Original Article

Association of Cytochrome P450 2E1 and N-Acetyltransferase 2 Genotypes with Serum Isoniazid Level and Anti-Tuberculosis Drug-Induced Hepatotoxicity: A Cross-Sectional Study

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What's Known

- Drug-metabolizing enzymes are associated with drug-induced liver toxicity.
- The extent of association between isoniazid-induced hepatotoxicity and slow acetylator phenotype varies in different populations.

What's New

- Hepatotoxicity was confirmed due to increased serum isoniazid levels in patients who are genetically slow acetylators. However, in contrast with previous studies, this was not the case for fast acetylators.
- Hepatotoxicity induced by fixed-dose combination drugs is likely lower than that of single anti-tuberculosis drug formulations.

Abstract

Background: Anti-tuberculosis drug-induced hepatotoxicity can result from genetic polymorphism of the isoniazid (INH) metabolizing enzyme. This study aimed to determine the effect of genetic polymorphism of N-acetyltransferase 2 (*NAT2*) and cytochrome P450 2E1 (*CYP2E1*) genes on serum isoniazid level and drug-induced hepatotoxicity.

Methods: A cross-sectional study was conducted on 120 patients (with and without hepatotoxicity) with pulmonary tuberculosis from June 2019 to April 2022 in Tehran (Iran). High-performance liquid chromatography was used to measure the serum concentration of INH and acetylisoniazid (AcINH). *NAT2* and *CYP2E1* genotypes were determined using polymerase chain reaction and restriction fragment length polymorphism methods. Data were analyzed using SPSS software (version 22.0) with independent two-sample *t* test, Chi square test, or Fisher's exact test. P<0.05 was considered statistically significant.

Results: A total of 40 patients showed hepatotoxicity. The risk of anti-tuberculosis drug-induced hepatotoxicity was significantly higher in patients who are slow acetylator (SA) phenotype than in rapid or intermediate acetylator (P<0.001). *NAT2**4/*4 genotypes were not found in patients with hepatotoxicity. The frequency of *NAT2**5 and *NAT2**6 haplotypes and serum INH concentration was significantly higher in patients with hepatotoxicity than in those without (P=0.003, P<0.001, and P<0.001, respectively). *NAT2**4 haplotype was correlated with protection against hepatotoxicity. A combination of SA and *CYP2E1* C1/C1 genotype was significantly associated with hepatotoxicity (P<0.001).

Conclusion: Hepatotoxicity in Iranian patients with tuberculosis was confirmed due to the presence of *NAT2* SA polymorphism. Determining *NAT2* and *CYP2E1* genotypes and/or INH concentration can be a valuable tool to identify patients susceptible to hepatotoxicity.

Introduction

Anti-tuberculosis drug-induced hepatotoxicity has long been a major clinical treatment issue and the cause of treatment failure

and mortality in patients with tuberculosis.[1, 2] The common treatment for tuberculosis is a six-month regimen, comprising of a two-month course of isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA), and ethambutol (EMB), followed by a four-month course of INH and RIF. In recent years, in patients weighing over 30 Kg, the use of fixed-dose combination (FDC) drugs has been replaced by separate anti-tuberculosis drug formulations.[3]

Studies suggested a link between drug-metabolizing enzymes and drug-induced hepatotoxicity.[4, 5] Enzymatic systems responding to anti-tuberculosis drugs include cytochrome P450 2E1 (*CYP2E1*), N-acetyltransferase 2 (*NAT2*), and glutathione S-transferase (*GST*). These are genetically polymorphic and exhibit a broad interindividual variation in drug metabolism, adverse effects, and malignancies such as cancer.[2, 6, 7] INH is mainly metabolized through *NAT2* into acetylisoniazid (AcINH), a phase two conjugating enzyme expressed in the liver, and then hydrolyzed to acetyl hydrazine. Direct hydrolysis of INH to hydrazine, a potent hepatotoxin, is another metabolic pathway that generates toxic metabolites. *NAT2* also metabolizes hydrazine into acetyl hydrazine and eventually into diacetyl hydrazine, which is a non-toxic compound.[8]

