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A study of N-acetyltransferase 2 gene polymorphisms in the Indian population and its relationship with serum isoniazid concentrations in a cohort of tuberculosis patients

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Abstract

The N-acetyltransferase 2 (*NAT2*) gene exhibits substantial genetic diversity, leading to distinct acetylator phenotypes among individuals. In this study, we determine *NAT2* gene polymorphisms in tuberculosis (TB) patients and analyze serum isoniazid (INH) concentrations across the various genotypes. An observational prospective cohort study involving 217 patients with pulmonary or extrapulmonary TB was carried out. The *NAT2* genotypes were identified using real-time polymerase chain reaction technology. INH concentrations at baseline and 2 hours post-dosing were estimated using high-performance liquid chromatography. The association between the acetylator status and INH concentrations was evaluated using odds ratios (OR), and the occurrence of adverse events across the different patient genotypes was also assessed. The genotype frequency of fast, intermediate, and slow acetylators was 7.37%, 39.17%, and 53.46%, respectively, while allele frequency was 27% for fast acetylators and 73% for slow acetylators. All the alleles followed the Hardy-Weinberg equilibrium. Patients with slow acetylator status had significantly increased serum INH concentrations 2 hours post-drug administration, followed by intermediate acetylators, as compared to fast acetylators. 69 (31.8%) patients developed adverse drug reactions post-therapy. Patients with slow acetylator status had the highest (OR: 9.66) risk of developing drug-induced hepatotoxicity, especially those with raised serum INH concentrations (OR: 1.34). Understanding the correlation between genetics and serum antitubercular drug levels in antitubercular drug-induced hepatotoxicity will provide valuable information to the medical community, minimizing the risk of adverse reactions and hospitalizations.

Key words: tuberculosis, serum isoniazid concentrations, NAT2 genetic polymorphisms, adverse drug reactions, drug-induced hepatotoxicity.

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Introduction

India faces a significant public health challenge with tuberculosis (TB), having one of the highest TB burdens worldwide. The annual incidence of TB in India is about 2.7 million cases, which constitutes a large portion of the global TB cases. The standard TB treatment regimen spans 6 months, starting with a 2-month intensive phase involving isoniazid (INH), rifampicin, ethambutol, and pyrazinamide, followed by a 4-month continuation phase with INH and rifampicin, sometimes including ethambutol [1]. Despite its effectiveness, treatment failures occur due to suboptimal drug levels, antibiotic resistance, and adverse events.

Common adverse drug reactions (ADRs) of antitubercular drugs (ATDs) include gastrointestinal issues like nausea and vomiting. Drug-induced hepatotoxicity is another risk, with incidence rates varying between 2-39% globally [2]. Studies indicate a higher incidence of drug-induced hepatotoxicity in the Indian population compared to Western countries. ADRs can reduce treatment adherence, leading to therapy failure, relapse, or drug resistance. INH and rifampicin are major contributors to hepatotoxicity in TB treatment.

INH is bactericidal, reducing the bacterial load by 90-95% within the first 2 days of treatment [3]. Its therapeutic range is 3-6 µg/mL. The N-acetyltransferase 2 (*NAT2*) gene, located on chromosome 8p22, encodes the N-acetyltransferase 2 enzyme, crucial for the metabolism of drugs like INH [4]. *NAT2* gene polymorphisms result in different acetylator phenotypes: fast (NAT2*4), intermediate, and slow (NAT2*5, NAT2*6, and NAT2*7) acetylators. Fast acetylators (FA) metabolize INH rapidly, risking treatment failure, and may need higher doses [5]. Slow acetylators (SA) metabolize INH slowly, increasing serum INH concentrations and the risk of hepatotoxicity. Identifying *NAT2* polymorphisms is essential for personalized medicine, influencing drug dosing, efficacy, and toxicity.

