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Genetic Interaction between *NAT2*, *GSTM1*, *GSTT1*, *CYP2E1*, and Environmental Factors Is Associated with Adverse Reactions to Anti-Tuberculosis Drugs

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Abstract

Background: Adverse drug reactions (ADRs) associated with anti-tuberculosis (anti-TB) drug regimens have considerable impact on anti-TB treatment, potentially leading to unsuccessful outcomes. Nevertheless, the risk factors that play a role in anti-TB drug-induced ADRs are not well established. It is well documented that genetic polymorphisms in drug-metabolizing enzymes (DMEs) result in considerably complex variability in anti-TB drug disposition. In addition, the impact of pharmacogenetic variation on the metabolism of anti-TB drugs may be modifiable by environmental exposure. Thus, an assessment of pharmacogenetic variability combined with biomarkers of environmental exposure may be helpful for demonstrating the effect of the gene-environment interaction on susceptibility to ADRs induced by anti-TB drug therapy.

Objective: The aim of the study was to investigate the impact of the interaction between environmental risk factors and pharmacogenetic polymorphisms in four common DMEs – N-acetyltransferase 2 (arylamine N-acetyltransferase) [NAT2], glutathione S-transferase theta 1 [GSTT1], glutathione S-transferase mu 1 [GSTM1], and cytochrome P450 2E1 [CYP2E1] – on commonly reported ADRs to first-line anti-TB drugs in 129 patients receiving homogeneous TB treatment.

Methods: TB patients monitored during drug treatment were divided into subgroups according to the presence or absence of ADRs. Additionally, the patients' clinical and demographic characteristics were collected in order to identify the environmental factors that are potential triggers for ADRs induced by anti-TB drug treatment. Pharmacogenetic variability was determined by gene sequencing, TaqMan® assays, or polymerase chain reaction.

Results: The findings of this study suggest that the *NAT2* slow acetylator haplotype, female sex, and smoking are important determinants of susceptibility to ADRs induced by anti-TB drugs. Patients carrying multiple, but not single, polymorphisms in the *NAT2*, *GSTM1*, *GSTT1*, and *CYP2E1* genes were found to have an increased risk of ADRs, as revealed by gene-gene interaction analysis. Moreover, we also identified meaningful gene-environment interaction models that resulted in the highest levels of ADR risk.

Conclusion: The study findings provide evidence of the clinical impact of the interaction between pharmacogenetic variability and environmental factors on ADRs induced by anti-TB drug therapy. Predictive pharmacogenetic testing and a comprehensive clinical history would therefore be helpful for identification and careful monitoring of patients at high risk of this complication.

Introduction

Tuberculosis (TB) is a common infectious disease, and adverse drug reactions (ADRs), such as hepatotoxicity, gastrointestinal discomfort, neurotoxicity, skin rash, and pruritus, have been of long-standing concern in relation to non-adherence to anti-TB treatment and the spread of the disease.[1,2] Moreover, considerable costs to the health care system and patient morbidity are also well documented and increase the negative consequences of anti-TB drug-induced ADRs.[3] ADRs are thus recognized as a serious clinical problem, and extensive efforts have been made to identify risk factors and assist in the prevention of ADRs.

Although the risk factors contributing to anti-TB drug-induced ADRs are not well elucidated, several lines of evidence support the idea that genetic variation in drug-metabolizing enzymes (DMEs) may have a strong influence on the inter-individual variability of anti-TB drug disposition and may therefore influence susceptibility to ADRs during drug therapy.[4] Acetylation of isoniazid, a cornerstone of regimens for the treatment and prevention of TB by phase II enzyme N-acetyltransferase 2 (NAT2), clearly illustrates this fact. Differences in isoniazid plasma concentrations are thought to be caused by polymorphisms in the *NAT2* gene.[5,6] In fact, two genetically determined profiles of isoniazid acetylation, characterized as 'fast' and 'slow', have been reported in individuals carrying different *NAT2* haplotypes.[7-9] The wild-type haplotype *NAT2**4 is considered fast, whereas the slow acetylator profile is determined by the presence of two defective *NAT2* alleles causing decreases in protein levels or activity.[10] Accumulation of anti-TB drugs or their toxic metabolites to potentially harmful concentrations in slow acetylators is the most widely accepted explanation for the impact of pharmacogenetic variability in *NAT2* on anti-TB drug-induced ADRs.[11]

Pharmacogenetic polymorphisms in other enzymes also related to isoniazid metabolism, such as glutathione S-transferases (GSTs) and cytochrome P450 (CYP) 2E1 (CYP2E1), are also presumed to act synergistically in contributing to susceptibility to ADRs.[12,13] For example, the acetylated form of isoniazid, acetylhydrazine, can undergo further metabolism by GSTs and CYPs to be converted into more toxic metabolites, such as reactive acetyl free radicals.[14,15] Patients carrying the multiple polymorphisms associated with increased phase I and truncated phase II enzyme levels and/or activity (such as slow NAT2 acetylation capacities) have been included in a group at high risk of developing anti-TB drug-induced ADRs.[11] Accordingly, it is possible that combined polymorphisms in *NAT2*, *GST*s, and *CYP2E1* may present potential risks of anti-TB drug-induced ADRs.

