useful new biomarkers for predicting anti-TB drug-induced hepatotoxicity.

Key words: Tuberculosis; Anti-tuberculosis drugs; Drug-induced hepatotoxicity; *NAT2*-haplotype; DNA-based diagnosis

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INTRODUCTION

Tuberculosis (TB) is a re-emerging infectious disease that was declared a global health problem by the World Health Organization in 1993[1]. Since there were 9 million new TB cases and approximately 2 million TB deaths in 2004, and more than 80% of all TB patients live in sub-Saharan Africa and Asia, the epidemiology and control of TB remain important public health issues[1,2]. However, the management of TB is associated with serious problems, including disease relapse in elderly patients, occurrence

in acquired immunodeficiency syndrome, development of adverse effects of anti-TB drugs, and increase in the prevalence of multidrug-resistant *Mycobacterium tuberculosis*[2-5]. In particular, poor compliance or non-compliance with anti-TB drugs because of adverse effects, such as hepatotoxicity, skin rash, drug fever, peripheral neuritis, eosinophilia, and/or hyperuricemia, may lead to decrease in the quality-of-life of TB patients and appearance of multidrug-resistant *M. tuberculosis*. An important focus of previous studies was drug-induced hepatotoxicity, because it constitutes a major and severe adverse effect in the treatment of tuberculosis. Although the common risk factors for hepatotoxicity include advanced age[6,7], gender[7-10], malnutrition[6,9], complications of diseases[8,10,11], and alcohol intake[6,8,12], genetic factors also have an important impact on the likelihood of the development of drug-induced hepatotoxicity. Case-control studies with candidate genes in the affected populations have identified several possible susceptibility genes, e.g., *N*-acetyltransferase 2 (*NAT2*)[13-17], cytochrome P450 2E1 (*CYP2E1*)[16,18], glutathione *S*-transferase M1 (*GSTM1*)[16,19], glutathione *S*-transferase T1 (*GSTT1*)[16,19], and HLA-DQA1/-DQB1[20].

We focused our research on *NAT2* as a candidate gene associated with drug-induced hepatotoxicity because *NAT2* is the main enzyme involved in isoniazid (INH) metabolism, and is expressed in the liver. Diminution or disturbance of *NAT2* activity could result in the accumulation of precursors, such as hydrazine and acetylhydrazine in the liver, leading to hepatotoxicity[21-23]. Furthermore, the degree of metabolism with regard to *NAT2* varies among individuals, suggesting that genetic variations contribute to the metabolic activation capacity. Although studies on the association between *NAT2* phenotype (slow acetylator)[24] and anti-TB drug-induced hepatotoxicity have been reported from Taiwan[15,18], India[6,16], and Japan[13,14,17], no study has examined the association between hepatotoxicity and haplotypes/diplotypes that are composed of single nucleotide polymorphisms (SNPs). In the present study, we report our findings of the association between *NAT2* haplotypes/diplotypes and anti-TB drug-induced adverse effects, especially hepatotoxicity, in Japanese TB patients.

MATERIALS AND METHODS

Subjects

The study subjects comprised of 100 patients with new onset of pulmonary TB treated with a INH- (400 mg/d) and rifampicin (RFP, 450 mg/d)-containing regimen for six or nine months, between the years of 2003 and 2005 (Table 1). All subjects were Japanese who were recruited randomly from four general health clinics in the Nagasaki area of Japan. The study protocol was approved by the Committee for the Ethical Issue on Human Genome and Gene Analysis in Nagasaki University, and written informed consent was obtained from each patient.

The diagnosis of pulmonary TB was made on the basis of symptoms, chest radiographic infiltrates, and presence of acid-fast bacilli on sputum smear and *M. tuberculosis* on sputum culture. Patients with liver cirrhosis, chronic and acute hepatitis, alcoholic liver disease, and other chronic liver diseases were excluded from the study.

Table 1 Characteristics of pulmonary TB patients included in the study

Characteristics	TB
Number of patients	100
Age range (yr)	22-94
Age (mean ± SD)	64.0 ± 17.4
Gender (male/female)	56/44
Body mass index (kg/m^2)	20.3 ± 2.9

Diagnosis of drug-induced adverse effects

Patients with TB were classified into the following two subgroups: those with adverse effects such as hepatotoxicity, skin rash, and eosinophilia, and those without any side effects. Drug-induced hepatotoxicity was defined according to the criteria of the International Consensus Meeting[25], i.e., development of a two-fold or more increase in serum alanine aminotransferase (ALT) level above the upper limit of the normal range: N ($\leqslant$ 42 IU/L), or a combined increase of over 2 N in serum aspartate aminotransferase (AST, $N \leqslant$ 33 IU/L) and total bilirubin (TB, $N \leqslant$ 1.5 mg/dL). The presence of > 450 eosinophils/mL was defined as eosinophilia.

