Genetic variants in antioxidant pathway: Risk factors for hepatotoxicity in tuberculosis patients

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Summary

Tuberculosis (TB) treatment can cause serious sequelae including adverse effects such as anti-TB drug-induced hepatotoxicity (ATDH). We performed a candidate gene-based association study between single nucleotide polymorphisms (SNPs) in 10 genes in the antioxidant pathway and ATDH susceptibility. The subjects comprised 100 Japanese patients with pulmonary TB who received a treatment regimen including isoniazid and rifampicin. Out of them, 18 patients had ATDH. Thirty-four tag SNPs in 10 genes were analyzed by PCR-restriction fragment length polymorphism or PCR-direct DNA sequencing. The frequencies of alleles and genotypes between patients with and without ATDH were compared in three different genetic models. Statistical analyses revealed that a C/C genotype at rs11080344 in *NOS2A*, a C/C genotype at rs2070401 in *BACH1*, and a G/A or A/A genotype at rs4720833 in *MAFK* independently conferred ATDH susceptibility. Remarkably, the association of the latter two tag SNPs with ATDH susceptibility was highly statistically significant ($P = 0.0006$) with an odds ratio of 9.730. This study is the first report to demonstrate that *NOS2A*, *BACH1*, and *MAFK* appear to be genetic

determinants of ATDH in Japanese patients with TB. Furthermore, a combination of *BACH1* and *MAFK* polymorphisms may be useful as new biomarkers to identify high-risk Japanese TB patients for ATDH.

Keywords: Tuberculosis, anti-tuberculosis drug-induced hepatotoxicity, antioxidant pathway, single nucleotide polymorphism (SNP), candidate gene-based association study

1. Introduction

Tuberculosis (TB) is a re-emerging infectious disease and was declared a global health problem by the World Health Organization in 1993.[1] In 2007, there were ~9.27 million new cases of TB and ~1.3 million TB-related deaths worldwide. As more than 80% of all TB patients live in Africa (31%) and Asia (55%), the epidemiology and control of TB remain important public health issues.[1] However, TB care involves serious issues, such as disease relapse in elderly patients, complications induced by acquired immunodeficiency syndrome, the occurrence of adverse effects of anti-TB drugs, and an increase in the prevalence of multidrug-resistant *Mycobacterium tuberculosis*.[1-3] In particular, because of the long treatment period of 6-9 months, adverse effects of anti-TB drugs (*e.g.*, hepatotoxicity, rash, fever, peripheral neuritis, eosinophilia, and hyperuricemia) may lead to a decline in the treatment effectiveness and quality of life for TB patients. Furthermore, adverse effects may lead to non-compliance with anti-TB drugs regimens, which may result in ineffective treatment, disease relapse, or the emergence of multidrug-resistant *M. tuberculosis*.[1,2]

One severe, sometimes fatal, adverse effect of anti-TB drugs is hepatotoxicity. A number of environmental risk factors (*e.g.*, advanced age,[4,5] gender,[5-8] malnutrition,[4,8] complications of diseases,[6,7,9] and alcohol intake[4,6]) are associated with susceptibility to anti-TB drug-induced hepatotoxicity (ATDH). Importantly, multiple genetic factors also impact the likelihood of ATDH. Previous candidate gene-based association studies revealed several possible ATDH-susceptibility genes, including *N*-acetyltransferase 2 (*NAT2*),[10-14] cytochrome P450 2E1 (*CYP2E1*),[12,15] glutathione *S*-transferase M1 (*GSTM1*),[12,16] glutathione *S*-transferase T1 (*GSTT1*),[12,16] and HLA-DQA1/-DQB1.[17]

Isoniazid (INH), a major drug used in the treatment of TB, is metabolized to acetylisoniazid by NAT2. Then, INH and acetylisoniazid are hydrolyzed to hydrazine and acetylhydrazine, respectively, in the liver. From an etiological perspective, the accumulation of these toxic metabolites, hydrazine and acetylhydrazine, in hepatocytes contributes to ATDH.[18-20] In addition, these hepatotoxic metabolites induced the excessive production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the rat hepatocytes after being metabolized by

enzymes including CYP2E1.[20,21] In particular, nitric oxide (NO), one of the RNS, is produced by NO synthase (NOS). In the presence of ROS/RNS, the inducible isoform of NOS (iNOS; coded by *NOS2A*) is upregulated by nuclear factor kappa B in the liver.[22,23]

Due to the biological imperative of protecting the cellular environment against oxidative stress, ROS/RNS are rapidly eliminated by antioxidant enzymes such as GSTs, NAD(P)H dehydrogenase quinone 1 (NQO1; coded by *NQO1*), and heme oxygenase 1 (HO1; coded by *HMOX1*) in humans.[24] The expression of these genes, which carry an antioxidant-responsive element (ARE) in their promoter regions is regulated by several transcriptional factors, including nuclear factor erythroid 2-related factor 2 (Nrf2; coded by *NFE2L2*), BTB and CNC homology 1 (Bach1; coded by *BACH1*), and the small Maf basic leucine zipper proteins (MafF, MafG, and MafK).[25-28] Heterodimers of Nrf2 and small Maf proteins bind to the ARE and upregulate the expression of antioxidant enzymes.[26] By contrast, a heterodimer complex of Bach1 and small Maf proteins downregulates antioxidant enzyme expression.[27,28] Furthermore, Kelch-like ECH-associated protein 1 (Keap1; coded by

