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ARTICLE

NAT2 Involed in the Susceptibility to Antituberculosis Drug-Induced Liver Injury

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ABSTRACT

Objective: To investigate whether the N-acetyltransferase 2 (NAT2) gene is involved in the development of susceptibility to antituberculosis drug-induced liver damage (ATDLI) in patients with pulmonary tuberculosis in the Han nationality. **Methods:** We retrospectively analyzed 300 cases of tuberculosis patients without liver damage (control group) and 221 cases of tuberculosis patients with liver damage after antituberculosis treatment (case group). After antituberculosis treatment, genetic polymorphisms of NAT2 were analyzed in those patients using MassARRAY method. **Results:** Of the 10 tagged SNPs selected, In the promoter area of NAT2, the frequencies of T allele in rs4646243 and A allele in rs4646246 were significantly higher in the patients with ATDLI than controls (0.569 vs. 0.483, p=0.0062 and 0.567 vs 0.487, p=0.0103). The A allele of rs1115784 in the intron area showed a significant association with the development of ATDLI (0.389 vs 0.305, p = 0.0043). The frequencies of the mutated genes T and A in rs1041983 and rs1799930 in the second exon region were significantly higher than those in the control group (0.491 vs 0.360, p<0.00001 and 0.336 vs 0.212, respectively; p<0.00001). Two monomer domains were found in the 10 tag SNP sites, haplotype ht [TGAA] in monomeric domain 1 and haplotype ht [TAG] in monomeric domain 2, both were significantly more likely to be detected in the liver injury group than in the control group(p=0.0038, p<0.001, respectively). Two haplotypes were also found on the NAT2 gene: haplotype ht [CGGG] in monomeric domain 1 and ht [CGG] in block 2, and their presence means a lower risk of liver damage. **Conclusion:** NAT2 genotypes might have significant association with the risk of ATDLI in the Chinese Han nationality. By detecting the NAT2 gene and its haplotype, we can screen patients with a higher risk of liver damage before anti-TB treatment and take measures for the protection of patients.

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1. Introduction

Overall, 5-15% of the estimated 1.7 billion people infected with M. tuberculosis will develop TB disease. However, among people infected with HIV, the probability of developing TB disease is much higher, other risk factors include under-nutrition, diabetes, smoking and alcohol consumption, etc. Currently, short-course chemotherapy for tuberculosis with isoniazid, rifampicin, ethambutol and pyrazinamide as the core is adopted[1]. A large number of clinical studies have shown that isoniazid, rifampicin and pyrazinamide are all likely to cause liver damage. Especially when they are used in combination, the incidence and severity of liver damage increase significantly[2-5]. Many risk factors are associated with the occurrence of hepatic damage caused by antituberculosis drugs, such as females, advanced age, HIV infection, hypoalbuminemia, alcoholism, hepatitis B or hepatitis C virus infection, and severe lung diseases with tuberculosis, etc[6-9]. Hepatic damage is the result of the complex interaction of these risk factors. However, when these risk factors are removed or balanced the risk of liver damage between different individuals is still very different, indicates that the individual's susceptibility may be the most important factor. In recent years molecular epidemiological studies have found a link between polymorphisms in certain drug-metabolizing enzyme genes and susceptibility to antituberculosis drug-induced liver damage [4,5,10], of which, phase II enzyme N-acetyltransferase 2 (NAT2) , the first enzyme in isoniazid metabolism, was considered as a target enzyme in the study of genetic polymorphisms and antituberculosis drug-induced liver damage[11,12].

In this report, using MassARRAY technology, a case-control study was conducted to investigate the relationship between NAT2 gene polymorphism and susceptibility to antituberculosis drug-induced liver damage in the Chinese Han nationality.