There are three genetic phenotypes for the *NAT2* enzyme, including rapid acetylator (RA), intermediate acetylator (IA), and slow acetylator (SA), which are principal hereditary traits. People with high *NAT2* activity (RA) have a lower risk of hepatotoxicity than those with low activity (SA).[9] Moreover, people with high INH acetylation capacity may develop hepatotoxicity. Therefore, it is hypothesized that other factors than *NAT2* gene polymorphism could be involved in hepatotoxicity.[8, 10, 11] The main reason for high hepatotoxicity in the SA state is assumed to be due to INH acetylation at a low rate, though it hydrolyzes directly to toxic hydrazine. Co-administration of RIF and INH not only enhances INH but also induces and increases hydrolysis of INH to hydrazine, ultimately increasing the risk of hepatotoxicity. In the RA state, INH is rapidly converted to non-toxic diacetyl hydrazine thus lowering hypersensitivity to hepatotoxicity.[9, 12] Studies investigating the relationship between hepatotoxicity and various concentrations of anti-tuberculosis drugs have demonstrated an increase in plasma concentration of anti-tuberculosis drugs on days 7 and 14, especially INH and RIF, such that the likelihood of hepatotoxicity is higher on these days.[10, 13] However, another study reported no association between serum concentrations of these drugs and the risk of hepatotoxicity in tuberculosis patients.[14] The most significant allelic alteration in *NAT2*-acetylating gene polymorphism is related to single nucleotide replacement at positions 481 (C to T, M1), 590 (G to A, M2), and 857 (G to A, M3).[6, 7]

Cytochrome P450 is a family of enzymes that catalyzes the oxidative biotransformation of most lipophilic drugs. *CYP2E1* enzyme is mainly expressed in the liver and is the major metabolic pathway of INH.[15] Acetyl hydrazine is oxidized by *CYP2E1* to form toxic mediators.[8, 16] A meta-analysis study on *CYP2E1 Rsa*I/*Pst*I and *Dra*I gene polymorphisms has reported that the C1/C1 genotype of these genes pose a higher risk of hepatotoxicity,[17] whereas another study found no significant correlation.[18] Other studies have reported that *CYP2E1 Rsa*I/*Pst*I*C1 and *CYP2E1 Dra*I*C alleles have high enzymatic activity, thereby increasing the production of hepatotoxins.[19-22]

The present study aimed to evaluate the relationship between genetic polymorphism of *NAT2* and *CYP2E1* with plasma concentration of INH and its metabolite (AcINH), and to determine their association with hepatotoxicity in Iranian patients with tuberculosis. To the best of our knowledge, this is the first study that examines the simultaneous effect of genetic polymorphism of these genes on INH and AcINH concentrations in tuberculosis patients receiving FDC drugs. We also assessed the effect of *NAT2* and *CYP2E1* gene mutations on drug-induced-hepatotoxicity.

Patients and Methods

A cross-sectional study was conducted on 120 (87 men and 33 women) Iranian patients with pulmonary tuberculosis at Masih Daneshvari Hospital (Tehran, Iran) from June 2019 to April 2022. The patients were divided into two groups, namely patients with anti-tuberculosis drug-induced hepatotoxicity and those without hepatotoxicity. The protocol of the study was approved by the Ethics Committee of Islamic Azad University, Science and Research Branch, Tehran, Iran (code: IR.IAU.SRB.REC.1397.105). Written informed consent was obtained from all patients.

Inclusion and Exclusion Criteria

Patients were evaluated for liver enzymes, especially alanine transaminase (ALT), aspartate transaminase (AST), and total bilirubin prior to administration of the drugs. The exclusion criteria were patients aged <16 years and >80 years; abnormal ALT, AST, and

total bilirubin; a history of alcohol consumption; chronic liver disease, hepatitis, autoimmune diseases, HIV, hypoxia, congestive heart failure, bacterial infection; any other factors causing abnormal serum transaminases and bilirubin levels; and the use of medications interacting with anti-tuberculosis drugs. Patients who did not meet the above-mentioned criteria and had a positive *Mycobacterium tuberculosis* culture were included in the study.[23, 24]