ADR risk may also be influenced by environmental stimuli such as smoking and alcohol intake.[16] Interindividual variability in ADR risk from exposure to exogenous risk factors depends in part on the individual's pharmacogenetic background, which influences, for example, how cells activate or detoxify xenobiotics. Thus, an assessment of pharmacogenetic variability combined with biomarkers of environmental exposure may be helpful for identification of gene-environment interactions related to susceptibility to ADRs induced by anti-TB drug therapy.[17]

We hypothesized that TB patients receiving a first-line anti-TB drug regimen and carrying susceptible pharmacogenetic polymorphisms and/or exogenous risk factors may have increased susceptibility to developing anti-TB drug-induced ADRs. In this study, we performed complete sequencing of *NAT2* and genotyping of selected polymorphisms in the *GSTM1*, *GSTT1*, and *CYP2E1* genes in order to investigate this relationship. The pharmacogenetic polymorphisms evaluated here have not been extensively studied in the Brazilian population. Moreover, most of the previous worldwide reports have been limited to an analysis of single-gene insights. The clinical and demographic characteristics of 129 patients undergoing anti-TB treatment were also collected in order to identify environmental factors and gene-environment interactions as potential triggers for ADRs induced by anti-TB drug treatment.

Methods

Subjects and Clinical Assessment

The study was conducted from August 2008 to December 2009 on a convenience sample of 129 TB patients monitored from the initiation of drug treatment at the Maria José de Magalhães Health Center (Itabuna, Brazil) and the Centro Especializado de Saúde III (CAE III; Ilhéus, Brazil). Male or female subjects aged 18 years or over, who had no previously described renal, allergic, or hepatic diseases and were not pregnant, were considered for the study. All patients were treated with the first-line anti-TB drug regimen isoniazid (300 mg/kg/day), rifampicin (300 mg/kg/day), and pyrazinamide (1500 mg/kg/day) for the first 2 months, and then isoniazid and rifampicin for a further 4 months. Patients who had no regular treatment adherence were excluded from the analysis. A trained physician performed monthly clinical examinations in accordance with the 2nd Brazilian Consensus on Tuberculosis.[18] Patients were divided into subgroups according to ADR presence (ADR+) or absence (ADR−). The study

defined ADRs as the presence of at least one of the following symptoms during the follow-up period: gastric, joint, neuromuscular, or skin reactions; and hepatotoxicity (in accordance with the criteria of drug-induced liver injuries developed by the international consensus meeting[19]). All recruited subjects completed a demographic and clinical questionnaire after signing the informed consent form approved by the Human Ethical Committee of Universidade Estadual de Santa Cruz (UESC; Ilhéus, Brazil) under protocol number 098/07.

Sample Collection and Genotyping

Peripheral blood (4 mL) was collected, and genomic DNA isolation was performed from white blood cells, using the FlexiGene DNA Kit (Qiagen, Valencia, CA, USA). The *NAT2* gene was amplified by polymerase chain reaction (PCR) using the following primers: 5′ AAAAGGGATTCATGCAGTAGA 3′ (external F); 5′ AAATAACGTGAGGGTAGAGAGG 3′ (external R); 5′ GTTAACAAATACAGCACTGGCA 3′ (internal F); and 5′ TGCCAGTGCTGTATTTGTTAAC 3′ (internal R). The PCR product was purified using the QIAquick PCR Purification Kit (Qiagen). *NAT2* gene sequencing was performed in an ABI 3130 Genetic Analyzer sequencer (Applied Biosystems, Foster City, CA, USA). Sequence alignment and identification of *NAT2* polymorphisms were performed using DNAStar SeqMan version 7.0.0 software. *GSTM1* and *GSTT1* null polymorphisms were genotyped by multiplex PCR in line with Magno et al.[20] The two functional *CYP2E1* single nucleotide polymorphisms (SNPs), rs3813867 (3739G>C or c2 allele) and rs6413432 (7632T>A or *Dra*I polymorphism), were selected according to their documented clinical impact and were identified by TaqMan® SNP genotyping assays in accordance with the Applied Biosystems protocol. At least 10% of the sample was assessed to ensure the accuracy of the genetic data collected.