Studies have reported that comorbid conditions such as HIV, diabetes, hypertension, and demographic factors like age, sex, body weight, and alcohol use can affect serum ATD levels [6-9]. Thus,

therapeutic drug monitoring of ATDs 2 hours post-drug administration becomes necessary as peak serum concentrations of ATDs are reached around this time [10]. Research has shown that individuals with SA status are more likely to experience INH-induced liver disorders, particularly in Asian populations. Nevertheless, inconsistent findings across studies have led to ongoing debate regarding this association. The influence of genetic polymorphisms on the hepatotoxic effects of ATDs in the Indian population, which is at a heightened risk for such adverse reactions, remains underexplored. To date, there are very few studies that have explored the genotype-phenotype association in Indian TB patients. This study thus aimed to genotype TB patients from Mumbai for variations in the *NAT2* gene. The study also compared serum INH concentrations across the different genotypes and evaluated the incidence of adverse events among the various genotypes following the initiation of ATD therapy.

Materials and Methods

Study design

This was a prospective observational cohort study.

Study participants

Patients with pulmonary or extrapulmonary TB of any gender aged between 18 to 65 years, who were already initiated or were to be initiated on ATDs as per National Tuberculosis Elimination Program program and whose liver function tests (LFT) were within the normal ranges and who were willing to participate were recruited from the Revised National Tuberculosis Control Program Center and Pulmonary Medicine Department OPD of our hospital and allied municipal hospitals. Patients diagnosed with multidrug-resistant TB, abnormal LFT, and pregnant or lactating women were not eligible to participate in the study.

Sample size estimation and sampling strategy

Considering that the drug-induced hepatotoxicity incidence rate ranges from 2% to 28% in TB patients receiving 1st line ATDs, a midpoint incidence rate of 15% was used to calculate the sample size. This calculation resulted in a sample size of 196 patients. To account for an anticipated 10% dropout rate, 217 patients with pulmonary or extrapulmonary TB were enrolled in the study

Study procedures

The study was commenced after receiving the approval from the Institutional Ethics Committee (approval no. ECARP/2019/140) in accordance with Indian Good Clinical Practice guidelines (2001), Declaration of Helsinki principles (2018), and ICMR National Ethical Guidelines for Biomedical and Health Research Involving Human Participants (2017). Patients fulfilling the eligibility criteria and ready to give written informed consent were requested to come to the Clinical Pharmacology Department for the study-related investigations. Detailed medical history along with co-morbid conditions, history of TB, family history of TB, and date of initiation of ATDs were documented. Also, patients were followed up till they completed the ATD course for the development of any adverse events, if any. 10 mL of blood was withdrawn from the patients who had consumed their ATD doses for at least 15 days, at 0 hours (before drug administration), of which 6 mL was collected in plain tubes for the estimation of the serum INH concentrations and LFT, and the remaining 4 mL was collected in ethylenediaminetetraacetic acid tubes for the *NAT2* genotyping. The compliance to ATD treatment was checked *via* telephonic follow-ups with the study patients on a monthly basis after the initiation of therapy till completion of treatment duration. A second blood sample of 6 mL was collected 2 hours after drug consumption to estimate peak serum INH concentrations. The blood samples were centrifuged at 3000 rpm for 15 minutes, and the serum obtained was separated and stored at -80°C until the samples were processed for serum INH concentrations.

Determination of *NAT2* genetic polymorphisms

Genomic DNA was isolated from whole blood using a phenol-chloroform method. Quantification of DNA was done using an Eppendorf Nanodrop spectrophotometer (Eppendorf, Hamburg, and was stored at -80°C until analysis. Three SNPs in the *NAT2* gene, NAT2*5 C481T (rs1799929), NAT2*6 G590A (rs1799930), and NAT2*7 G857T (rs1799931) were analyzed using Taqman SNP genotyping assay on Applied Biosystem QuantStudio3 real-time PCR system (Applied Biosystem, Foster City, CA, USA) [11]. The reaction mixture for the TaqMan genotyping assay included the TaqMan genotyping master mix, assay probes, and 20 ng of genomic DNA. The polymerase chain reaction (PCR) protocol consisted of the following stages: a pre-read stage at 60°C for 30 seconds, an initial holding stage at 95°C for 10 minutes, followed by 45 cycles of denaturation at 95°C for 15 seconds, and annealing/extension at 65°C for 1 minute. The post-PCR read stage was conducted at 60°C for 30 seconds. Alleles were determined using Quant Studio design and analysis software, which identifies the probe labels with either VIC or fluorescein amidites reporter dyes.