Administration of Anti-tuberculosis Drugs

As the first-line treatment, four anti-tuberculosis drugs were administered, namely INH (75 mg), RIF (150 mg), EMB (275 mg), and PZA (400 mg). Patients weighing under 50 Kg received three FDC tablets, between 50 Kg and 80 Kg received four FDC tablets, and those over 80 Kg received five FDC tablets. Hepatotoxicity was defined as ALT and AST serum levels more than three times the normal range in patients with symptoms of hepatotoxicity or ALT and AST serum levels more than five times and bilirubin serum levels more than three times the normal range in patients with no symptoms of hepatotoxicity.[25] Assessment duration was the first two weeks from drug administration until the onset of hepatotoxicity. In this period, there is no possibility of genomic modification. Serum ALT, AST, and bilirubin levels were assessed between day 7 and day 14 of hospitalization by collecting blood samples at 6 AM. If hepatotoxicity was confirmed, genetic tests were performed on the same day of blood sampling and the concentration of isoniazid and its metabolite were measured two hours after taking the FDC drug. Otherwise, in the case of non-hepatotoxicity, the tests were performed on day 14.[14] For each patient, demographic information as well as the status of alcohol consumption, drug use, smoking history, comorbidities, viral infection status, and other medical data were collected. Beyond day 14, serum AST, ALT, and total bilirubin levels of all patients were monitored on a weekly basis during the first month and then monthly until the end of treatment, i.e., no signs and symptoms of hepatotoxicity.

Blood Samples and DNA Extraction

Blood samples collected from patients were transferred into two tubes. The first tube contained ethylenediaminetetraacetic acid (EDTA) for DNA extraction, and the second tube contained heparin gel to separate blood plasma and measure INH and AcINH concentrations. The gel-containing tubes were immediately centrifuged, and the plasma was poured into a 1.5 mL microtube and stored at -70 °C. From the EDTA tubes, DNA was extracted on the same day using a DNA extraction kit (Genet Bio Co., South Korea) and stored at -20 °C.

Genetic Polymorphisms of NAT2

The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique was used for genotyping of *NAT2*.[6, 26] The primers used in PCR amplification were P1 (5′-gtcacacgaggaaatcaaatgc-3′) and P2 (5′-gttttctagcatgaatcactctgc-3′). All primers were purchased from Metabion International AG (Germany), and a 1:10 dilution with distilled water was prepared for analysis. PCR was performed using 1,250 µL Taq™ DNA Polymerase Master Mix (Ampliqon, Denmark) consisting of Taq DNA polymerase buffer, dNTPs, and $MgCl_2$ (1.5 µm). In addition, 12.5 µL of Master Mix, 0.1 µL of each P1 and P2 primer, 8.5 µL of distilled water, and 2 µL of human genomic DNA were mixed. The PCR temperature cycling conditions were initial denaturation at 90 °C for 2 min, followed by 30 cycles of 30 sec at 94 °C, 30 sec at 64.5 °C, 1 min at 72 °C, and final elongation at 72 °C for 5 min. The PCR product (1211 bp) from the *NAT2* gene was confirmed by electrophoresis on 1.5% agarose gel prepared in TBE buffer (89 mM Tris-borate, 2 mM EDTA, pH=8), stained with YTA safe stain (Invitrogen, USA), and visualized under ultraviolet light.

For the analysis of polymorphisms in nucleotides 481, 590, and 857, 10 µL of specific amplified DNA was digested separately with 5 U of *Kpn*I (481 C>T), *Taq*I (590 G>A), and *Bam*HI (857 G>A) endonucleases in appropriate conditions for 2-4 hr. *Kpn*I enzyme was purchased from Thermo Fisher-Maxwell (USA), and *Taq*I and *Bam*HI enzymes were procured from Fermentas Inc. (USA). A 1.2-kb PCR product was used for the 481 and 857 nucleotides. To analyze point mutations at site 590, a semi-nested PCR was performed with a mixture of 12.5 µL of Master Mix, 2 µL of each of P3 and P4 primer, 0.5 µL of Mg, 0.5 µL of DMSO, 6.5 µL of distilled water, and 1 µL: of 1.2 bp DNA product using a 1.2-kb PCR product with primers P3 (5'-CCTGGACCAAATCAGGAGAG3') and P4 (5'-GCAAGGAACAAAATGATGTGG-3'). PCR conditions were adjusted as follows: initial denaturation at 95 °C for 5 min, followed by 11 denaturation cycles at 94 °C for 30 sec, annealing at 56 °C for 1 min, extension at 72 °C for 1 min, and final extension at 72 °C for 1 min. The nested PCR product (190 bp) was digested with 5 U of *Taq*I (590). Temperature incubation conditions were at 37 °C for 15 min for *Kpn*I and 65 °C for 60 min for *Taq*I. Digest products were analyzed on 3% agarose gel prepared in

a TBE buffer. The bands were stained with a YTA-safe stain and then examined. Wild-type and mutant alleles were recorded according to bands presented in table 1 and figure 1 (a-c) to estimate the frequency of slow and fast stabilizers.[26] Different genotypes of *NAT2* were determined, and their frequency was estimated.