2. Research Objects and Methods

2.1 Research Objects

A total of 528 patients with primary and recurrent tuberculosis who met the inclusion criteria from May 2010 to March 2016 were recruited. After a retrospective analysis, the patients were divided into two groups, including 228 patients in the case group, who had liver damage after receiving first-line antituberculosis treatment (2HRZE/4HR). Antituberculosis drug-induced liver damage is defined as asymptomatic or hepatic symptoms after taking antituberculosis drugs, such as loss of appetite, nausea, and vomiting, etc., and includes at least one of the following conditions: (1) Serum AST and/or ALT is more than 2 times the upper limit of normal (ULN) (or > 80 U/L), (2) Any increase in ALT, AST is accompanied by progressively elevated bilirubin (>2.5 mg/dl). A total of 300 tuberculosis patients taking the same antituberculosis drugs without drug-induced liver damage were selected as the control group. All patients included in the study were required to meet the following criteria: normal liver function tests at the beginning of chemotherapy and exclusion of any other factors that may cause liver damage, such as malnutrition, HIV infection, alcohol abuse, viral hepatitis, liver disease, cardiac insufficiency, and no use of other drugs that may cause liver damage, etc. During the course of treatment, the enrolled patients were asked to be closely monitored for changes in their liver function.

2.2 Methods

2.2.1 DNA Extraction from Human Peripheral Blood Mononuclear Cells

A peripheral blood sample of the above-mentioned tuberculosis patient was collected. DNA of the mononuclear cells in those samples was extracted using the whole genome DNA extraction kit (Tiangen Biotech, Beijing,China) according to the instructions and were immediately stored in a refrigerator at -20°C.

2.2.2 Selection of NAT2 Gene SNPs and Detection of Their Polymorphisms

According to the gene polymorphism published in the public SNP database and related literature reports, 10 tag SNP loci from the NAT2 gene of the Chinese Han nationality, were screened using haploview4.2 software. See Table 1 for details. Mass spectrometry (MassARRAY, Sequenom, USA) was used to detect the genotype of each SNP locus.

2.2.3 Statistical Analysis

Using SPSS 13.0 and haploview 4.2 software, t-test, x2 test and other statistical methods were used to analyze the basic parameters of the case group and the control group. Pearson's chi-square test or Fisher's exact test was used to analyze the distribution of genotypes and alleles at each site in the case and control groups. The test level is α=0.05.

3. Results

3.1 Analysis of the Basic Characteristics of the Two Groups

There was no statistically significant difference in gen-

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der and age between the case group and the control group. The values of alanine aminotransferase (ALT), aspartate aminotransferase (AST), direct bilirubin (DBIL) and total bilirubin (TBIL) before administration were all within the normal range. Hardy-Weinberg equilibrium in control group, MAF test of all data, the percentage of non-deletional genotypes in the locus, and other gene locus related information are shown in Table 1.

Table 1. The basic of the 10 SNP loci

Name	Position	ObsHET	PredHET	HWpval	%Geno	MAF	Alleles
rs4646243	18291889	0.498	0.499	0.2496	99.4	0.48	T:C
rs4271002	18292548	0.281	0.286	1	100	0.173	G:C
rs4646246	18292941	0.495	0.499	0.3619	99.2	0.479	A:G
rs1115784	18299690	0.466	0.45	0.5332	99.8	0.341	G:A
rs1041983	18302075	0.483	0.486	0.0191	99.8	0.416	C:T
rs1801280	18302134	0.087	0.084	1	99.8	0.044	T:C
rs1799929	18302274	0.083	0.08	1	100	0.042	C:T
rs1799930	18302383	0.382	0.39	0.1749	99.8	0.265	G:A
rs1208	18302596	0.084	0.08	1	99.8	0.042	A:G
rs1799931	18302650	0.247	0.247	0.9113	100	0.144	G:A

Notes:

• Position: Position of the locus on the chromosome;

• %Geno: The percentage of non-deleted genotypes on the locus for all samples (the minimum value is 75%, less than this value is considered to have failed the test);

• MAF: The frequency of the last allele at this site (minimum value is 0.001, less than this value is considered to have failed the test);

• Alleles: major and minor alleles at the locus.