Determination of NAT2 polymorphisms

Genomic DNA was extracted from peripheral blood leukocytes of each patient using the DNA Extracter WB-Rapid Kit (Wako, Osaka, Japan), according to the manufacturer's protocol. SNPs of *NAT2* deposited in the SNP-database[26] were determined with PCR-restriction fragment length polymorphism (RFLP) method as described previously[27,28]. PCR was performed in a 25-μL reaction mixture containing 20 ng of genomic DNA, 20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 200 μmol/L dNTP, 0.4 μmol/L each of sense and antisense primers, and 1.5 U Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) with a DNA thermal cycler, GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA), according to the following protocol: initial denaturation at 95℃ for 5 min; 35 cycles of denaturation at 95℃ for 30 s, annealing at 55℃ for 30 s, and extension at 72℃ for 30 s; and a final extension at 72℃ for 5 min. Subsequently, the PCR product was digested by restriction enzyme (TaKaRa Biomedical, Shiga, Japan) for detection of each SNP. A SNP, C282T, was detected by digestion with *Fok* I. Likewise, C481T, G590A, and G857A were detected by *Kpn* I, *Taq* I, or *Bam* HI, respectively. These fragments were subjected to electrophoresis on a 2% agarose gel, and visualized with UV transilluminator (Alpha Innotech, San Leandro, CA, USA) after ethidium bromide (Invitrogen) staining. Haplotypes were determined to be based on a combination of four SNPs (Table 2)[26,28].

Statistical analysis

Data obtained are shown as mean ± standard deviation (SD). Clinico-pathological parameters were compared between TB patients with and without adverse effect, using the Mann-

Table 2 Five Haplotypes composed of four SNPs in *NAT2*

Haplotype	SNP			
	C282T	C481T	G590A	G857A
*NAT2*4*	-	-	-	-
*NAT2*6A*	+	-	+	-
*NAT2*7B*	+	-	-	+
*NAT2*11*	-	+	-	-
*NAT2*13*	+	-	-	-

Plus or minus symbols for C282T, C481T, G590A, and G857A indicate the presence or absence of SNPs.

Whitney *U* test, χ^2 test with Yates' correction, and Fisher's exact test. Expected allele frequencies were calculated from respective single allele frequencies according to the Hardy-Weinberg equilibrium. The observed and expected allele frequencies were compared by a χ^2 test using SNP Alyze 6.0 standard (Dynacom Inc., Chiba, Japan). To evaluate odds ratio (OR) with 95% confidence interval (95% CI) for the susceptibility to anti-TB drug-induced adverse effects, haplotype and diplotype frequencies were compared between TB patients with and without adverse effects, using multivariate logistic regression analysis. A *P* value of 0.05 or less was considered statistically significant. SPSS 14.0 (SPSS Japan Inc., Tokyo, Japan) program package was used for all statistical analyses.

RESULTS

Frequency of drug-induced adverse effects, and clinico-pathological parameters for susceptibility to the effects

Out of the 100 TB patients enrolled in the study, 50 (50%) patients had anti-TB drug-induced adverse effects, 18 hepatotoxicity, 25 skin rash, and 34 eosinophilia. There were no differences in the clinical characteristics and baseline laboratory data (before chemotherapy) between TB patients with and without adverse effects (Table 3). However, eosinophilia developed less frequently in female patients than male patients ($P = 0.0186$). During TB chemotherapy, patients with hepatotoxicity had 8-times higher serum levels of ALT and AST than those without hepatotoxicity ($P < 0.0001$). Likewise, during therapy, ALT values and eosinophil counts were significantly higher in patients with skin rash compared to those without skin rash ($P = 0.0245$ and $P = 0.0058$, respectively). Moreover, eosinophils in patients with eosinophilia were increased in number compared with those without this complication ($P < 0.0001$).

NAT2-haplotype susceptible to adverse effects

In the 100 TB patients examined, we identified three haplotypes composed of four SNPs (Table 4). One haplotype, "*NAT2*4*" is a wild-type (major type), while the other haplotypes are variants (minor types). Distribution of SNPs and haplotypes among patients corresponded well with the Hardy-Weinberg equilibrium, implying that our samples had a homogeneous genetic background, and was consistent with previous observations[13,14,17]. However, since the frequencies of two haplotypes, *NAT2*11* and *NAT2*13*, were very low, they were not used for further statistical analysis.