Biochemical Analysis

Serum aspartate aminotransferase (AST) levels, serum alanine aminotransferase (ALT) levels [assayed by the Reitman and Frankel method], and total bilirubin levels [assayed by the Sims-Horn method] were documented both prior to and 30 days after the initiation of anti-TB drug treatment. Doles® reagent kits (Doles Reagentes Ltda, Goiânia, Brazil) were used in the biochemical analysis, in accordance with the manufacturer's instructions. Serum levels considered to be abnormal were in line with the 2nd Brazilian Consensus on Tuberculosis.[18]

Statistical Analysis

A comparison of all quantitative variables between patients who experienced and those who did not experience ADRs was performed using the student's t-test. The categorical variables – allele, genotype, and haplotype associations – were evaluated by χ^2 tests followed by computation of odds ratios (ORs) and 95% confidence intervals (CIs). SPSS version 15.0 software was used for statistical analysis. PHASE version 2.1.1 software[21] was used for *NAT2* haplotype construction. Gene-gene and gene-environment interactions were detected by an open-source multi-factor dimensionality reduction (MDR) software package (version 2.0.0),[22] which has proven to be a useful method for identifying interactions in small populations.[23] Observed genetic frequencies were compared with expected frequencies, and Hardy-Weinberg equilibrium was tested using Haploview version 4.2 software.[24] All tests were two-tailed, and the level of significance for all statistical results was set at $p < 0.05$.

Results

Clinical Characteristics

All 129 patients (aged 18–84 years) recruited into this study had good treatment adherence. Of these, 54 (41.9%) developed ADRs in the course of the 6-month first-line anti-TB drug therapy. Since only one single case of drug-induced hepatotoxicity was reported, we grouped it as a gastrointestinal symptom. Gastrointestinal symptoms (44.4%) and neuromuscular symptoms (27.7%) were thus the most common ADRs in this sample (table I). Environmental and demographic parameters were analyzed in order to evaluate the relative strengths of the non-genetic factors important to ADRs induced by anti-TB drugs. The results of a simple analysis of sex and smoking habits showed that patients who were female or smokers had an increased risk of anti-TB drug-induced ADRs (OR 3.41, $p = 0.001$, and OR 2.86, $p = 0.005$, respectively) [table I]. Age, self-assessed skin color, and alcohol intake did not clearly influence the risk of anti-TB drug-induced ADRs. Although the patients showed a small rise in AST and ALT transaminase levels 30 days after the initiation of drug therapy, there were no significant differences in serum AST and ALT levels between the ADR– and ADR+ groups (figure 1a and 1b). The average increases in AST and ALT levels in ADR– patients were 30.2% and 26.8%, respectively. In ADR+ patients, AST and ALT levels increased by 17.4% and 31.1%, respectively. Likewise, bilirubin levels were similar in both patient groups 30 days after anti-TB drug treatment, and no significant alteration in these

Table I. Characteristics of patients with and patients without adverse drug reactions to anti-tuberculosis drugs

Characteristic	Total sample (n [%])	ADR status		OR [95% CI]	p-Value[a]
		ADR– (n [%])	ADR+ (n [%])		
N	129 [100]	75 [58.1]	54 [41.9]		
Sex					
Male	81 [62.8]	56 [69.1]	25 [30.9]	3.41 [1.52, 7.73]	***0.001***
Female	48 [37.2]	19 [39.6]	29 [60.4]		
Age					
<36 years	66 [51.2]	39 [59.1]	27 [40.9]	1.08 [0.51, 2.31]	0.82
≥36 years	63 [48.8]	36 [57.1]	27 [42.9]		
Skin color					
Black/mixed race	109 [84.5]	63 [57.8]	46 [42.2]	1.32 [0.48, 3.60]	0.59
Other	20 [15.5]	12 [60.0]	8 [40.0]		
Alcohol intake					
No	87 [68.0]	54 [62.1]	33 [37.9]	1.72 [0.76, 3.89]	0.15
Yes	41 [32.0]	20 [48.8]	21 [51.2]		
Smoking					
No	59 [46.1]	42 [71.2]	17 [28.8]	2.86 [1.29, 6.40]	***0.005***
Yes	69 [53.9]	32 [46.4]	37 [53.6]		
Reaction site					
Gastrointestinal			24 [44.4]		
Neuromuscular			15 [27.7]		
Joint			13 [24.1]		
Skin			11 [20.4]		

a Significant associations are indicated by bold italic text.

ADR = adverse drug reaction; **ADR–** = absence of ADR; **ADR+** = presence of ADR; **CI** = confidence interval; **OR** = odds ratio.

levels was found during the first 30 days of drug therapy (an increase of 2.58% in ADR– patients and a decrease of 0.54% in ADR+ patients) [figure 1c].

Genetic Analysis

The allele, genotype, and haplotype frequencies for the *NAT2*, *GSMT1*, *GSTT1*, and *CYP2E1* genes in TB patients in this study are summarized in tables II–IV. All of the studied polymorphisms were in Hardy-Weinberg equilibrium (data not shown), except for one in *CYP2E1*.