3.2 Analysis of Individual SNP and ATDLI

The differences in the distribution of genotypes and alleles at each site between the case group and the control group were analyzed using the Pearson chi-square test or Fisher's exact test. The results are shown in Table 2. As can be seen from the chart, there are statistically significant differences in the alleles of the NAT2 gene at the five SNP loci in the case group and the control group: rs4646243, rs4646246, rs1115784, rs1041983, and rs1799930. Among the five SNPs in NAT2, the T allele in the promoter region rs4646243 and the A allele in rs4646246 were significantly more frequently expressed in patients with antituberculous drug-induced hepatic impairment than in the control group (0.569 vs 0.483, p=0.0062 and 0.567 vs 0.487, p=0.0103, respectively), indicating that they both increased the risk of liver damage. In rs1115784, which was located in the first intron region, the frequency of its A allele in the case group was also higher than that in the control group (0.389 vs 0.305, p = 0.0043). The frequency of T allele in rs1041983, located in the second exon region, was significantly higher in the case group than in the control group (0.491 vs 0.360, p<0.00001). However, this SNP change does not cause codon changes, and the expression product is a tyrosine-containing protein. Rs1799930 is also located in the second exon region of the NAT2 gene. The frequency of A allele is not only significantly higher in the case group than in the control group (0.336 vs 0.212, p<0.00001), but also this mutation makes the 197th code of the NAT2 gene changed from arginine to glutamine, The tertiary structure of proteins may therefore be altered.

Table 2. Differences in distribution of genotypes and alleles between case group and control group

Site	Alleles	Number of cases (case,control)	Frequency (case,control)	χ^2	P
rs4646243	T	257:195, 288:308	0.569, 0.483	7.504	0.0062
rs4271002	C	79:375, 103:497	0.174, 0.172	0.01	0.9206
rs4646246	A	255:195, 290:306	0.567, 0.487	6.59	0.0103
rs1115784	A	176:276, 183:417	0.389, 0.305	8.165	0.0043
rs1041983	T	222:230, 216:384	0.491, 0.360	18.247	1.94E-05
rs1801280	T	436:18, 570:28	0.960, 0.953	0.318	0.573
rs1799929	C	436:18, 574:26	0.960, 0.957	0.088	0.767
rs1799930	A	152:300, 127:473	0.336, 0.212	20.543	5.83E-06
rs1208	A	437:17, 571:27	0.963, 0.955	0.382	0.5363
rs1799931	A	68:386, 84:516	0.150, 0.140	0.2	0.6545

3.3 Analysis of Linkage Disequilibrium

In a certain population, the frequency at which two alleles at different loci appear on the same chromosome is higher than the expected random frequency. This phenomenon is defined as linkage disequilibrium. A non-random combination of certain alleles of this different locus is often inherited together. By using D'/r^2 and other methods to investigate the linkage disequilibrium between loci, it was found that there are 2 monomer domains in 10 sites of NAT2 gene, and there is a linkage disequilibrium in some sites in each monomer domain. The specific results are shown in Fig. 1. Above the monomer domain, we marked the rs number of the locus, which makes it easy to see which specific rs-sites have haplocells.

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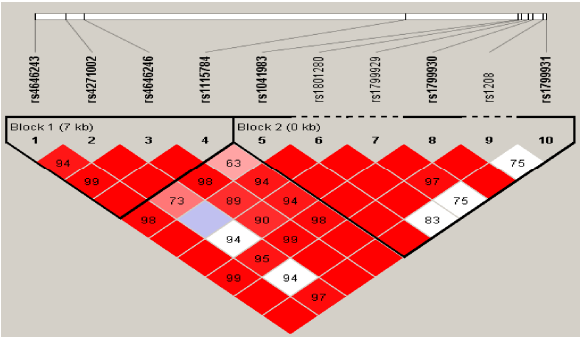

Figure 1. Pairing LDs between SNPs in a gene cluster

The value of each square represents the correlation coefficient of the tag's top-left and top-right SNPs. No data on the grid represents D'=1. A bold square represents haplotype formation in that particular SNP site.

3.4 Haplotype Analysis

A set of single nucleotide polymorphisms that are related to each other in a specific region of a chromosome and tend to be inherited globally to offspring, were known as haplotypes. For the monomer domain detected in the above linkage disequilibrium analysis, The distribution ratios of haplotypes in each monomer domain in the case group and the control group were calculated separately, Pearson's chi-square test was used to examine the association between overall genetic haplotype and disease ($p<0.05$). The results are shown in Table 3. Four haplotypes closely related to antituberculosis drug-induced hepatic impairment were found in two monomer domains consisting of 10 SNPs. Among them, the haplotype ht [TGAA] located in monomeric domain 1 and the haplotype ht [TAG] located in monomeric domain 2 appeared more often in the case group than in the control group ($p=0.0043$ and $p<0.0001$, respectively). Two haplotypes were also found on the NAT2 gene and were associated with a lower risk of liver damage: haplotype ht [CGGG] in monomeric domain 1 and ht [CGG] in monomeric domain 2.