Determination of serum concentrations of isoniazid

Serum samples were analyzed for INH concentrations using high-performance liquid chromatography (HPLC). Stock solutions of INH and the internal standard (β-naphthoflavone) were prepared by dissolving each in methanol to achieve a concentration of 1 mg/mL, which were then diluted to create working standard solutions. Serum samples from both standards and patients were spiked with the internal standard. Subsequently, 450 µL of acetonitrile was added, and the mixture was vortexed and centrifuged at 10,000 rpm for 15 minutes. The supernatant was then injected into the HPLC system, equipped with a C18 column and a mobile phase composed of acetonitrile, potassium dihydrogen phosphate buffer, and trifluoroacetic acid, monitored at a wavelength of 205 nm. The chromatographic run time was 8 minutes at a flow rate of 1.0 mL/min at room temperature. INH and the internal standard had retention times of 2.5 minutes and 7.9 minutes, respectively. This method was validated and exhibited linearity with a correlation coefficient (r) of 0.99 within the concentration range of 2-10µg/mL. The therapeutic range for INH is 3-6µg/mL.

Determination of serum liver enzymes

Serum obtained from the plain tube was used for LFT (serum bilirubin, serum glutamate oxaloacetate transaminase, and serum glutamate pyruvate transaminase) using commercially available Meril kits on a fully automated biochemistry analyzer (Meril Diagnostics Pvt. Ltd, Vapi, India).

Statistical analysis

Data analysis was conducted using GraphPad InStat software (Version 3.06). For non-normally distributed data, the median and interquartile range have been reported. The Kruskal-Wallis post-hoc test was used to examine differences in serum INH concentra-

tions at 0 and 2 hours across the various genotypes. Allelic and genotypic frequencies were determined through the counting method and assessed for Hardy-Weinberg equilibrium using the Chi-square test. The odds ratio for the relationship between genotype, drug-induced hepatotoxicity, and serum INH concentration at 2 hours was calculated.

Results

Clinical demographics of enrolled patients

A total of 217 patients with pulmonary and extrapulmonary TB, with a median age ranging from 20 to 37 years and a predominance of female patients, were enrolled in the cohort.

The majority of the patients had consumed ATD for at least 15 days [75% (163 patients)] prior to study participation, and the remaining 25% of patients (n=54) who were yet to be initiated on ATD therapy were requested to come for participation in the study after taking the ATD for a minimum period of 15 days.

The median duration of treatment in patients with TB depended on the site of the infection. The duration of treatment was 6 months in case of pulmonary TB and 12-18 months in cases of extrapulmonary TB, like central nervous system TB or Pott's spine. If issues of compliance or resistance were detected in these patients, the duration was prolonged till there was clinical improvement and/or *Mycobacterium tuberculosis* was not detected in their samples.

No difference in weight and age was recorded amongst the 3 acetylators groups. 69 (31.8%) TB patients exhibited adverse effects due to ATDs. Among the various adverse events documented, the majority of TB patients (n=26) with SA and intermediate acetylators (IA) (n=16) developed drug-induced hepatotoxicity (deranged transaminases), followed by gastrointestinal disturbances in 11 and 6 patients with SA and IA. No significant difference in the median INH dose was observed across the acetylator status. Significant increase in serum INH concentrations at 0 hours and 2 hours was observed in the SA group in comparison to the FA group, and increased INH level at 2 hours was observed in the SA group when compared to IA (*Supplementary Table 1*).