KEAP1) binds to Nrf2 in the cytoplasm, thus suppressing its translocation to the nucleus and inhibiting the expression of antioxidant enzymes under non-oxidative stress.[29] Conversely, exportin 1 (Xpo1; coded by *XPO1*) binds to and exports Bach1 from the nucleus to the cytoplasm, which leads to the activation of antioxidant enzyme expression, eventually resulting in the elimination of ROS/RNS.[30] Therefore, the dysregulation of the activator arm (including Nrf2/small Mafs/Xpo1) and the repressor arm (including Bach1/small Mafs/Keap1) in the antioxidant pathway may contribute to the occurrence of ATDH in humans.

Here, we conducted a candidate gene-based association study by selecting several targets involved in both the activator and repressor arms of the antioxidant pathway to assess as putative ATDH-susceptibility genes. The purpose of this study was to investigate whether polymorphisms of these target genes are associated with ATDH susceptibility in Japanese patients with pulmonary TB, and whether such polymorphisms could be used as new genetic biomarkers to identify Japanese TB patients at high-risk for developing ATDH.

2. Patients and methods

2.1. Patients

The subjects comprised 100 unrelated Japanese patients with new onset of pulmonary TB with a treatment regimen including INH (400 mg/day) and rifampicin (RFP; 450 mg/day) for 6-9 months between 2003 and 2005 (Table 1). All of the patients were randomly enrolled from three general hospitals in Nagasaki, Japan. The study protocol was approved by the Ethics Committee dealing with the Human Genome and Gene Analysis at Nagasaki University, and written informed consent was obtained from each patient.

The diagnosis of pulmonary TB was made on the basis of three clinical criteria: 1) the presence of symptoms; 2) compatible chest radiographic infiltrate findings; and 3) the presence of acid-fast bacilli on sputum smear and *M. tuberculosis* on sputum culture.[31] Patients with liver cirrhosis, acute hepatitis, chronic hepatitis, alcoholic liver disease, or other chronic liver diseases were excluded from this study.

2.2. Definition of ATDH

ATDH was defined according to the criteria of the International Consensus Meeting.[32] Specifically, patients classified as having ATDH presented with serum alanine aminotransferase (ALT) levels that were ≥ 2-fold above the upper limit of the normal range (normal ≤ 42 IU/L), or had a combined increase of over 2-fold in serum aspartate aminotransferase (AST, normal ≤ 33 IU/L) and total bilirubin (normal ≤ 1.5 mg/dL) levels during the course of TB treatment.

2.3. Preparation of genomic DNA

Genomic DNA was extracted from whole blood samples using a QuickGene DNA Whole Blood Kit S (Fujifilm, Tokyo, Japan) with a QuickGene-800 (Fujifilm) according to the manufacturer's protocol.

2.4. Selection of tag SNPs in candidate target genes

All of the single nucleotide polymorphisms (SNPs) in *NOS2A* (GenBank accession number: NM_000625; MIM 163730) located on chromosome 17q11.2-q12; *NQO1* (GenBank accession number: NM_000903; MIM 125860) located on chromosome 16q22.1; *HMOX1* (GenBank accession

number: NM_002133; MIM 141250) located on chromosome 22q12-q13.1; *NFE2L2* (GenBank accession number: NM_006164; MIM 600492) located on chromosome 2q31; *BACH1* (GenBank accession number: NM_206866; MIM 602751) located on chromosome 21q22.11; *MAFF* (GenBank accession number: NM_012323; MIM 604877) located on chromosome 22q13.1; *MAFG* (GenBank accession number: NM_002359; MIM 602020) located on chromosome 17q25.3; *MAFK* (GenBank accession number: NM_002360; MIM 600197) located on chromosome 7p22.3; *KEAP1* (GenBank accession number: NM_203500; MIM 606016) located on chromosome 19p13.2; and *XPO1* (GenBank accession number: NM_003400; MIM 602559) located on chromosome 2p15 were obtained using the Japanese data in Tokyo (Rel 24/phaseII Nov08, on NCBI B36 assembly, dbSNP b126) available on the International HapMap website.[33] Candidate tag SNPs were selected from all SNPs in the each chromosomal region including 2-kb upstream with priority in minor alleles with a frequency of more than 20% in the International HapMap data. Subsequently, genotyped tag SNPs among the candidate tag SNPs were determined using the Haploview 4.1 software program.[34] Since there were

no SNPs within *MAFG*, this gene was excluded from genotyping in this study.

2.5. Genotyping of tag SNPs in each gene

Genotyped tag SNPs were analyzed by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) or PCR-direct DNA sequencing method. The polymorphic region was amplified by PCR with a GeneAmp PCR System 9700 thermal cycler (Life Technologies, Carlsbad, CA, USA) using 20 ng genomic DNA in a 25-μL reaction mixture containing 0.8X GoTaq Green master mix (Promega, Madison, WI, USA) and 15 pmol each of forward and reverse primers (Table 2). The amplification protocol consisted of initial denaturation at 95°C for 2 minutes, followed by 30 or 35 cycles of denaturation at 95°C for 30 seconds, annealing for 30 seconds at appropriate temperature for the primer pair (Table 2), and extension at 72°C for 30 seconds, and final extension at 72°C for 5 minutes.