Genetic Polymorphisms of CYP2E1

A combination of PCR and RFLP was used for genotyping *CYP2E1*.[23, 27] Forward (F) 5′-TTCATTCTGTCTTCTAACTGG-3′ and reverse (R) 5′-CCAGTCGAGTCGACATTGTCA-3′ primers were amplified by PCR using extracted genomic DNA as the template. To this end, 12.5 µL of the prepared Master Mix, 1 µL of each of the F and R primers, 8.5 µL of distilled water, and 5 µL of the extracted DNA were mixed. PCR conditions were initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation

at 94 °C for 45 sec, annealing at 63 °C for 45 sec, elongation at 72 °C for 45 sec, and final elongation at 72 °C for 5 min. Next, 5 µL of PCR product, 1 µL of *Rsa*I enzyme (Thermo Fisher-Maxwell, USA), 2 µL of buffer, and 15 µL of distilled water were mixed and incubated at 37 °C for 37 min. The PCR product was digested, and the *CYP2E1* alleles (C1/C1, 360 and 50 bp; C2/C2, 410 bp; C1/C2, 410, 360, and 50 bp) were electrophoresed on a 2% agarose gel and stained with YTA safe stain (figure 1d).

Measurement of INH and AcINH Concentrations

INH was purchased from Sigma-Aldrich (USA). AcINH was synthesized by reacting INH with acetic anhydride as described in a previous study.[28] The analytical grades were phosphoric acid, acetic anhydride, trichloroacetic acid, glacial acetic acid (Merck, Germany), and hexane-1-sulfonic acid sodium salt (Sigma-Aldrich, USA).

Table 1: Restriction endonucleases used to detect various polymorphisms in the *NAT2* gene

SNPs	Recognition site	Restriction endonuclease	Fragment size (bp)		
481 C>T (Rs1799929)	GGTAC'C	*Kpn*I	CC	CT	TT
			662,549	1211,662,549	1211
857 G>A (Rs1799931)	G'GATCC	*Bam*HI	GG	GA	AA
			925,286	1211,925,286	1211
590 G>A (Rs1799930)	T'CGA	*Taq*I	GG	GA	AA
			109,88	197,109,88	197
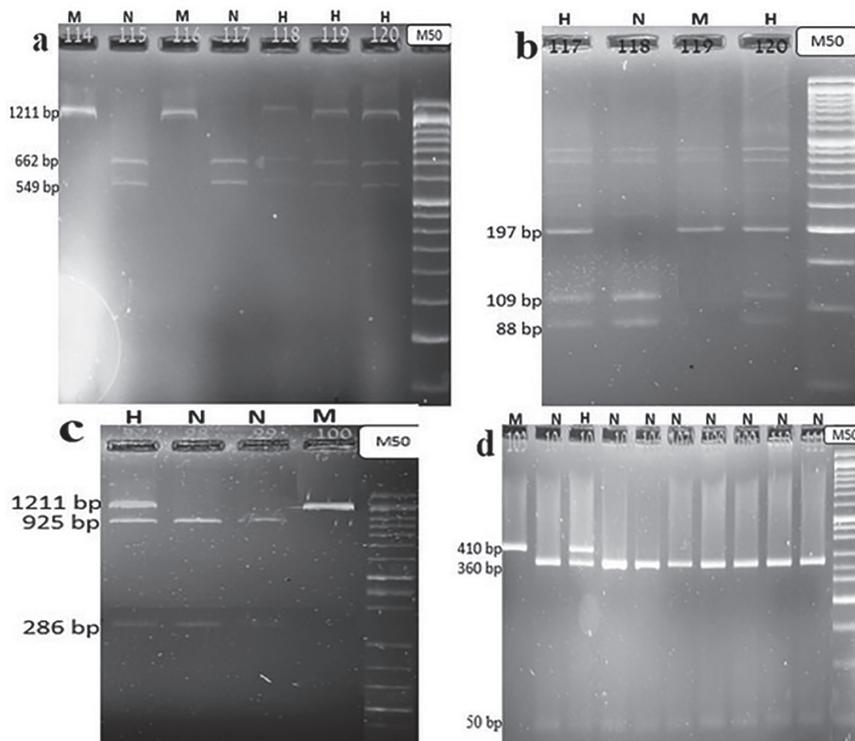
Figure 1: Electrophoresis of each restriction enzyme digest genotype of N-acetyltransferase 2 (*NAT2*) and cytochrome P450 subtype 2E1 (*CYP2E1*) assessed using polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP). (a) PCR-RFLP analysis of polymorphisms at 481 nt C>T (*Kpn*I), (b) 590 nt G>A (*Taq*I), (c) 857 nt G>A (*Bam*HI) in *NAT2* gene, (d) 1293 nt (*Rsa*I) in *CYP2E1* gene. W: Wild type; H: Heterozygous; M: Mutant. The PCR product was confirmed by electrophoresis and stained with YTA safe stain. 50 bp DNA Ladder was used as the molecular weight marker. The resolution of the figure was set to 300 DPI.