Allelic and genotypic frequencies of the *NAT2* polymorphism

In the present cohort of TB patients residing in Mumbai, four alleles, *i.e.*, NAT2*4 (Wild allele), NAT2*5 C481T (rs1799929), NAT2*6 G590A (rs1799930), NAT2*7 G857A (rs1799931) of the *NAT2* gene were studied. The minor allele frequency (MAF) for all the alleles was calculated (*Supplementary Table 2*). The allele frequency for wild and mutant alleles of NAT2*5 C481T, NAT2*6 G590A, and NAT2*7 G857A are 66%, 67%, 92% and 34%, 33%, 8% respectively. 10 different genotypes from the *NAT2* four alleles were observed in the study. The genotype frequently observed in this cohort was NAT2*5/*6 followed by NAT2*4/*5, NAT2*4/*6, NAT2*6/*6 and NAT2*5/*5. Fast, intermediate, and SA had genotype frequency of 7.37%, 39.17% and 53.46% respectively. The allele frequency for FA was 27% and 73% for SA. All the alleles followed the Hardy-Weinberg equilibrium (*Supplementary Table 3*).

Association between serum isoniazid concentration and *NAT2* genotype

The patients with SA showed maximum and significantly increased serum INH concentrations post 2 hours of drug administration, followed by intermediate acetylators (Figure 1). Whereas patients with FA showed the lowest median serum INH concentration. Across the various genotypes of the *NAT2* gene, NAT2*5/*7 showed maximum median INH concentration post 2 hours of drug administration (13.36µg/mL), followed by NAT2*5/*5 and NAT2*7/*7 (*Supplementary Table 3*).

Antitubercular drugs induced adverse drug reactions

Patients consuming ATDs were followed up till they completed therapy. Whenever any patient developed any adverse reaction/s during the study period, the details of the ADR were captured from the date of occurrence to resolution and the management of the same. In the present cohort, the average duration for the development of ADRs was between 15 and 45 days. Of the 217 TB patients enrolled in the study, 69 (31.8%) developed ADRs (Figure 2). Of these, 42 (61%) reported drug-induced

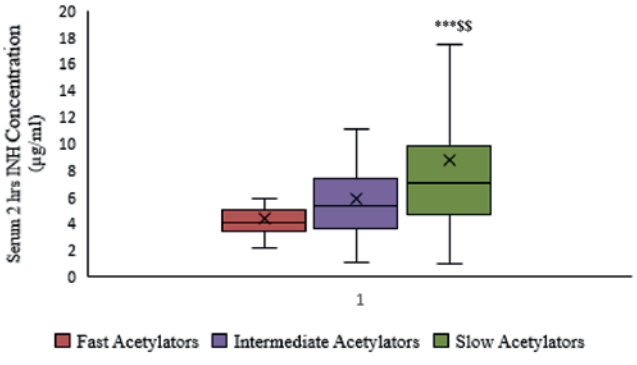

Figure 1. Serum isoniazid (INH) concentrations among the NAT2 acetylator genotype groups. The data is displayed using a box and whisker plot format depicting median and interquartile range. Significance levels are indicated as follows: ***p<0.001 in comparison to Fast Acetylators and $$p<0.01in comparison to Intermediate acetylators, utilizing the Kruskal-Wallis test followed by Dunn's multiple comparison test.

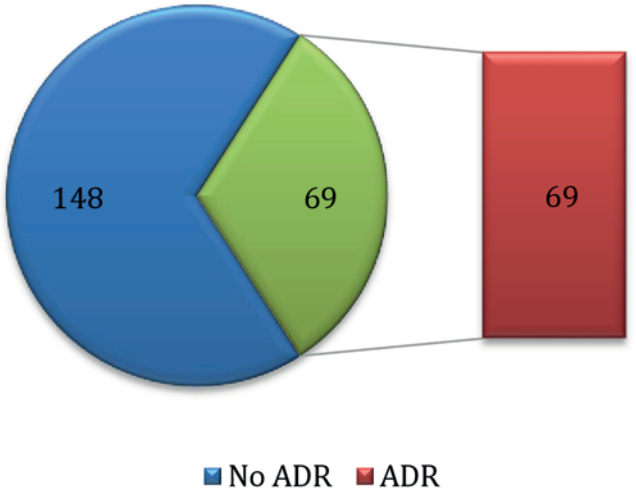

Figure 2. Adverse drug reactions (ADRs) developed in tuberculosis patients receiving antitubercular drugs (n=69). The data is represented as the number of patients.

hepatotoxicity, followed by gastrointestinal disturbances in 19 (28%), skin-related issues in 5 (7%), blurred vision in 2 (3%), and 1(1%) patient developed convulsions. (Figure 3) These adverse effects were resolved on stoppage of medications. Resolution of adverse effects of ATD in our patients was confirmed by monitoring the liver enzymes in case of drug-induced hepatitis, and in case of other adverse effects, the patients were monitored clinically with reference to the respective specialty physician, wherever needed.