With respect to the RFLP method, the PCR products were digested with each restriction enzyme (Table 2), separated by electrophoresis on a

6% to 12% polyacrylamide gel (Nacalai Tesque, Kyoto, Japan) or a 2% ME-agarose gel (Nacalai Teque), stained with ethidium bromide, and visualized with an ultraviolet transilluminator (Alpha Innotech Co., San Leandro, CA, USA).

With respect to the direct DNA sequencing method, the PCR products were treated with ExoSAP-IT (Amersham Pharmacia Biotech, Plscataway, NJ, USA) and cycle sequenced using a BigDye Terminator v3.1 Cycle Sequencing FS Ready Reaction Kit (Life Technologies). The cycle sequencing was hot-started at 96°C for 30 seconds, followed by 25 cycles of denaturation at 96°C for 10 seconds, annealing at 50°C for 5 seconds, and extension at 60°C for 4 minutes using 1 pmol PCR forward primer or reverse primer. After the sequencing reaction solutions were purified using Sephadex G-50 superfine columns (Amersham Pharmacia Biotech), the samples were dried and sequenced with an ABI Prism 3100 Genetic Analyzer (Life Technologies).

2.6. Statistical analysis

Data are expressed as the means ± standard deviations. The clinical

characteristics and laboratory data were compared between patients with and without ATDH by Mann-Whitney *U* test, chi-square test, or Fisher's exact test using the IBM SPSS Statistics 19 software package (IBM Japan, Tokyo, Japan). In order to determine whether each SNP was in the Hardy-Weinberg equilibrium, chi-square test with Yates' correction was performed using the SNP Alyze 7.0 standard software package (Dynacom Inc., Chiba, Japan). The frequencies of alleles and genotypes between patients with and without ATDH were compared by chi-square test or Fisher's exact test in three different genetic models: the multiplicative; the minor allele dominant; and the minor allele recessive, using the SNP Alyze 7.0 standard software package. Univariate analyses allowed the selection of a set of genetic risk factors for ATDH that were identified based upon their statistically significant association. Then, a comparison of these putative risk factors between patients with and without ATDH was carried out by multivariate logistic regression analysis using the IBM SPSS Statistics 19. The odds ratio (OR) with 95% confidence interval (CI) was calculated using the IBM SPSS Statistics 19. A *P* value of less than 0.05 was considered to be statistically significant.

2.7. Genetic test using genetic polymorphisms as biomarkers

Finally, in order to evaluate the genetic polymorphisms which indicated a close association with ATDH susceptibility independently by multivariate logistic regression analysis, a genetic test using these genetic polymorphisms as biomarkers was performed. As sensitivity implicates the proportion of actual positives in a binary classification test, it was calculated according to the formula: the number of the TB patients with ATDH possessing these genetic polymorphisms divided by that of TB patients with ATDH. On the other hand, specificity implies the proportion of actual negatives. Therefore, we used the following formula: the number of the TB patients without ATDH not possessing these genetic polymorphisms divided by that of TB patients without ATDH.

3. Results

3.1. Comparison of the clinicopathological parameters between TB patients with and without ATDH

Out of the 100 TB patients enrolled in this study, 18 patients had

hepatotoxicity. The TB treatment of all of the patients with ATDH was interrupted, subsequently leading to desensitization therapy. There were no significant differences in the clinical characteristics and laboratory data between patients with and without ATDH (Table 3).

3.2. Association between tag SNPs and susceptibility to ATDH

The distributions of alleles and genotypes at tag SNPs in each gene were identified and compared between patients with and without ATDH by chi-square test or Fisher's exact test in three different genetic models (Table 4). The distributions of all tag SNPs among TB patients corresponded well to the Hardy-Weinberg equilibrium, implying that the patient base had a homogenous genetic background.

Two tag SNPs, rs11080344 in *NOS2A* and rs11125883 in *XPO1*, were significantly associated with a lack of ATDH susceptibility in two genetic models. With respect to rs11080344 in *NOS2A*, the frequencies of a minor T allele in the multiplicative model and a heterozygous C/T genotype or minor homozygous T/T genotype in the minor allele dominant model were significantly decreased in patients with, as

compared to patients without, ATDH ($P = 0.043$, OR = 0.424 and $P = 0.044$, OR = 0.348, respectively; Table 4). Conversely, a major C allele and its homozygous C/C genotype indicated susceptibility to ATDH ($P = 0.043$, OR = 2.357 and $P = 0.044$, OR = 2.872, respectively). With respect to rs11125883 in *XPO1*, the frequencies of a minor C allele in the multiplicative model and a heterozygous A/C genotype or minor homozygous C/C genotype in the minor allele dominant model were significantly lower in patients with, as compared to patients without, ATDH ($P = 0.031$, OR = 0.416 and $P = 0.026$, OR = 0.312, respectively; Table 4). By contrast, a major A allele and its homozygous A/A genotype increased susceptibility to ATDH ($P = 0.031$, OR = 2.407 and $P = 0.026$, OR = 3.201, respectively).