Table 3. Relationship between haplotype and liver damage

Haplotypes	Frequency	Number of cases (case,control)	Frequency (case,control)	χ^2	P
Domain 1					
CGGG	0.475	193.0 : 259.0, 307.0 : 293.0	0.427, 0.512	7.409	0.0065
TGAA	0.341	176.0 : 276.0, 183.0 : 417.0	0.389, 0.305	8.165	0.0043
TCAG	0.168	77.0 : 375.0, 100.0 : 500.0	0.170, 0.167	0.026	0.8726
Domain 2					
CGG	0.584	231.4 : 222.6, 384.0 : 216.0	0.510, 0.640	18.083	2.11E-05
TAG	0.265	152.6 : 301.4, 127.0 : 473.0	0.336, 0.212	20.553	5.80E-06
TGA	0.144	68.0 : 386.0, 84.0 : 516.0	0.150, 0.140	0.2	0.6545

4. Discussion

In the process of isoniazid metabolism, isoniazid is first acetylated to acetyl isoniazid by NAT2 and then hydrolyzed to produce monoacetyl hydrazine, which is then catalyzed by cytochrome P450 2E1 and oxidized to a hydroxylamine form, and this intermediate metabolite can cause liver damage[13]. In the study of pharmacokinetics, the phenotypes of NAT2 were divided into fast acetylated genotypes, mid-acetylated genotypes, and slow acetylated genotypes depending on the rate of acetylation. This classification is mainly related to genetic polymorphisms, and studies have shown that the different NAT2 genotypes have a significant effect on their metabolic capacity[14-16]. The fast-acetylated genotypes convert isoniazid to acetylcholine significantly faster than slow-acetylated genotypes, and by further acetylation, acetyl hydrazine can be converted to diacetyl hydrazine, which makes acetyl hydrazine, an intermediate metabolite with strong hepatotoxicity, not accumulates, thereby increasing detoxification efficiency. On the contrary, in the slow acetylation type, the formation of a non-toxic derivative of diacetyl hydrazine from acetyl hydrazine may be hindered, which favors the formation of more toxic monoacetyl guanidine derivatives through cytochrome P450 2E1-mediated metabolism, causing liver damage.

Studies have evaluated the relationship between NAT2 genotypes and the risk of hepatitis. Through the study of 224 tuberculosis patients treated with antituberculous drugs, Huang et al.[17] reported that the incidence of drug-induced liver damage was 14.7%, and the incidence of severe liver damage was 6.3%. In the slow acetylation genotypes, the risk factors for drug-induced liver damage in the NAT2*6/6 and NAT2*6/7 genotypes were as high as 4.02. Compared with fast-acetylated genotypes, slow-acetylated genotypes have a high incidence of drug-induced liver damage and are more prone to severe drug-induced liver damage. The study concluded that NAT2 slow acetylation genotypes are important susceptibility factors for the development of antituberculosis drug-induced hepatic impairment. In India, Bose et al.[5] studied 218 patients with tuberculosis, including 41 patients with hepatic impairment after treatment, and the results showed that the appearance of NAT2*5/*7 and NAT2*6/*7 genotypes was significantly higher in patients with hepatic impairment than in patients without liver damage. The investigation of Cho et al.[18] pointed out that