NAT2

NAT2 sequencing and haplotype pair reconstruction revealed 27 *NAT2* haplotypes from 22 SNPs. Of the 90 samples sequenced for *NAT2*, 58.9% were classified as fast acetylators, 37.8% as slow acetylators, and 3.3% as non-defined, in accordance with the consensus gene nomenclature for *NAT2*[25] (tables II and IV). The wild-type rapid-acetylator haplotype *NAT2**4 was the haplotype most frequently present in the sample (25.6%), followed by the slow-acetylator haplotypes *NAT2**5B and *NAT2**5G (16.7% and 10.6%, respectively). Three patients were unclassified and were considered to be carriers of novel *NAT2* haplotypes on the basis of a mismatch with the official *NAT2* gene nomenclature[25] (table III). The X haplotype, which is composed of single point mutations at 282C>T and 481C>T, is possibly a fast acetylator, since both mutations are synonymous and fall outside the regulatory sequence of the *NAT2* gene. On the other hand, the Y and Z haplotypes may potentially determine NAT2 slow acetylation, due to the presence of single point mutations at 341T>C, 434A<C, and 590G>A, which are associated with the amino acid changes I114T, Q145P, and R197Q, respectively.

Since most (96%) of the acetylator profiles of the *NAT2* haplotypes identified in this study have already been reported, the impact of the NAT2 acetylator phenotype on the risk of anti-TB drug-induced ADRs was investigated. After dividing

the sample into carriers of slow or fast *NAT2* haplotypes, a positive association between carriers of slow *NAT2* acetylator haplotypes and anti-TB drug-induced ADRs was observed (OR = 3.2, p = 0.009) [table IV]. We did not find differences in the distributions of single *NAT2* SNPs between the ADR– and ADR+ groups (data not shown).

GSTM1 and *GSTT1*

Results from *GSTM1* and *GSTT1* genotyping showed that 23.8% and 34.0% of the 88 genotyped patients were carriers of the respective null polymorphisms. However, none of these null polymorphisms was found to alter the risk of anti-TB drug-induced ADRs (table IV).

CYP2E1

The GG wild type genotype (rs3813867) and the AT heterozygote genotype (rs6413432) in the *CYP2E1* gene were the most common genotypes in the overall sample (n = 86) and in both patient groups (table IV). Interestingly, no single patient was found to carry the CC or AA genotypes of the rs3813867 and rs6413432 SNPs, respectively. According to comparative analysis, the genotype and allele frequencies of rs6413432 did not differ between patients who experienced ADRs and those who did not. The rs3813867 SNP was not in Hardy-Weinberg equilibrium and was therefore excluded from this and subsequent analysis (data not shown).

Gene-Gene and Gene-Environment Interaction

We applied the MDR method[22] in order to explore the impact of the gene-gene and gene-environment interactions with pharmacogenetic polymorphisms, sex, and smoking on prediction of ADR risk. Because of sample-size limitations, and on the basis of previous positive associations, we limited the analysis to three specific interaction categories (A–C). According to the MDR analysis, the best models in each category are shown in figure 2a in descending order from 1 to 3. Category A shows gene-gene interactions only. In this model, we only inputted individual simultaneous carriers of two *NAT2* slow haplotypes, since we preliminarily identified this condition as increasing the risk of anti-TB drug-induced ADR to higher levels than in subjects who carry only one *NAT2* slow haplotype (data not shown). Under these conditions, the four-way A1 interaction found between *NAT2* slow + *GSTM1* null, *GSTT1* null + rs6413432 (*CYP2E1*) exhibited the highest cross-validation consistency (CVC) [10/10], although the testing-balanced accuracy (TBA) of the A2 (two-way) and A3 (three-way) models were higher (61% and 51%). Categories B (smoking) and C (female sex) denote gene-environment interactions that only include the identified environmental risk factors. We found that simultaneous genetic alterations in *NAT2*, *GSTM1*, and *GSTT1* (B1) or *NAT2* and *GSTT1* (C1) were the most significant models of