Another set of tag SNPs, rs2070401 in *BACH1* and rs4720833 in *MAFK*, showed ATDH susceptibility in one genetic model. With respect to rs2070401 in *BACH1*, the frequency of a minor homozygous C/C genotype in the minor allele recessive model was significantly increased in patients with, as compared to patients without, ATDH ($P = 0.018$, OR = 16.200; Table 4). With respect to rs4720833 in *MAFK*, a heterozygous G/A

genotype or minor homozygous A/A genotype in the minor allele dominant model was significantly more prevalent in patients with, as compared to patients without, ATDH ($P = 0.037$, OR = 3.162; Table 4).

No significant differences were observed in the frequencies of the other alleles and genotypes between TB patients with and without ATDH (Table 4).

3.3. Gene interactions among ATDH-susceptible genotypes

Gene interactions among the four genotypes that showed significant association with ATDH were analyzed between patients with and without ATDH. Multivariate logistic regression analysis indicated that three variable genetic risk factors–the C/C genotype at rs11080344 in *NOS2A*, the C/C genotype at rs11080344 in *BACH1*, and the G/A or A/A genotype at rs4720833 in *MAFK*–independently contributed to ATDH susceptibility ($P = 0.036$, OR = 3.601; $P = 0.021$, OR = 29.144; and $P = 0.014$, OR = 5.724, respectively; Table 5).

Furthermore, in order to better predict TB patients at high-risk for ATDH, we tested combinations of the three independent genetic risk

factors (*NOS2A, BACH1*, and *MAFK* genotypes). The most effective prediction panel included both the *BACH1* and *MAFK* genotypes, which are repressors in the antioxidant pathway. Remarkably, the presence of either the C/C genotype at rs2070401 in *BACH1* or the G/A or A/A genotype at rs4720833 in *MAFK* was strongly associated with ATDH susceptibility ($P = 0.0006$, OR = 9.730, with a 95% CI of 2.100–45.08; Table 6). The sensitivity and specificity of this test were estimated at 88.9% and 54.9%, respectively.

4. Discussion

To our knowledge, this is the first demonstration of an association between *NOS2A*, *BACH1*, and *MAFK* polymorphisms and ATDH susceptibility in Japanese patients with pulmonary TB. In particular, the possession of the C/C genotype at rs11080344 in *NOS2A*, the C/C genotype at rs2070401 in *BACH1*, and the G/A or A/A genotype at rs4720833 in *MAFK* independently conferred susceptibility to ATDH. Although the presence of the C/C genotype at rs2070401 in *BACH1* resulted in a high odds ratio (OR = 16.200) for ATDH, the sensitivity of

this test was very low, as the number of patients with ATDH carrying this genotype was only 3 (16.7%; data not shown). By contrast, the presence of either the C/C genotype at rs2070401 in *BACH1* or the G/A or A/A genotype at rs4720833 in *MAFK* was significantly associated with ATDH at $P = 0.0006$ with an odds ratio of 9.730. In addition, the number of patients with ATDH carrying one of these genotypes was 16 (88.9% in Table 6), indicating that these genotypes may serve as more accurate biomarkers. These findings suggest that *NOS2A*, *BACH1*, and *MAFK* are genetic determinants for a predisposition to the onset and/or development of ATDH in Japanese TB patients. However, the number of TB patients in this study was relatively small. Therefore, further studies on a larger number of Japanese TB patients and on other ethnic populations will be necessary to confirm the associations we observed between *NOS2A*, *BACH1*, and *MAFK* polymorphisms and ATDH. The inclusion of other ethnicities will be important to broaden the utility of our results as different populations will often have distinct allele and genotype frequencies.

Under normal conditions, the transcriptional repressor Bach1

forms heterodimers with small Maf proteins including MafK, which are involved in the repressor arm in the antioxidant pathway. The heterodimer then binds to the ARE in the promoter region of antioxidative stress genes, and initially suppresses the expression of antioxidant enzymes such as GSTs, NQO1, and HO1 (Figure 1).[27,28] Conversely, under conditions of oxidative stress, Bach1 dissociates from the ARE, allowing Nrf2/small Mafs to bind to the ARE and activate antioxidant enzyme expression,[35] eventually leading to the elimination of ROS (Figure 1). Furthermore, the knockdown of Bach1 in human keratinocytes specifically upregulates the expression of HO1.[36]

From a molecular genetics perspective, the location of rs2070401 SNP, which was strongly associated with ATDH susceptibility in this study, in the 3'-untranslated region of *BACH1* may change the stability and half-life of *BACH1* mRNA. Therefore, it seems reasonable to speculate that the C/C genotype at rs2070401 in *BACH1* may enhance the stability and prolong the half-life of *BACH1* mRNA. Such a modification would likely result in an increase in Bach1 protein, leading to an enhanced suppression of antioxidant enzyme expression. This process may reduce the

effectiveness of protective mechanisms against ROS in the liver, resulting in accumulation of ROS in hepatocytes, the development of persistent inflammation, and eventual hepatotoxicity through mitochondrial DNA damage and mitochondrial dysfunction.[37-39] Although this represents an attractive model to relate *BACH1* polymorphisms with ATDH susceptibility, the actual molecular mechanisms involved remain unknown.