Acetonitrile and water were of high-performance liquid chromatography grade (Merck, Germany).

Sample Preparation and Calibration

The heparinized plasma samples were prepared, and their calibration scheme was performed as described in a previous study.[29]

Statistical Analysis

Discrete variables were expressed as numbers and percentages. Continuous variables were expressed as mean±SD. Independent two-sample *t* test, Chi square test, or Fischer's exact test was used for univariate analysis. The association between *NAT2* gene polymorphism in tuberculosis patients and polymorphism in the *CYP2E1* gene with hepatotoxicity were assessed as odds ratio (OR) and confidence interval (CI). Data were analyzed using SPSS software version 22.0 for Windows (New York, USA). $P < 0.05$ was considered statistically significant.

Results

Of the 120 tuberculosis patients, 40 showed hepatotoxicity. Out of the 40 patients, 31 had serum ALT and AST levels more than three times the normal range at day 10 of hospitalization, a reason to temporarily discontinue the INH. After discharge and at the next visit, the remaining nine patients showed hepatotoxicity. There was no significant difference between the two study groups in terms of age, sex, BMI (kg/m^2), diabetes, blood pressure, and smoking habit. Before the initiation of the treatment, no significant difference was observed in serum ALT (P=0.225), AST (P=0.553), and total bilirubin (P=0.215) levels between the groups. However, these factors significantly increased after treatment in patients with hepatotoxicity compared to those without (P<0.001) (table 2).

NAT2 Genetic Polymorphism in Patients with and without Hepatotoxicity

The results of the genetic analysis indicated that out of 40 patients with hepatotoxicity, eight (20%) were genotyped as IA and 32 (80%) as SA. However, none of the patients with hepatotoxicity were found to be genotyped as RA. The risk of anti-tuberculosis drug-induced hepatotoxicity was significantly higher in SA than in RA and IA (78% vs. 10.1%, OR: 31.57, 95% CI=11.16-89.26, P<0.001) (table 3). Therefore, the risk of hepatotoxicity of SA was 7.72 times higher than the two other acetylators, and no risk was observed for the RA phenotype.

The frequency of *NAT2*4 haplotypes was significantly higher in patients without than in those with hepatotoxicity (P<0.001). In contrast, the frequency of *NAT2*5 (P=0.003) and *NAT2*6 (P<0.001) haplotypes was significantly higher in patients with hepatotoxicity than in those without. The frequency of the *NAT2*7 haplotype was not significantly different between the two groups (P<0.999) (table 4).

CYP2E1 Genetic Polymorphism in Patients with and without Hepatotoxicity

A total of 107 patients were genotyped as C1/C1, 12 as C1/C2, and one as C2/C2 for *CYP2E1* polymorphism. There was no significant difference in *CYP2E1* C1/C1 genotype, *CYP2E1* C1/C2 genotype, and *CYP2E1* C2/C2 genotype (P<0.999) between the two groups (table 5).

Table 2: Demographic characteristics of tuberculosis patients with and without hepatotoxicity

Variables		With hepatotoxicity (n=40)	Without hepatotoxicity (n=80)	P value
Sex	Male	25 (62.5)	62 (77.5)	0.083
	Female	15 (37.5)	18 (22.5)	
Age (years)		55.10±19.59	49.83±15.90	0.144
BMI (kg/m^2)		21.83±3.86	21.46±3.99	0.628
Diabetes		7 (17.5)	16 (20.0)	0.743
Blood pressure		7 (17.5)	7 (8.8)	0.226
Smoking		14 (35.0)	36 (45.0)	0.295
Baseline value	ALT (U/L)	27.6±15.3	20.7±14.1	0.225
	AST (U/L)	22.3±12.5	21.9±13.7	0.553
Total bilirubin (mg/dL)		0.6±0.5	0.6±0.7	0.215
During treatment*	Peak ALT (U/L)	156.68±53.42 (86-2067)	25.36±14.21 (8-151)	<0.001
	Peak AST (U/L)	147.70±114.46 (62-4173)	26.90±13.35 (15-82)	<0.001
	Peak total bilirubin (mg/dL)	1.17±0.67 (0.3-9.1)	0.54±0.29 (0.1-1.7)	<0.001