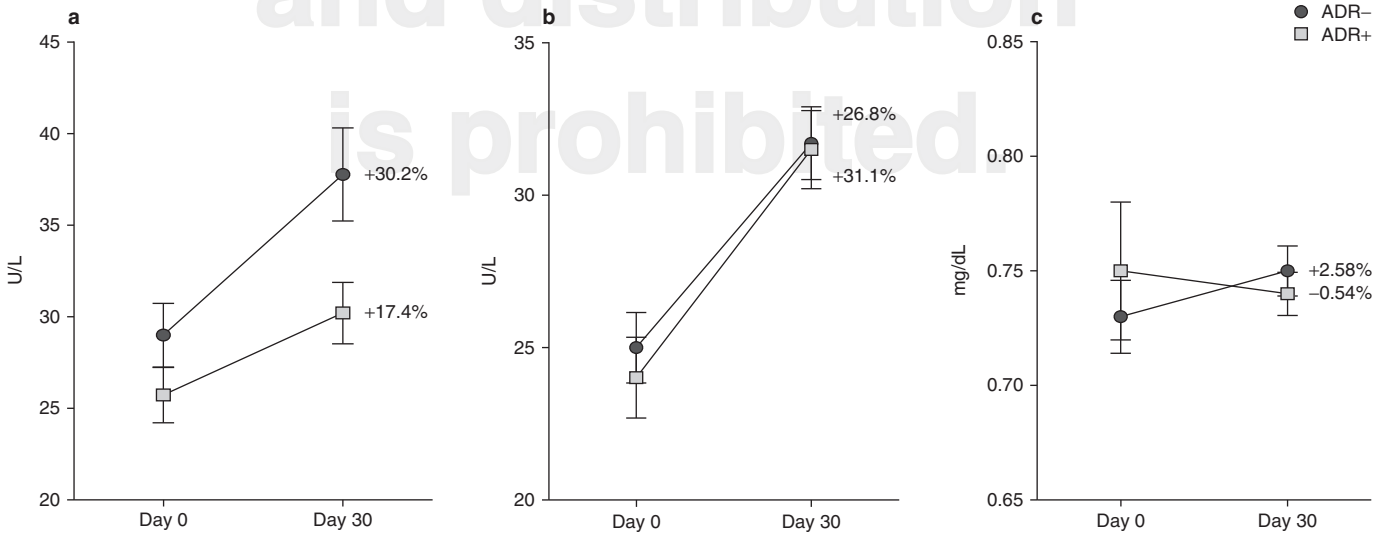

Fig. 1. Changes in (**a**) serum aspartate aminotransferase, (**b**) serum alanine aminotransferase, and (**c**) serum bilirubin levels within the first 30 days of anti-tuberculosis drug therapy in patients with and patients without adverse drug reactions. The ratio changes between day 0 (prior to drug therapy) and day 30 (30 days after initiation of drug therapy) are expressed in percentage values. As shown in graphs (**a**) and (**b**), aspartate aminotransferase and alanine aminotransferase levels had increased slightly after 30 days of monitoring, but no significant difference was detected between patients with and without adverse drug reactions. As shown in graph (**c**), we did not find any significant differences in bilirubin levels between day 0 and day 30 after treatment, or between patients with and patients without adverse drug reactions. **ADR–** = absence of adverse drug reactions; **ADR+** = presence of adverse drug reactions.

Table II. Frequencies of N-acetyltransferase 2 (arylamine N-acetyltransferase) haplotypes and profiles of N-acetyltransferase 2 acetylation in 90 tuberculosis patients

NAT2 haplotype	n [%]	Acetylator status	n [%]
*4	46 [25.56]	Fast	37 [41.1]
*5B	30 [16.67]	Slow	22 [24.4]
*5G	19 [10.56]	Slow	19 [21.1]
*6A	12 [6.67]	Slow	9 [10.0]
*6B	10 [5.56]	Slow	9 [10.0]
*5C	8 [4.44]	Slow	6 [6.7]
*12A	8 [4.44]	Fast	8 [8.9]
*13A	6 [3.33]	Fast	5 [5.6]
*17	6 [3.33]	Slow	6 [6.7]
*11A	5 [2.78]	Fast	5 [5.6]
*5D	5 [2.78]	Slow	5 [5.6]
*12C	3 [1.67]	Fast	3 [3.3]
*7B	3 [1.67]	Slow	3 [3.3]
*6E	2 [1.11]	Slow	2 [2.2]
*6D	2 [1.11]	Slow	2 [2.2]
*6C	2 [1.11]	Slow	2 [2.2]
*14B	2 [1.11]	Slow	2 [2.2]
*5K	2 [1.11]	Non-defined	2 [2.2]
*5J	1 [0.56]	Slow	1 [1.1]
*5E	1 [0.56]	Slow	1 [1.1]
*5A	1 [0.56]	Slow	1 [1.1]
*14A	1 [0.56]	Slow	1 [1.1]
*12B	1 [0.56]	Fast	1 [1.1]
*6J	1 [0.56]	Non-defined	1 [1.1]
Unclassified	3 [1.67]	Non-defined	3 [3.3]
Total	**180 [100]**		

NAT2= N-acetyltransferase 2 (arylamine N-acetyltransferase).

gene-environment interaction, increasing the ADR risk in smokers and female patients, respectively.

The next step in our analysis was to evaluate the specific risk in the best models found for gene-gene and gene-environment interaction (A1, B1, and C1). As shown in figure 2b, the gene-gene or gene-environment interaction models revealed the most significant increase in ADR risk, particularly when compared with those found in the single-gene or environmental analysis.

Discussion

This study evaluated the influence of environmental factors and pharmacogenetic polymorphisms on commonly reported ADRs to first-line anti-TB drugs in homogenously treated TB patients. ADRs associated with anti-TB drug regimens have considerable impact on anti-TB treatment, potentially leading to unsuccessful outcomes and prolongation of the intensive treatment phase.[26] Almost half (41.9%) of the patients enrolled in this study were found to be ADR+ (table I), suggesting that better understanding of the risk factors for anti-TB drug-induced ADRs is a critical supplement to diagnosis in order to prevent iatrogenic injuries.