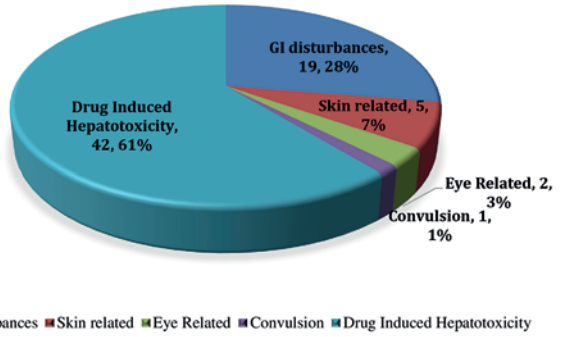

Figure. 3 Distributions of adverse drug reactions reported in a group of tuberculosis patients. The data is represented as the number of patients and the percentage. GI, gastrointestinal.

Association between drug-induced hepatotoxicity and *NAT2* acetylator status

Drug-induced hepatotoxicity is defined according to the guidelines established by the American Thoracic Society [12,13]. The odds ratio shows that the risk of developing drug-induced hepatotoxicity was highest in patients with SA status (9.66) compared to FA, followed by individuals with a combination of IA and SA status. Under the recessive model, SA demonstrated a 1.53 times greater risk for drug-induced hepatotoxicity compared to the combined group of fast and intermediate acetylators (*Supplementary Table 4*).

Association between isoniazid concentration 2 hours post-drug administration and *NAT2* acetylator status

As per reported literature, it is documented that the therapeutic range of serum INH is 3-6µg/mL. Thus, the association between *NAT2* acetylators and serum INH concentrations at 2 hours post-drug administration was evaluated by considering serum INH concentrations below and above the therapeutic range. While considering the recessive model, it was noted that patients with SA had a 1.4 times higher risk of higher serum INH concentration in comparison to fast and intermediate acetylators. Similarly, when considering the dominant model, patients with SA also demonstrated 1.34 times the risk of raised serum INH concentrations when compared to FA (*Supplementary Table 4*).

Discussion

INH undergoes hepatic metabolism through the enzyme NAT2. Variations in the *NAT2* gene can significantly affect the metabolic rate of INH, thereby influencing both its therapeutic efficacy and the likelihood of adverse effects. This study aimed to analyze the genotypic and allelic frequencies of *NAT2* mutations in a group of TB patients undergoing antitubercular treatment. Additionally, we measured serum INH concentrations 2 hours post-drug administration to investigate the correlation between *NAT2* genotype and INH metabolism.

While standard antitubercular therapy is generally effective, managing ADRs remains a significant challenge. The role of genetic polymorphisms in ATD-induced hepatotoxicity is not fully established in the Indian population, who have a higher susceptibility to such adverse effects. Consequently, patients were monitored throughout the treatment period to detect any emerging toxicities.

The median dose of INH used in this study was 5.0 mg/kg of body weight, aligning with the World Health Organization 2022 guidelines, which recommend a dosage range of 4-6 mg/kg/day for TB patients [14]. This study examined the genotypes NAT2*5 C481T, NAT2*6 G590A, and NAT2*7 G857A in a group of TB patients using real-time PCR technology. The MAF observed for NAT2*5, NAT2*6, and NAT2*7 were 34%, 33%, and 8%, respectively. These allele frequencies vary among different populations globally, including those in India and other countries.