BMI: Body mass index; ALT: Alanine transferase; AST: Aspartate transaminase; Reference interval (ALT: 0-40 U/L, AST: 0-40 U/L, Total bilirubin: 0.2-1.5 mg/dL). For both groups, continuous variables were compared using an independent two-sample *t* test, and categorical variables using Chi square test or Fisher's exact test. Data are expressed as mean±SD, n (%), or mean±SD (range). *P<0.05 was considered statistically significant.

Table 3: Genotype frequencies and odds ratio for *NAT2* gene polymorphism in tuberculosis patients with or without hepatotoxicity

Phenotypes	Genotypes	N (%)	With hepatotoxicity (n=40)	Without hepatotoxicity (n=80)	OR (95% CI)	P value
RA	NAT2*4/*4	10 (8.3)	0	10 (12.5)	0.16 (0.02-1.25)	0.030
IA	NAT2*4/*5	45 (37.5)	5 (12.5)	40 (50.0)	0.14 (0.05-0.40)	<0.001
	NAT2*4/*6	23 (19.2)	3 (7.5)	20 (25.0)	0.24 (0.07-0.88)	0.022
	NAT2*4/*7	1 (0.8)	0	1 (1.3)	0.98 (0.09-11.08)	<0.999
	IA subtotal	69 (57.5)	8 (20.0)	61 (76.3)	0.08 (0.03-0.20)	<0.001
SA	NAT2*5/*5	16 (13.3)	12 (30.0)	4 (5.0)	8.14 (2.42-27.35)	<0.001
	NAT2*5/*6	12 (10.0)	11 (27.5)	1 (1.3)	29.97 (3.70-242.48)	<0.001
	NAT2*5/*7	5 (4.2)	2 (5.0)	3 (3.8)	1.35 (0.22-8.43)	<0.999
	NAT2*6/*6	6 (5.0)	6 (15.0)	0	16.20 (1.92-136.66)	0.001
	NAT2*6/*7	1 (0.8)	1 (2.5)	0	4.05 (0.36-46.01)	<0.999
	NAT2*7/*7	1 (0.8)	0	1 (1.3)	0.98 (0.09-11.08)	<0.999
	SA subtotal	41 (34.1)	32 (80.0)	9 (11.3)	31.57 (11.16-89.26)	<0.001

N: Number; RA: Rapid acetylator; SA: Slow acetylator; IA: Intermediate acetylator; CI: Confidence interval; *NAT2*: N-acetyltransferase 2; OR: Odds ratio; For both groups, categorical variables were compared using Chi square test or Fisher's exact test. Data are expressed as mean±SD or n (%). P<0.05 was considered statistically significant.

Table 4: Distribution of *NAT2* haplotypes in tuberculosis patients with and without hepatotoxicity

Haplotype	With hepatotoxicity (n=40)	Without hepatotoxicity (n=80)	OR (95% CI)	P value
*NAT2*4*	8 (10.0)	81 (50.6)	0.108 (0.049-0.240)	<0.001
*NAT2*5*	42 (52.5)	52 (32.5)	2.296 (1.325–3.977)	0.003
*NAT2*6*	27 (33.8)	21 (13.1)	3.372 (1.757–6.473)	<0.001
*NAT2*7*	3 (3.7)	6 (3.8)	1.000 (0.243–4.107)	<0.999

CI: Confidence interval; *NAT2*: N-acetyltransferase 2; OR: Odds ratio. For both groups, categorical variables were compared using Chi square test or Fisher's exact test. Data are expressed as n (%). P<0.05 was considered statistically significant.