Although most anti-TB drugs exhibit ADRs in a synergistic manner, multiple anti-TB drugs are frequently used in combination in order to enhance therapeutic efficacy. The most commonly used anti-TB drugs, which include isoniazid, rifampicin, and pyrazinamide, are metabolically inactivated by DMEs such as NAT2, CYPs, and GSTs. Many recent studies have shown that these enzymes are highly polymorphic and are subject to pronounced interindividual variability in expression and activity.[27] During anti-TB therapy, high levels of drug exposure, coupled with inefficient clearing owing to genetic polymorphisms, are thought to provide a significant susceptibility factor in the development of ADRs. For instance, carriers of slow *NAT2* haplotypes are known to acetylate isoniazid and its metabolites (which are precursors of toxic intermediates) more slowly than rapid acetylators and may therefore be prone to higher accumulation rates of isoniazid toxic metabolites and risk of ADRs.[28] The results of this study corroborate current hypotheses that NAT2 acetylator status may be a risk factor for ADRs induced by anti-TB drugs. Indeed, we found that carriers of the slow *NAT2* haplotypes have an increased risk of

Table III. Novel N-acetyltransferase 2 (arylamine N-acetyltransferase) haplotypes found in this Brazilian sample of tuberculosis patients

New haplotype	Single nucleotide polymorphism													
	190 C>T	191 G>A	282 C>T	341 T>C	434 A>C	458 C>T	481 C>T	499 G>A	590 G>A	600 A>G	803 A>G	838 G>A	845 A>C	857 G>A
X	–	–	+	–	–	–	+	–	–	–	–	–	–	–
Y	–	–	+	+	+	–	+	–	+	–	–	–	–	–
Z	–	–	+	+	–	–	–	–	+	–	+	–	–	–

– indicates wild type; + indicates mutant.

Table IV. Genetic frequencies and statistical associations between the absence and the presence of adverse drug reactions in tuberculosis patients who were genotyped

Genotype or allele	Total sample (n [%])	ADR status		χ^2	OR [95% CI]	p-Value[a]
		ADR– (n [%])	ADR+ (n [%])			
NAT2 acetylator[b]						
Fast	52 [58.9]	34 [72.3]	18 [45.0]	6.72	3.2 [1.2, 8.6]	***0.009***
Slow	35 [37.8]	13 [27.7]	22 [55.0]			
GSTM1[c]						
Wild	64 [64.2]	33 [73.3]	31 [72.1]	0.02	1.1 [0.4, 4.2]	1.00
Null	24 [23.8]	12 [26.7]	12 [27.9]			
GSTT1[c]						
Wild	58 [66.0]	28 [62.2]	30 [69.8]	0.55	0.7 [0.3, 1.7]	0.501
Null	30 [34.0]	17 [37.8]	13 [30.2]			
CYP2E1 rs3813867[d]						
CC	75 [87.2]	39 [86.6]	36 [87.8]	0.02	0.9 [0.2, 3.2]	0.874[e]
CG	11 [12.7]	6 [13.3]	5 [12.2]			
GG	0 [0.0]	0 [0.0]	0 [0.0]			
C	80 [93.6]	42 [93.3]	38 [93.9]	0.02	0.9 [0.2, 3.1]	0.878[e]
G	6 [6.4]	3 [6.6]	3 [6.1]			
CYP2E1 rs6413432[d]						
AA	6 [6.8]	3 [6.7]	3 [7.3]	0.01	0.9 [0.2, 4.8]	0.908[e]
AT	80 [93.1]	42 [93.3]	38 [92.7]			
TT	0 [0.0]	0 [0.0]	0 [0.0]			
A	46 [53.4]	24 [53.3]	22 [53.6]	<0.00	1.0 [0.5, 1.8]	0.967[e]
T	40 [46.6]	21 [46.7]	19 [46.4]			

a p-Values for two-tailed χ^2 tests (one significant association is indicated by bold italic text).

b Ninety patients were genotyped for *NAT2*, but three of them (3.3%) could not be defined as either fast or slow acetylators.

c Eighty-eight patients were genotyped for *GSTM1* and for *GSTT1*.

d Eighty-six patients were genotyped for *CYP2E1*.

e The p-values shown for *CYP2E1* are for the genotype and allele associations, respectively.

ADR = adverse drug reaction; **ADR–** = absence of ADR; **ADR+** = presence of ADR; **CI** = confidence interval; ***CYP2E1*** = cytochrome P450 2E1; ***GSTM1*** = glutathione S-transferase mu 1; ***GSTT1*** = glutathione S-transferase theta 1; ***NAT2*** = N-acetyltransferase 2 (arylamine N-acetyltransferase); **OR** = odds ratio.

developing ADRs (OR = 3.2, p = 0.009) [table IV]. These results are consistent with previous studies, which reported that the *NAT2* slow acetylator profile was an important modulator of susceptibility to anti-TB drug-induced ADRs.[29-31]

Previous studies using single-locus analysis have reported an association between polymorphisms in *GSTM1*, *GSTT1*, and *CYP2E1* and anti-TB drug-induced ADRs.[32,33] However, we found no single polymorphism influence on ADR risk. This discrepancy in our results may be due to differences in the frequencies of the alleles investigated in our sample. For example, although the distribution of the *CYP2E1* rs3813867 genotypes found here is similar to that previously reported for African American populations in the PubMed SNP database (dbSNP), the highest frequency found for the *CYP2E1* rs3813867 AT heterozygotes was unexpected.[34] These data possibly provide evidence of the genetic admixture occurring in Brazil.[35] Further studies should therefore be conducted with a larger sample size and with one that is fully balanced across various genotypes, in order to better evaluate the relationship between these SNPs and ADRs during anti-TB drug treatment.