Multivariate logistic regression analyses revealed that the frequency of a variant haplotype "*NAT2*6A*", which is composed of two SNPs (C282T and G590A), was significantly increased in TB patients with hepatotoxicity, compared with those without hepatotoxicity ($P = 0.001$, OR = 3.535, 95% CI: 1.648-7.585) (Table 4). By contrast, the frequency of the wild-type (major) haplotype, "*NAT2*4*", was significantly lower in TB patients with hepatotoxicity than those without hepatotoxicity ($P < 0.001$, OR = 0.265). There were no significant differences in the frequency of *NAT2*-haplotypes between TB patients with and without skin rash or eosinophilia (Table 4).

NAT2-diplotype susceptible to adverse effects

We identified six diplotypes composed of three haplotypes (Table 5). Distributions of the diplotypes in our study population were consistent with previous observations [13,14,17]. Of a total of 18 TB patients with hepatotoxicity, 3 (16.6%) had a diplotype, "*NAT2*6A/*7B*"; using multivariate logistic regression analyses, the frequency was significantly higher than in patients without hepatotoxicity (2/82, 2.4%; $P = 0.029$, OR = 8.000, 95% CI: 1.230-52.023) (Table 5). On the other hand, the frequency of another diplotype, "*NAT2*4/*4*", was significantly lower in TB patients with hepatotoxicity than those without hepatotoxicity ($P = 0.032$, OR = 0.272). There was no difference in the frequency of *NAT2*-diplotypes between TB patients with and without skin rash or eosinophilia (Table 5).

DISCUSSION

We have shown that a variant haplotype, *NAT2*6A*, of *NAT2* is associated with susceptibility to anti-TB drug-induced hepatotoxicity, and a wild-type (major) haplotype, *NAT2*4*, is associated with non-susceptibility to hepatotoxicity. These findings suggest that *NAT2* is one of the genetic factors responsible for predisposition to anti-TB drug-induced hepatotoxicity. However, since the number of TB patients in the present study was relatively small, it remains to be confirmed whether this association can be reproduced in a larger number of Japanese TB patients with and without hepatotoxicity as well as in other ethnic populations. Although previous reports have shown a positive association in Japanese TB patients between drug-induced hepatotoxicity and *NAT2* variants with phenotypic activities of *NAT2*, such as rapid, intermediate, and slow acetylators[13,14,17], the present study is the first report demonstrating an association with *NAT2*-haplotype variation.

Three *NAT2* haplotypes, *NAT2*5B*, *NAT2*6A*, and *NAT2*7B*, are believed to be associated with slow acetylators[24,29,30]. We did not detect *NAT2*5B* in our samples, probably because of its low frequency in the Japanese population as described in our previous study[28]. Since *NAT2* is the main enzyme involved in the metabolism of INH and *NAT2*6A* is functionally related to the low activity of *N*-acetylation in the INH metabolic pathway[30], TB patients possessing *NAT2*6A* may fail to metabolize toxic substances, such as hydrazine and acetylhydrazine, generated by INH metabolism in the liver, which therefore accumulate in the body, leading to drug-induced hepatotoxicity[21-23,31,32].

Table 3 Clinical characteristics and laboratory data of TB patients with or without adverse effect