Table 5: Risk of anti-tuberculosis drug-induced hepatotoxicity and genetic polymorphism in *CYP2E1*

CYP2E1 Rsal polymorphism	With hepatotoxicity (n=40)	Without hepatotoxicity (n=80)	OR (95% CI)	P value
C1/C1	36 (90.0)	71 (88.8)	1.41 (0.33-3.96)	<0.999
C1/C2	4 (10.0)	8 (10.0)	1.00 (0.28-3.54)	<0.999
C2/C2	0	1 (1.3)	0.98 (0.09-11.08)	<0.999
C1	46 (92.0)	150 (93.8)	0.77 (0.23-2.56)	0.746
C2	4 (8.0)	10 (6.3)	1.30 (0.39-4.36)	0.746

OR: Odds ratio; CI: Confidence interval; *CYP2E1*: Cytochrome P450 2E1. For both groups, categorical variables were compared using Chi square test or Fisher's exact test. Data are expressed as n (%). P<0.05 was considered statistically significant.

Table 6: The risk of anti-tuberculosis drug-induced hepatotoxicity associated with both *CYP2E1* genotype and acetylator status

Acetylator status	CYP2E1 genotype	With hepatotoxicity (n=40)	Without hepatotoxicity (n=80)	OR (95% CI)	P value
RA	c1/c1	0	8 (9.9)	0.20 (0.02-1.62)	0.038
	c1/c2 or c2/c2	0	2 (2.5)	0.64 (0.06-6.37)	<0.999
IA	c1/c1	7 (17.5)	56 (70.0)	0.09 (0.04-0.23)	<0.001
	c1/c2 or c2/c2	1 (2.5)	5 (6.3)	0.39 (0.04-3.41)	0.663
SA	c1/c1	29 (72.5)	7 (8.8)	27.49 (9.71-77.84)	<0.001
	c1/c2 or c2/c2	3 (7.5)	2 (2.5)	3.16 (0.51-19.74)	0.332

OR: Odds ratio; CI: Confidence interval; RA: Rapid acetylator; SA: Slow acetylator; IA: Intermediate acetylator. For both groups, categorical variables were compared using Chi square test or Fisher's exact test. Data are expressed as n (%). P<0.05 was considered statistically significant.

Combined Risk Associated with NAT2 and CYP2E1 Genetic Polymorphisms

The risk associated with a combination of different *CYP2E1* genotypes and the status of *NAT2* acetylators showed that SA with *CYP2E1* C1/C1 genotype in patients with hepatotoxicity was significantly different from patients without (P<0.001), indicating a higher prevalence of hepatotoxicity in this group of the Iranian population (table 6).

Serum INH and AcINH Levels based on NAT2 Phenotype

The mean concentration of INH (μg/mL) was highest in the SA phenotype and lowest in the RA phenotype. However, AcINH concentration (μg/mL) and AcINH/INH ratio were reversed in both groups. In the SA phenotype, the concentration of INH in patients with hepatotoxicity was significantly higher than those without (5.36±1.20 vs. 3.70±1.12, P<0.001), while AcINH concentration (1.13±0.59 vs. 2.69±0.83, P<0.001) and AcINH/INH ratio (0.21±0.11 vs. 0.80±0.36, P<0.001) were significantly lower in patients with hepatotoxicity than those without. There was no significant difference in INH and AcINH concentrations between the two groups in terms of RA and IA phenotypes (data not shown, P>0.05).

Effect of CYP2E1 Polymorphism on the INH and AcINH Concentrations

There was no significant difference between the patients with *CYP2E1* C1/C1, *CYP2E1* C1/C2, and C2/C2 genotypes in terms of mean INH and AcINH concentrations (data not shown, P=0.17 and P=0.21, respectively).

Discussion

Replacement of FDC therapy with multiple single-drug treatments is one of the best strategies to ensure the effectiveness of anti-tuberculosis drugs. However, the combined use of these drugs, especially INH, is of concern, as it may cause severe hepatotoxicity.[7, 26, 30] Recent studies have mainly focused on the association of *NAT2* with *CYP2E1* polymorphisms and INH concentration for the prevention of hepatotoxicity through separate administration of anti-tuberculosis drug formulations. This study examined the simultaneous association of *NAT2* and *CYP2E1* polymorphisms with INH and AcINH concentrations and anti-tuberculosis drug-induced hepatotoxicity, particularly through the administration of FDC drugs.