The small Maf proteins (MafF, MafG, and MafK) are transcription factors which localize to the nucleus. These proteins dimerize with the CNC family proteins including Nrf2 and Bach1 (Figure 1).[26-28] Oxidative stress induces the removal of Bach1/small Maf heterodimers from the ARE. In their place, heterodimers of Nrf2 and small Maf proteins bind, leading to the activation of antioxidant enzyme expression (Figure 1). Therefore, our data suggest that at least two distinct pathways may be affected in ATDH. One involves downregulation of Nrf2. The other concerns upregulation of Bach1. Since rs4720833 SNP is located in intron 1 of *MAFK*, the G/A or A/A genetic genotype of this SNP may act as a repressor for its expression (dimerization with Nrf2) and/or as an activator for its expression (dimerization with Bach1), resulting in the

observed association with ATDH susceptibility. However, the presence of enhancers, repressors, or non-coding RNAs around rs4720833 SNP in intron 1 of *MAFK* remains unknown. Furthermore, the downregulation of Nrf2 or upregulation of Bach1 in the patients with ATDH possessing the G/A or A/A genotype at rs4720833 in *MAFK* needs to be verified. However, since both *BACH1* and *MAFK* conferred ATDH susceptibility, the repressor arm (Bach1/MafK/Keap1) in the antioxidant pathway may play a critical role in the stress response processes, especially in ATDH.

Another possible mechanism connecting our candidate susceptibility genes for ATDH involves the inflammatory response. Transgenic overexpression of MafK in T cells decreased T-cell proliferation and IL-2 proinflammatory cytokine secretion.[40] Therefore, the diminished expression of MafK due to the G/A or A/A genetic variant in rs4720833 may increase IL-2 secretion, resulting in persistent inflammation and eventually leading to hepatotoxicity.

A third possible mechanism explaining our data incorporates iNOS, a key molecule for ATDH pathogenesis. Hepatotoxicity is decreased both in animals treated with the iNOS inhibitor and in *iNOS*-knockout mice.[41,42]

Furthermore, NO, which is produced by iNOS, accelerates the cytotoxic effects of NO itself and reacts with the superoxide anion radical $O_2^{\cdot -}$, thereby generating a new product, peroxynitrite, which is a potent oxidant.[43] The overproduction of peroxynitrite inactivates antioxidant enzymes by oxidizing thiols and the methionine residue,[43,44] leading to a decrease in the protection of hepatocytes from oxidative stress. Therefore, the C/C genotype at rs11080344 in *NOS2A* may affect the expression of *NOS2A* and subsequently lead to a gain-of-function of iNOS activity. From a molecular genetics perspective, as rs11080344 SNP is located in intron 11, this genetic alteration may activate the enhancers and non-coding RNAs, or may inactivate the repressors, resulting in the gain-of-function of iNOS. The activated iNOS in the patients possessing the C/C genotype at rs11080344 in *iNOS* may result in the overproduction of NO, and the consequent diminution of antioxidant enzyme activity. The reduction of antioxidant enzymes in hepatocytes may lead to an increased susceptibility to ATDH.

In conclusion, *NOS2A*, *BACH1*, and *MAFK* appear to be genetic determinants of ATDH in Japanese patients with TB. Furthermore, a

combination of *BACH1* and *MAFK* polymorphisms, which are repressors in the antioxidant pathway response to ROS, may be useful as new biomarkers for identifying Japanese TB patients at high risk for developing ATDH.

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Tables

Table 1: The clinical characteristics of TB patients in this study.

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

Body mass index was calculated on following formula:

body weight (kg) / height X height (m)

Abbreviation: SD, standard deviation.

Table 2: Information regarding genotyping of tag SNPs.

Gene	tag SNP	Location	Sequence of forward primer (5' to 3')
NOS2A	rs10459953	exon 1	CACCTTCTCTCTGTAGGCAG
	rs3794764	intron 5	TTCCAGTCAGCACCAAAGCC
	rs12944039	intron 11	TGCACACGTCAGACAAGGAC
	rs11080344	intron 11	GGGCATCTGTCAGCTTTGTG
	rs2314810	intron 11	AGGCAGTGGAAGGACACAGT
	rs3729966	intron 12	GACTGGATTTGGCTGGTCCC
	rs944722	intron 20	CACGTCTCAGGTTCTCTCAC
	rs2255929	intron 23	GGCACTGAAGAGGACAGGAG
	rs3794756	intron 25	GGCATCAATGAAGGCAGTCC
NQO1	rs2917669	promoter	ACCCTAGGGGAACTCAGAGG
	rs689452	intron 3	GGCTACAGGAGATGGAATGC
	rs1800566	exon 6	TTCTCTAGTGTGCCTGAGGC
	rs10517	3'-UTR	ACCTGGCCCTTGCAATCTTC
HMOX1	rs2071746	promoter	GGATTCCAGCAGGTGACATT
	rs2071749	intron 3	TCACCTTCCCCAACATTGCC
	rs5755720	intron 4	AGCTATGAACCCACCACAGG