Clinical data	Hepatotoxicity		*P*	Skin rash		*P*	Eosinophilia		*P*
	Present (*n* = 18)	Absent (*n* = 82)		Present (*n* = 25)	Absent (*n* = 75)		Present (*n* = 34)	Absent (*n* = 66)	
Age (mean ± SD)	60.8 ± 17.7	64.7 ± 17.3	0.3942	63.6 ± 18.1	64.7 ± 17.3	0.9028	63.3 ± 19.5	64.4 ± 16.3	0.7598
Gender (M/F)	9/9	47/35	0.6081	12/13	44/31	0.3640	25/9	31/35	0.0186
Body mass index (kg/m^2)	19.6 ± 2.3	20.5 ± 3.1	0.2721	19.8 ± 2.5	20.6 ± 3.1	0.2626	19.8 ± 2.9	20.6 ± 3.0	0.2684
Baseline values									
ALT (IU/L)	18.0 ± 10.4	21.1 ± 16.6	0.4527	23.1 ± 25.8	19.7 ± 10.4	0.3392	25.9 ± 24.4	17.8 ± 6.8	0.6571
AST (IU/L)	29.1 ± 26.8	26.8 ± 23.3	0.7188	31.3 ± 39.2	25.9 ± 15.9	0.3268	35.4 ± 38.5	23.0 ± 7.8	0.4277
TB (mg/dL)	0.48 ± 0.19	0.64 ± 0.43	0.1115	0.61 ± 0.44	0.61 ± 0.39	0.9874	0.62 ± 0.44	0.61 ± 0.38	0.9490
Creatinine (mg/dL)	0.64 ± 0.13	0.88 ± 1.10	0.3482	0.72 ± 0.28	0.87 ± 1.13	0.5121	0.82 ± 0.47	0.84 ± 1.17	0.9092
Eosinophils (/μL)	105.1 ± 120.6	115.5 ± 121.8	0.7434	104.7 ± 93.7	116.6 ± 129.3	0.6750	141.3 ± 133.4	99.3 ± 112.6	0.1011
During TB chemotherapy									
Peak ALT (IU/L)	316.2 ± 281.7	40.0 ± 19.5	< 0.0001	147.8 ± 281.7	61.6 ± 88.1	0.0245	129.6 ± 284.1	59.2 ± 75.4	0.3885
Peak AST (IU/L)	294.5 ± 353.6	36.7 ± 21.5	< 0.0001	139.7 ± 222.9	73.1 ± 128.9	0.1325	116.3 ± 175.9	76.0 ± 149.3	0.2107
Peak TB (mg/dL)	1.20 ± 1.16	0.74 ± 0.53	0.0101	0.78 ± 0.47	0.84 ± 0.77	0.7039	0.92 ± 0.89	0.77 ± 0.58	0.3241
Peak Creatinine (mg/L)	0.76 ± 0.13	0.96 ± 1.23	0.4742	0.81 ± 0.13	0.97 ± 1.28	0.5316	0.87 ± 0.31	0.96 ± 1.36	0.7098
Peak Eosinophils (/μL)	692.4 ± 929.1	461.1 ± 844.7	0.4538	668.4 ± 773.9	447.5 ± 885.1	0.0058	1028.1 ± 1327.9	232.1 ± 114.9	< 0.0001

Table 4 Distributions of *NAT2*-haplotypes in TB patients with and without adverse effect

Haplotype	Hepatotoxicity					Skin rash					Eosinophilia				
	Present (%)	Absent (%)	OR	95% CI	*P*	Present (%)	Absent (%)	OR	95% CI	*P*	Present (%)	Absent (%)	OR	95% CI	*P*
*NAT2*4*	16 (44.4)	120 (73.2)	0.265	0.129-0.546	< 0.001	34 (68.0)	102 (68.0)	1.00	0.503-1.987	1.000	45 (66.2)	91 (68.9)	0.880	0.472-1.642	0.688
*NAT2*6A*	14 (38.9)	29 (17.7)	3.535	1.648-7.585	0.001	12 (24.0)	31 (20.7)	1.22	0.570-2.607	0.609	15 (22.0)	28 (21.2)	1.052	0.517-2.138	0.889
*NAT2*7B*	6 (16.7)	15 (9.1)	2.235	0.818-6.104	0.117	4 (8.0)	17 (11.3)	0.70	0.226-2.170	0.537	8 (11.8)	13 (9.9)	1.227	0.482-3.124	0.667
Total number	36	164				50	150				68	132			

Table 5 Distribution of *NAT2*-diplotypes in TB patients with and without adverse effect

Diplotype	Hepatotoxicity					Skin rash					Eosinophilia				
	Present (%)	Absent (%)	OR	95% CI	*P*	Present (%)	Absent (%)	OR	95% CI	*P*	Present (%)	Absent (%)	OR	95% CI	*P*
*NAT2*4/*4*	4 (22.2)	42 (51.2)	0.272	0.083-0.897	0.032	12 (48.0)	34 (45.3)	1.113	0.449-2.757	0.817	14 (41.2)	32 (48.5)	0.744	0.322-1.717	0.488
*NAT2*4/*6A*	7 (38.9)	23 (28.1)	1.632	0.564-4.726	0.366	7 (28.0)	23 (30.7)	0.879	0.323-2.394	0.801	11 (32.4)	19 (28.9)	1.183	0.484-2.894	0.713
*NAT2*4/*7B*	1 (5.6)	13 (15.9)	0.312	0.038-2.555	0.278	3 (12.0)	11 (14.7)	0.793	0.203-3.108	0.740	6 (17.6)	8 (12.1)	1.554	0.492-4.909	0.453
*NAT2*6A/*6A*	2 (11.1)	2 (2.4)	5.000	0.655-38.152	0.121	2 (8.0)	2 (2.7)	3.174	0.423-23.812	0.261	1 (2.9)	3 (4.5)	0.636	0.064-6.360	0.700
*NAT2*6A/*7B*	3 (16.6)	2 (2.4)	8.000	1.230-52.023	0.029	1 (4.0)	4 (5.3)	0.74	0.079-6.945	0.792	2 (5.9)	3 (4.5)	1.313	0.209-8.257	0.772
*NAT2*7B/*7B*	1 (5.6)	0 (0)	-	-	-	0 (0)	1 (1.3)	-	-	-	0 (0)	1 (1.5)	-	-	-
Total number	18	82				25	75				34	66			