In line with previous studies,[10, 19, 21, 24] our results showed that anti-tuberculosis drugs caused more hepatotoxicity in the SA phenotype with C1/C1 genotype, and increased INH concentration intensified drug-induced toxicity. However, other studies do not support our findings.[2, 14, 18, 20] Among various risk factors, acetylator status is the most significant susceptibility risk factor for anti-tuberculosis drug-induced hepatotoxicity.[21, 24, 31] *NAT2* genotype were shown to be the most critical factor for increased INH in tuberculosis patients.[32] Our results showed that the risk of anti-tuberculosis drug-induced hepatotoxicity was

20% for IA and 80% for SA. The RA phenotype was not a risk factor for hepatotoxicity. These findings confirm that patients with SA phenotype are at higher risk of hepatotoxicity. The risk of hepatotoxicity among patients with RA phenotype is rare, and in the case of IA phenotype, it is low. In contrast, some previous studies reported that patients with IA or RA phenotype were at higher risk of hepatotoxicity.[10, 19, 21, 33]

In line with the results of a previous study, the frequency of the *NAT2*4 haplotype in our patients was higher than in other haplotypes.[6] Similar to other studies,[24, 34, 35] our results showed the protective effect of *NAT2*4 haplotype against hepatotoxicity. We also found that the frequency of *NAT2*6 followed by the *NAT2*5 haplotype was higher in patients with hepatotoxicity than in those without. This is indicative of the significant effect of these two haplotypes on hepatotoxicity, effective use of *Taq*I and *Kpn*I restriction enzymes in *NAT*2 genotyping, and detection of single nucleotide polymorphisms. Homozygous point mutations had the highest risk of hepatotoxicity at position 590, followed by position 481. *Rsa*I is the most studied *CYP2E1* genetic polymorphism in relation to anti-tuberculosis drug-induced hepatotoxicity and hepatocellular carcinoma. Previous studies reported a significant association between the C1/C1 genotype and the risk of anti-tuberculosis drug-induced hepatotoxicity.[20, 36] However, in line with the results of previous studies conducted in Korean, Chinese, and Indian populations,[21, 24, 27, 34] we did not observe such association (P<0.999).

Given the risk of hepatotoxicity associated with both *NAT2* acetylator and *CYP2E1* genotype status, a significant risk of hepatotoxicity is anticipated in patients having SA phenotype with C1/C1 genotype (P<0.001). This finding was in line with those reported in other studies.[21, 34] We also analyzed the risk of hepatotoxicity associated with both the status of *NAT2* acetylator and serum INH and AcINH levels. The results showed that the mean INH concentration was highest in the SA phenotype and lowest in the RA phenotype. In addition, INH concentration was significantly higher in patients with hepatotoxicity than those without (P<0.001). Therefore, it can be concluded that a high concentration of INH in the SA phenotype is a factor that significantly affects hepatotoxicity. Except for one study,[14] this effect was confirmed in other studies.[10, 13]

The results of the present study showed no association between *CYP2E1* polymorphism and INH and AcINH concentrations. The mean concentration of INH or AcINH in patients with the C1/C1 genotype was not significantly associated

with C1/C2 or C2/C2 genotype (P=0.17 and P=0.21, respectively). The discrepancy between our results and those reported in other studies can be attributed to our use of FDC anti-tuberculosis drugs. Based on the results, in addition to reducing the risk of unnecessary prescription and hospitalization, this drug may have less toxicity in RA and IA phenotypes. A comparison between the administration of FDC and single-drug treatments in different groups of tuberculosis patients is recommended to confirm our findings.

As a limitation of the study, only 8% of our patients had RA phenotypes. Therefore, more such patients are required to determine the extent of hepatotoxicity. Another issue was related to the administration of FDC drugs (e.g., other anti-tuberculosis drugs combined with INH), which may quantitatively and qualitatively alter drug metabolism and thereby affect hepatotoxicity. RIF not only increases INH but also induces hydrolysis of INH to hydrazine, causing a biphasic effect on *CYP2E1* activity (inhibition following induction).[12] Therefore, it has a significant impact on increasing the risk of hepatotoxicity as well as pharmacokinetic and pharmacodynamic drug-drug interactions.[37] As a final note, we could not evaluate the serum concentrations of all drugs. A patient with SA phenotype may also slowly metabolize other anti-tuberculosis drugs.

Conclusion

SA phenotype was associated with significantly higher serum INH concentration and a higher risk of anti-tuberculosis drug-induced hepatotoxicity. Therefore, the determination of *NAT2* genotype to identify SA or measuring serum INH concentration could aid in more personalized treatment of tuberculosis patients with lower-dose and dose-adjusted prescription drugs, resulting in the reduction of hepatotoxicity and hospitalization.

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Authors' Contribution

NP: Preparation of the initial draft of the manuscript. All authors contributed to material preparation, data collection, and analysis, and revising the manuscript for important intellectual content. They have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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