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in the study of 132 patients with tuberculosis in South Korea (18 cases of drug-induced hepatotoxicity), the risk of liver damage in patients with the NAT2 slow acetylation genotype is 3.8 times higher than in patients with fast acetylation, suggesting that the NAT2 genotype can be used as an effective predictor of antituberculosis drug-induced liver damage. Through the study of 50 tuberculosis patients in Iran, the results of Khalili et al.[19] also confirmed that the frequency of NAT2 slow acetylation genotypes in antituberculosis drug-induced hepatic impairment is much higher than that of fast acetylation genotypes. Wang Jinhe et al.[20] explored NAT2 gene polymorphisms in 32 patients with antituberculous drug-induced hepatic impairment and 35 patients without liver damage. The results of the study also proved that the NAT2 genotype is highly related to isoniazid and rifampin-induced liver disease, and moreover, the 857 codon mutation may be one of the susceptibility genotypes of hepatic toxicity in TB patients. However, the above studies only emphasize the relationship between the acetylation phenotype and liver damage in NAT genotypes, and most studies only verified the relationship between base mutations in the exon region of the NAT2 gene and liver damage.[21,22]

In this study, we screened out the target locus according to the genotype frequency in the SNP of the Chinese population reported in the public SNP database. Most of these sites are in the promoter and exon regions, and a few are in the intron region. Then, the frequency of each SNP locus allele in the case group and the control group was measured by the MassARRAY method. Further, we analyzed the association of these SNP loci and haplotypes with antituberculous drug-induced hepatic impairment. When analyzing NAT2, we found -9905C>T (rs4646243), -8853 G > A (rs4646246) in the promoter region, and -2098G>A (rs1115784) in the first intron, and in the second exon, 282C>T (rs1041983), 590G>A (rs1799930), are all significantly associated with antituberculosis drug-induced liver damage. The relationship between the first three SNP polymorphisms and antituberculosis drug-induced liver damage has not been reported before. Kim et al.[23] proposed that in the Korean population, if A replaces T at -9796 in the promoter of NAT2 gene, is closely related to antituberculosis drug-induced hepatitis, it is closely related to the occurrence of antituberculosis drug-induced hepatitis. Furthermore, in vitro experiments showed that the activity of luciferase containing the A allele was reduced, so it is believed that carrying this variant allele in the promoter will reduce NAT2 transcriptional activity. We speculate that the -9905 C>T, -8853 G>A mutation also reduces the expression of the NAT2 gene by reducing its transcriptional activity, thereby increasing the risk of liver damage. The reason for the correlation between the first intron -2098 G>A mutation and drug-induced liver damage has yet to be confirmed. The mutation of 282 C>T, 590 G>A in the second exon (according to the international term consensus, also known as NAT2*6) increased the risk of occurrence of antituberculosis drug-induced liver damage, which is consistent with the results of Possuelo et al.[24]. In the study by Possuelo et al., 282 T genotypes and NAT2*6/6 genotypes were more susceptible to antituberculosis drug-induced liver damage, with risk factors of 4.3 and 5.7, respectively ($P<0.01$). The research of Huang et al.[17] also supports this view. However, the change of 282 C>T in coding region does not cause codon change, and the exact mechanism of the TT genotype prone to antituberculosis drug-induced hepatic impairment is still unclear. It may be because this base change causes a spatial conformational change in the NAT2-encoded protein, resulting in a change in protein function. The change in coding region 590 G>A caused the 197 codon of the NAT2 gene to be changed, and the amino acid at this position in the encoded protein was changed from arginine to glutamine. This leads to changes in the structure and function of functional proteins, resulting in a reduction in the ability of NAT2 enzymes to metabolize, thereby increasing the risk of drug-induced liver damage[15,23]. In addition, we also found 4 haplotypes closely related to antituberculosis drug-induced hepatic impairment in NAT2, of which 2 haplotypes increased the risk of occurrence of hepatic impairment in antituberculosis drugs: haplotypes in monomeric domain 1 ht [TGAA] and haplotype ht [TAG] located in monomer domain 2. While the other two haplotypes: haplotype ht [CGGG] in monomeric domain 1 and ht [CGG] in monomeric domain 2 are suggestive of a lower risk of liver damage, and they may play an important role in predicting the risk of drug related liver damage.

5. Conclusion

In summary, we used MassARRAY method to analyze 10 SNP sites in NAT2 and found 5 SNP sites and 4 haplotypes closely related to antituberculosis drug-induced liver damage. Therefore, we should be able to screen for NAT2 genotypes and haplotypes in TB patients using the MassARRAY method to predict the possibility of liver damage in that particular patient before taking antituberculosis drugs, and thus provide a scientific basis for individualized use of liver protection drugs.

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