The underlying mechanism for the link between pharmacogenetic polymorphisms and ADRs in anti-TB drug therapy is still not fully understood. However, it is currently believed that studies addressing multi-gene, rather than single-gene, polymorphisms in DMEs related to the biotransformation pathway of anti-TB drugs may help to uncover the susceptibility factors

a

Model	Interaction	TBA	CVC	p-Value
	Gene-gene interaction			
A1	*NAT2* slow + *GSTM1/GSTT1* null + rs6413432	0.44	10/10	0.001
A2	*NAT2* slow + *GSTT1* null	0.61	8/10	0.005
A3	*NAT2* slow + *GSTM1/GSTT1* null	0.51	5/10	0.005
	Gene-environment interaction (smoking)			
B1	*NAT2* slow + *GSTM1/GSTT1* null + smoking	0.65	10/10	$<10^{-4}$
B2	*NAT2* slow + smoking	0.68	10/10	0.005
B3	*NAT2* slow + *GSTT1* null + smoking	0.46	6/10	0.001
	Gene-environment interaction (sex)			
C1	*NAT2* slow + *GSTT1* null + female sex	0.66	10/10	10^{-4}
C2	*NAT2* slow + female sex	0.56	10/10	0.005
C3	*NAT2* slow + *GSTT1* null + female sex	0.58	6/10	$<10^{-4}$

b

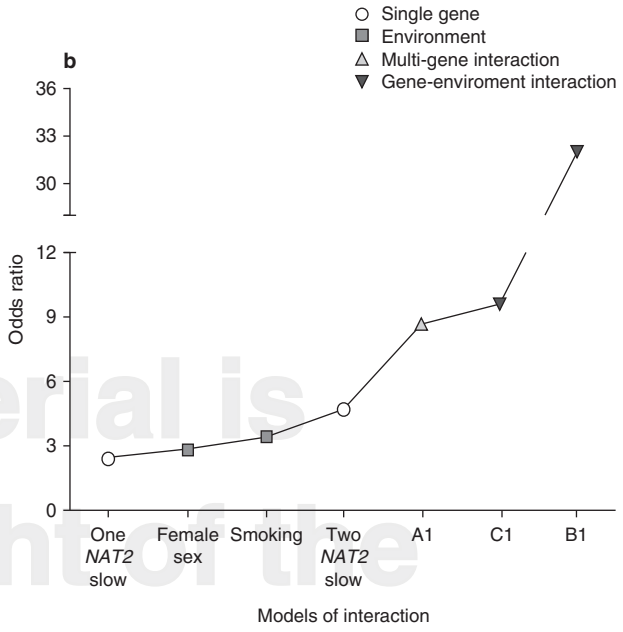

Fig. 2. Gene-gene and gene-environment interactions. (**a**) A multi-factor dimensionality reduction method[20] was applied to analyze the impact of three specific interaction categories on adverse drug reactions to anti-tuberculosis drugs: A, gene-gene interaction; B gene-smoking interaction; C, gene-sex interaction. In each category, the best models according to multi-factor dimensionality reduction analysis are shown in descending order from 1 to 3, and the best models for each category (A1, B1, and C1) are indicated in bold italic text. (**b**) The odds ratios found in this study provide evidence that gene-environment interaction models (B1 and C1) are more reliable than multi-gene, single-gene, or environmental associations in identifying high-risk factors that influence adverse drug reactions to anti-tuberculosis drugs. **CVC** = cross-validation consistency; ***GSTM1*** = glutathione S-transferase mu 1; ***GSTT1*** = glutathione S-transferase theta 1; ***NAT2*** = N-acetyltransferase 2 (arylamine N-acetyltransferase); **TBA** = testing-balanced accuracy.