NFE2L2	rs2886161	intron 1	ACAATCCCAATGAAGACTGGG
	rs4243387	intron 1	TCAGACCTACACCTTGGCAG
	rs6726395	intron 1	AACCAACCCTCATGAGCTGG
	rs2001350	intron 1	CTGGATGTGGTTCCTATGCC
BACH1	rs2300301	intron 1	GATTATTGAGAAGGCAGCTGG
	rs1153285	intron 1	TTGACTTGGTATTACTGTGGG
	rs2070401	3'-UTR	TTGGCAGCGTCTTGAAAGCC
MAFF	rs2413508	promoter	ACCAGGGTGGTCAGGAAATG
	rs2267373	intron 1	GAAGGCAGGAGCTGTGATTC
	rs2235264	intron 2	TGGACCAATGTGGAGAGAGG
	rs4821765	intron 2	TGGACCAATGTGGAGAGAGG
MAFK	rs4720833	intron 1	CGCGGAGAATAGAAGTGGAA
	rs3808337	intron 1	CCTTCCAAAAGCAAGCTGTC
KEAP1	rs1048290	exon 4	GTTTCACCCCAGGATGGTAG
	rs11545829	exon 5	CCAAGGACGTAGATTCTCCC
XPO1	rs7606167	intron 4	AGATCTTCAGCAGAAGTGACC
	rs11125883	intron 21	GTTGTGTGAGAGCTAAACTG
	rs1050567	3'-UTR	ACAGCATGTGGGTATTTGTCG

Abbreviations: PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; 3'-UTR, 3'-untranslated region.

(Continued)

Sequence of reverse primer (5' to 3')	Annealing temperature (°C)	Cycle number	Analytical method	Restriction enzyme
CAACTCTCTGGATGGCATGG	60	30	PCR-RFLP	*Eco*52 I
CATATGCACGTGCTGACCAC	60	35	PCR-RFLP	*Bsr* I
ATAGGCATAAGCCACGGTGC	60	30	PCR-RFLP	*Mnl* I
CCTTGTCTGACGTGTGCATG	60	35	PCR-RFLP	*Fok* I
ACTCAGCATTTGCCTGGTGC	60	35	Sequencing	–
TGAGGTGCACACACACACAC	62	35	Sequencing	–
AACAGTCCCAGCCCATAAGG	60	35	PCR-RFLP	*Alw*26 I
AGGGATCTGGGGTTTAGGAG	64	35	Sequencing	–
ATCAGAGGGGCTCTTCTGTC	60	30	PCR-RFLP	*Hae* III
TGTCAACTGCAAGGGCAGTC	62	30	Sequencing	–
TCCTCCTACCTGTGATGTCC	62	30	Sequencing	–
TCAAAGAGGCTGCTTGGAGC	60	35	PCR-RFLP	*Hin*f I
AATGCACCACAAGAGGGCAG	62	35	PCR-RFLP	*Nco* I
CTGTCCCCTTGGGACTTGAT	62	40	Sequencing	–

AAACCTCTCTGGCTAGGCTG	60	35	PCR-RFLP	*Tsp*E I
ACCCCACCAAATAGCCAAGC	60	35	PCR-RFLP	*Taq* I
TGCTGTCAAGGGTAAGAGTTG	60	30	Sequencing	–
GCTGTGACAGTGCAGATGAG	60	30	PCR-RFLP	*Eco*91 I
AATTCAGACCTGCCCTGAGG	60	30	Sequencing	–
CTCCAGGGAAGGATTAAAGG	60	30	Sequencing	–
ATGACTTTCCTAAGGCACTGC	64	30	PCR-RFLP	*Vsp* I
GATTACAAATACATgTGGGGG	60	30	Sequencing	–
AGTAGCTGACACCCTGCTTC	62	30	PCR-RFLP	*Tsp*E I
TTGGCCACTCTCTCCTCATC	62	35	Sequencing	–
CTGGCTTCCTGTTTCTCTGG	62	35	Sequencing	–
TTCCAGGAAAGAAGGGGAAG	60	35	Sequencing	–
TTCCAGGAAAGAAGGGGAAG	60	35	Sequencing	–
CGAAGTCGAGATTGCAGTGA	60	30	Sequencing	–
TCCAACCCCAGTACTTCGAG	60	30	Sequencing	–
TTACAGGCATGAGCCATCGC	64	35	PCR-RFLP	*Alw*26 I
GTAACCACAGAAGCCCTGGA	60	30	Sequencing	–
GGCACCGTAATACTGGCTAC	60	30	PCR-RFLP	*Pag* I

GAGATTTTGGTGCACCTGTC	58	35	Sequencing	–
CGATTCAGTCCAAGAGGTGC	60	30	PCR-RFLP	*Taq* I

Table 3: Comparison of the clinical characteristics and baseline laboratory data between TB patients with and without ATDH.