A variant diplotype, *NAT2*6A/*7B*, is associated with susceptibility to hepatotoxicity ($P = 0.029$). Although another *NAT2*-diplotype, *NAT2*6A/*6A*, showed a trend towards susceptibility to hepatotoxicity, the results were statistically not significant ($P = 0.121$). However, if a larger number of subjects were analyzed, *NAT2*6A/*6A* as well as *NAT2*6A/*7B* may demonstrate a significant association with hepatotoxicity. Both of these diplotypes are homozygous for variant haplotypes and indicate phenotypically slow acetylators. Therefore, it is likely that some of the slow acetylators who are variant homozygotes possessing the *NAT2*6A* haplotype have susceptibility to anti-TB drug-induced hepatotoxicity. In this context, the results of the present study with regard to *NAT2*-haplotypes/diplotypes are comparable to those of previous reports on the association between *NAT2* phenotypic variation and hepatotoxicity[13-17,32]. Conversely, a wild-type homozygote, *NAT2*4/*4*, is associated with non-susceptibility and resistance to hepatotoxicity.

In conclusion, the haplotypes, *NAT2*4* and *NAT2*6A*, are new biomarkers for predicting drug-induced hepatotoxicity, and may prove useful in achieving optimal treatment of individual TB patients.

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COMMENTS

Background

Tuberculosis (TB) is a re-emerging infectious disease and has been declared a global health problem by the WHO. Adverse effect of anti-TB drugs including

isoniazid (INH) has become a serious problem in the management of tuberculosis. Risk factors associated with the development of adverse effects include both clinical and genetic factors. Recently, genome-wide screening and candidate gene-based association studies have been launched to identify the possible susceptibility genes sensitive to anti-TB drugs.

Research frontiers

Association studies with candidate gene-based approach in Asian and Caucasian patients have identified several possible susceptibility genes, e.g., *N*-acetyltransferase 2 (*NAT2*), cytochrome P450 2E1, glutathione *S*-transferase M1, glutathione *S*-transferase T1, and HLA-DQA1/-DQB1.

Innovations and breakthroughs

There are several reports on the association between *NAT2* polymorphisms and adverse effects, especially hepatotoxicity, of anti-TB drugs from Japan, Taiwan, and India. However, *NAT2* polymorphisms have been analyzed as phenotypic activities of *NAT2*, such as rapid, intermediate, and slow acetylators, but not as *NAT2*-haplotypes. The present study has shown that some phenotypically slow acetylators who are variant homozygotes possessing *NAT2*6A* haplotype have increased susceptibility to anti-TB drug-induced hepatotoxicity. This is the first report on the association with *NAT2*-haplotypes and hepatotoxicity in Japanese TB patients.

Applications

Our findings can be used for DNA-based diagnosis of TB patients before initiating treatment with anti-TB drugs, using *NAT2*6A* as a biomarker. Since patients possessing *NAT2*6A* haplotype have higher susceptibility to anti-TB-drug-induced hepatotoxicity, such individuals should be treated by reducing the dose of INH from 400 to 200 mg, in order to achieve optimal results.

Terminology

NAT2 is the main enzyme in the INH metabolism, and is expressed in the liver. Single nucleotide polymorphism (SNP) is a DNA sequence variation which occurs when a single nucleotide in the genome differs in paired chromosomes of an individual. Haplotype is a combination of alleles at multiple linked loci that are transmitted together. A second interpretation is that a haplotype is a set of SNPs on a single chromatid that is statistically associated. Such information is very valuable in investigating the genetics behind common diseases. Restriction fragment length polymorphism (RFLP) is a laboratory technique designed to distinguish differing nucleotide sequences from two related contexts.

Peer review

This study is well performed and the subject matter is very interesting.

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