involved in reported ADRs.[36] We addressed this proposal here, and our findings are also consistent with this hypothesis. As previously discussed, we did not find any significant change in ADR risk when single-SNP analyses were carried out. On the other hand, the MDR approach clearly identified the interesting contribution of specific models of gene-gene interaction to ADR susceptibility (figure 2a). According to our findings, there is an increasing risk of ADRs in carriers of the combination of *NAT2* slow, *GSTM1*, *GSTT1*, and *CYP2E1* rs6413432 polymorphisms (model A1, TBA 44%, CVC 10/10, OR 8.67, p = 0.001). Interestingly, patient susceptibility to ADRs associated with this gene-gene interaction model was even higher than that observed in the *NAT2* slow acetylator haplotype (table IV and figure 2b). The *NAT2* slow haplotype has similarly been reported to raise the risk of anti-TB drug-induced ADR nearly threefold.[29] Although the SNPs in *GSTM1*, *GSTT1*, and *CYP2E1* have not demonstrated changes in ADR risk, the results of this study revealed that when they are combined with *NAT2* slow haplotypes, as in the model A1 gene-gene interaction, the ADR risk is increased more than eight times (figure 2b). Seen together, these data support the idea that some of these polymorphism effects may be undetectable through single-locus methodology, suggesting that the haplotype and gene-gene interaction approaches have become a central theme and provide a much better picture of the influence of genetic variability on human diseases. If more genes and polymorphisms with a putative role in anti-TB drug metabolism were included, other interaction models might emerge. On the other hand, an alternative explanation for this finding might be attributed to the inclusion of all heterogeneous ADRs as one single group, which may potentially mask certain tissue-specific associations. Furthermore, a comprehensive analysis considering types and severity of ADRs would provide a better view of the impact of pharmacogenetic variation on ADR risk.

One of the strongest points of this study was the performance of a full sequencing analysis of the *NAT2* coding region. This approach not only enabled us to cover all *NAT2* SNPs but also increased the accuracy of haplotype reconstruction from the high-coverage data. In the particular case of the *NAT2* gene, haplotyping rather than single SNP analysis was critical in identifying NAT2 acetylation status and therefore in estimating the clinical impact of NAT2 pharmacogenetic variability. Furthermore, *NAT2* sequencing enabled the identification of three new haplotypes, here described for the first time (table III). We hypothesized that two of these (haplotypes Y and Z) may

potentially determine a modification in NAT2 acetylation activity, due to their predicted amino acid changes. However, these novel *NAT2* variants are present at low frequencies (<2%) and therefore may not substantially influence phenotype prediction in population studies (table II).

Among the environmental risk factors, smoking and female sex were also found to be risk factors for susceptibility to ADRs related to anti-TB drugs (table I). Although results may vary according to the sample, most studies have also reported similar associations. It is speculated that individual exposure to tobacco may stimulate the activity of the same type of DMEs induced by anti-TB drugs, especially isoniazid. Therefore, patients who have a reduced capacity to detoxify and who are simultaneously exposed to tobacco and anti-TB drugs might form more reactive metabolites and subsequently have an increased risk of ADRs.[37] It remains unclear why the female sex experiences a high incidence of ADRs.[38] Although sex, smoking, and *NAT2* slow have independently shown a positive effect on ADR risk (tables I and IV), the increased ADR risk attributable to these was more significant when taking gene-environment interactions into account (figure 2b). For example, the B1 model appears to identify smokers who carry a combination of *NAT2* slow, *GSTM1*, and *GSTT1* polymorphisms as being at much higher risk of ADRs. These individuals' risk level is at least nine times higher when compared, for example, with that of smokers without these risk alleles. This additive model of gene-environment interaction indicates that the impact of environmental factors, such as smoking, and endogenous host elements, such as pharmacogenetic variation, on the risk of ADRs to anti-TB drugs, are partly associated. In accordance with this meaningful interaction, several studies have consistently reported variations in DME genes as toxicological susceptibility markers.[17]

In contradiction of previously reported associations, the analysis in this study did not detect any difference in transaminase and bilirubin levels between the ADR+ and ADR– patient groups within the first 30 days of anti-TB drug therapy. We do not discard the hypothesis that the ADR onset time in this sample might be the same or longer that the 38 or 56 days reported in other cohort studies.[39,40] Thus, the biochemical analysis 30 days after treatment initiation probably did not allow sufficient time for elevation of serum transaminase and bilirubin levels in ADR+ patients. Furthermore, this result may vary according to the drug treatment scheme. Together, these data suggest that monitoring only the serum levels of liver biomarkers during the beginning of first-line anti-TB drug therapy is not necessarily useful in predicting future vulnerability to ADRs.

Conclusion

Our findings suggest that the *NAT2* slow acetylator haplotype, female sex, and smoking are important determinants of susceptibility to ADRs induced by anti-TB drugs. The study found a positive association between multiple, but not single, polymorphisms in the *NAT2*, *GSTM1*, *GSTT1*, and *CYP2E1* genes and increased risk of anti-TB drug-induced ADRs. Finally, the results also indicate that endogenous host elements, such as pharmacogenetic polymorphisms, could interact with environmental factors in modifying ADR risk. For example, gene-environment interaction analysis identified meaningful models that increased ADR risk to a greater extent than those found independently in multi-gene or environmental associations (figure 2b). The results of this study will be helpful for understanding the pharmacogenetic basis of ADR susceptibility. We believe that the development of predictive pharmacogenetic testing and the use of comprehensive clinical histories might be helpful for identification of patients at high risk of this complication. We therefore suggest that patients carrying the risk factors identified in this study be carefully monitored during anti-TB drug treatment.

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