Characteristics	ATDH		*P* value*
	Present	Absent	
Number	18	82	
Age, mean ± SD (years)	60.8 ± 17.7	64.7 ± 17.3	0.39
Gender (male/female)	9/9	47/35	0.61
[%]	[50.0/50.0]	[57.3/42.7]	
Body mass index (kg/m^2)	19.6 ± 2.3	20.5 ± 3.1	0.27
Lean (BMI < 18.5/18.5 < BMI)	6/10	18/55	0.35
[%]	[37.5/62.5]	[24.7/75.3]	
Alcoholism (+/–)	5/12	21/59	0.77
[%]	[29.4/70.6]	[26.3/73.7]	
Hepatic diseases (+/–)	2/16	12/68	1
[%]	[11.1/88.9]	[15.0/85.0]	
HBs (+/–)	0/14	1/74	1
[%]	[0/100]	[1.3/98.7]	
HCV (+/–)	0/14	4/61	1

[%]	[0/100]	[6.2/93.8]	
Number of anti-TB drugs	3.5 ± 0.6	3.6 ± 0.6	0.32
INH (mg/kg)	6.8 ± 1.4	6.3 ± 1.4	0.25
RFP (mg/kg)	9.1 ± 1.6	8.8 ± 1.8	0.54
PZA (+/−)	10/8	53/29	0.59
[%]	[55.6/44.4]	[64.6/35.4]	
Number of concomitant drugs	3.9 ± 2.0	5.0 ± 2.9	0.14
AST (IU/L)	29.1 ± 26.8	26.8 ± 23.3	0.72
ALT (IU/L)	18.0 ± 10.4	21.1 ± 16.6	0.45
T-bil (mg/dL)	0.48 ± 0.19	0.64 ± 0.43	0.11
Albumin (g/dL)	3.51 ± 0.68	3.64 ± 0.64	0.46
γ-GTP (IU/L)	32.4 ± 22.3	43.2 ± 58.4	0.45
ALP (IU/L)	289.1 ± 103.5	298.7 ± 104.8	0.73
LAP (IU/L)	58.3 ± 9.1	62.7 ± 18.6	0.70
LDH (IU/L)	194.9 ± 58.0	195.2 ± 62.1	0.98
S-creatinine (mg/dL)	0.64 ± 0.13	0.88 ± 1.10	0.35
Eosinophil (/μl)	105.1 ± 120.6	115.5 ± 121.8	0.74
Platelet ($\times 10^4$/μl)	31.8 ± 11.0	27.7 ± 9.3	0.11

Hemoglobin (g/dL)	11.7 ± 2.1	12.6 ± 1.7	0.054

*Characteristics were statistically compared by Mann-Whitney U test or chi-square test.

Abbreviations: ATDH, anti-tuberculosis drug-induced hepatotoxicity; SD, standard deviation; BMI, body mass index; HBs, hepatitis B surface; HCV, hepatitis C virus; INH, isoniazid; RFP, rifampicin; PZA, pyrazinamide; AST, aspartate aminotransferase; ALT, alanine aminotransferase; T-bil, total bilirubin; γ-GTP, γ-glutamyl transpeptidase; ALP, alkaline phosphatase; LAP, leucine aminopeptidase; LDH, lactate dehydrogenase; S-creatinine, serum creatinine; IU, international unit.

Table 4: Allele and genotype comparison in three genetic models between TB patients with and without ATDH.

Gene	tag SNP	Major > minor	Multiplicative model	
			OR (95% CI)	*P* value*
NOS2A	rs10459953	C > G	0.680 (0.328 – 1.412)	0.299
	rs3794764	G > A	0.844 (0.364 – 1.955)	0.692
	rs12944039	G > A	0.645 (0.296 – 1.406)	0.268
	rs11080344	C > T	0.424 (0.182 – 0.988)	0.043
	rs2314810	G > C	0.829 (0.336 – 2.044)	0.683
	rs3729966	C > T	0.964 (0.449 – 2.071)	0.926
	rs944722	T > C	1.033 (0.450 – 2.373)	0.939
	rs2255929	A > T	0.826 (0.379 – 1.799)	0.630
	rs3794756	C > T	1.000 (0.435 – 2.300)	1.000
NQO1	rs2917669	C > T	1.077 (0.500 – 2.319)	0.850
	rs689452	C > G	1.047 (0.487 – 2.253)	0.906
	rs1800566	C > T	1.103 (0.525 – 2.316)	0.796
	rs10517	C > T	1.195 (0.568 – 2.512)	0.639
HMOX1	rs2071746	T > A	0.674 (0.327 – 1.389)	0.283

	rs2071749	G > A	1.889 (0.896 – 3.983)	0.092
	rs5755720	A > G	1.610 (0.771 – 3.364)	0.203
NFE2L2	rs2886161	C > T	0.818 (0.387 – 1.729)	0.599
	rs4243387	T > C	0.774 (0.315 – 1.903)	0.576
	rs6726395	G > A	0.784 (0.353 – 1.742)	0.549
	rs2001350	A > G	0.947 (0.399 – 2.251)	0.903
BACH1	rs2300301	A > G	0.959 (0.462 – 1.993)	0.911
	rs1153285	G > A	1.455 (0.695 – 3.045)	0.318
	rs2070401	T > C	2.063 (0.933 – 4.560)	0.070
MAFF	rs2413508	C > G	1.635 (0.789 – 3.388)	0.183
	rs2267373	T > C	1.689 (0.817 – 3.490)	0.155
	rs2235264	A > G	1.959 (0.937 – 4.095)	0.071
	rs4821765	T > C	0.931 (0.355 – 2.441)	0.885
MAFK	rs4720833	G > A	0.669 (0.312 – 1.433)	0.299
	rs3808337	T > C	0.711 (0.333 – 1.519)	0.377
KEAP1	rs1048290	G > C	1.412 (0.684 – 2.917)	0.350
	rs11545829	C > T	1.305 (0.625 – 2.725)	0.477
XPO1	rs7606167	G > C	1.174 (0.534 – 2.533)	0.684

rs11125883	A > C	0.416 (0.184 – 0.939)	0.031
rs1050567	G > A	1.327 (0.622 – 2.830)	0.464

*Alleles and genotypes in three genetic models were compared by chi-square test or Fisher's exact test.