Kumar *et al.* reported frequencies of 29%, 39%, and 7% for NAT2*5, NAT2*6, and NAT2*7, respectively, in the Indian population [15]. In North India, the frequencies were 27.6%, 42.8%, and 8.4%, respectively, aligning with our study's MAF. Internationally, frequencies for NAT2*6 and NAT2*7 were 22.4% and 13.2% in South Korea, 28.5% and 2.9% in Caucasians, and 26.0% and 2.8% in Egyptians [16-18].

In our study, the genotype frequencies of slow, intermediate, and FA were 53.46%, 39.17%, and 7.37%, respectively. These figures are similar to those reported in the South Indian population (58%, 35%, and 7%). Yadav *et al.* observed frequencies of 62% for SA, 34% for IA, and 4% for FA in Eastern Uttar Pradesh [19]. Conversely, Rana *et al*. found a higher prevalence of FA (37.05%) and a lower frequency of SA (19.52%) [20]. Jain *et al.* also noted a higher prevalence of FA and IA and a lower prevalence of SA in North India, which differed from our study [21]. The frequency of SA observed in our study is comparable to that of the American [22] (55.9%) and UK Caucasian (66.1%) populations [23]. These variations highlight the genetic heterogeneity among different populations, suggesting that results from one group may not apply to others.

INH achieves peak concentration around 2 hours post-drug administration with a therapeutic range of 3-6 µg/mL [10]. Hence, in our study, serum INH concentrations, post 2 hours of drug administration, were analyzed in all patients. A significant difference in serum INH concentrations was documented across the 3 acetylators' status, with SA showing the maximum serum concentration in comparison to FA. Similar trends have been reported in other studies, and our results are in concordance with the reported results. Higher INH concentrations in individuals with *NAT2* SA or IA genotypes may lead to an increased risk of drug toxicity [15,24-27]. SA and IA metabolize INH at a slower rate, potentially resulting in the accumulation of the drug in the body when standard doses are administered. Differences in an individual's response to drugs can lead to ADRs, ineffective treatments, or increased susceptibility to other diseases. Similarly, genetic variations in the *NAT2* gene can influence how different populations metabolize INH.

The relationship between *NAT2* genotypes, serum INH concentrations, and drug-induced hepatotoxicity has been extensively documented in numerous studies [4,24-26]. In our study, 26% of SA developed drug-induced hepatotoxicity, aligning with the reported incidence range of 2-39% across various countries [2]. The Indian sub-population, however, has shown a higher incidence of hepatotoxicity with ATDs as compared to Western populations [28,29]. Our study has its limitations. In the present study, we have only looked at some of the *NAT2* alleles. Additionally, there are other genes involved in INH metabolism. Also, serum levels of INH metabolites were not estimated in the study.

Conclusions

Knowledge of the genetic determinants that affect drug metabolism is essential to predict how individuals will respond to different medications, which will help in guaranteeing effective therapy. Investigating the genetic mechanisms underlying antitubercular drug-induced hepatotoxicity and correlating these findings with serum drug levels is clinically important. This knowledge can provide the medical community with critical insights before initiating therapy, thus minimizing the risk of adverse reactions and hospitalizations.

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Online supplementary material:

Supplementary Table 1. Clinical data of tuberculosis patients as per their acetylator status.
Supplementary Table 2. Allelic and genotypic frequencies of NAT2 *polymorphism in tuberculosis patients (n=217).*
Supplementary Table 3. Allelic, genotype frequency, and serum INH concentrations of NAT2 *acetylators in Indian tuberculosis patients (n=217).*
Supplementary Table 4. Relationship between NAT2 *acetylator and development of drug induced hepatoxicity and serum INH concentrations.*

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Conflict of interest: the authors declare that there is no conflict of interest.

Ethics approval and consent to participate: the study protocol was reviewed and approved by the Institutional Ethics Committee (approval no. ECARP/2019/140 dated 23/01/20).

Informed consent: written informed consent to participate was obtained from all the study participants.

Patient consent for publication: consent for publication was obtained from all the study participants.

Availability of data and materials: all data generated or analyzed during this study are included in this published article. The data used to support the findings of this study are available from the corresponding author upon request.

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