Abbreviations: ATDH, anti-tuberculosis drug-induced hepatotoxicity; OR, odds ratio; CI, confidence interval.

(Continued)

Dominant model		Recessive model	
OR (95% CI)	*P* value*	OR (95% CI)	*P* value*
0.541 (0.186 – 1.577)	0.256	0.733 (0.218 – 2.461)	0.773
1.129 (0.404 – 3.158)	0.817	2.353 (0.202 – 27.456)	0.452
1.447 (0.519 – 4.038)	0.479	5.000 (0.655 – 38.153)	0.147
0.348 (0.122 – 0.995)	0.044	0.312 (0.038 – 2.555)	0.455
0.855 (0.301 – 2.427)	0.768	0.870 (0.040 – 18.910)	1.000
0.800 (0.286 – 2.241)	0.671	1.583 (0.293 – 8.570)	0.632
0.882 (0.316 – 2.460)	0.810	1.549 (0.152 – 15.811)	0.554
0.708 (0.255 – 1.970)	0.508	1.014 (0.200 – 5.149)	1.000
0.579 (0.198 – 1.691)	0.314	5.267 (0.969 – 28.625)	0.070
1.250 (0.448 – 3.486)	0.669	0.807 (0.163 – 4.002)	1.000
1.191 (0.427 – 3.320)	0.739	0.807 (0.163 – 4.002)	1.000
1.347 (0.460 – 3.946)	0.586	0.807 (0.163 – 4.002)	1.000
1.565 (0.536 – 4.575)	0.411	0.807 (0.163 – 4.002)	1.000
2.593 (0.692 – 9.715)	0.147	1.167 (0.293 – 4.650)	0.731
2.60 (0.850 – 7.957)	0.087	2.438 (0.411 – 14.463)	0.294

0.645 (0.214 – 1.942)	0.553	0.363 (0.077 – 1.713)	0.231
1.006 (0.353 – 2.864)	0.992	0.478 (0.100 – 2.284)	0.512
0.742 (0.253 – 2.175)	0.586	0.745 (0.084 – 6.600)	1.000
0.550 (0.194 – 1.559)	0.256	1.440 (0.353 – 5.869)	0.699
0.781 (0.266 – 2.291)	0.652	1.583 (0.293 – 8.570)	0.632
0.541 (0.186 – 1.577)	0.256	2.318 (0.626 – 8.583)	0.244
1.417 (0.484 – 4.147)	0.524	2.533 (0.570 – 11.268)	0.203
1.645 (0.590 – 4.590)	0.339	16.200 (1.577 – 166.387)	0.018
1.417 (0.484 – 4.147)	0.524	2.769 (0.813 – 9.430)	0.138
2.035 (0.664 – 6.235)	0.208	1.718 (0.531 – 5.555)	0.348
2.069 (0.548 – 7.805)	0.276	2.842 (0.946 – 8.545)	0.067
0.703 (0.228 – 2.168)	0.538	14.140 (0.552 – 362.100)	0.180
3.162 (1.033 – 9.686)	0.037	0.237 (0.013 – 4.296)	0.344
2.100 (0.719 – 6.131)	0.169	0.630 (0.073 – 5.468)	1.000
1.364 (0.406 – 4.581)	0.773	1.778 (0.585 – 5.399)	0.363
1.170 (0.412 – 3.323)	0.768	1.844 (0.513 – 6.632)	0.464
1.050 (0.379 – 2.913)	0.925	1.622 (0.392 – 6.711)	0.449
0.312 (0.109 – 0.896)	0.026	0.444 (0.093 – 2.116)	0.515

0.907 (0.327 – 2.517)	0.851	3.619 (0.904 – 14.50)	0.078

Table 5: The gene interactions among genotypes for ATDH susceptibility.

Factor	Factor comparison*	
	OR (95% CI)	*P* value
C/C genotype at rs11080344 in *NOS2A*	3.601 (1.084 – 11.963)	0.036
C/C genotype at rs2070401 in *BACH1*	29.144 (1.656 – 513.038)	0.021
G/A or A/A genotype at rs4720833 in *MAFK*	5.724 (1.419 – 23.087)	0.014
A/A genotype at rs11125883 in *XPO1*	3.267 (0.993 – 10.745)	0.051

*The factors were statistically analyzed by multivariate logistic regression analysis.

Abbreviations: ATDH, anti-tuberculosis drug-induced hepatotoxicity; OR, odds ratio; CI, confidence interval.

Table 6: The combination effect of genotypes for ATDH susceptibility.

Factor	ATDH		Factor comparison*	
	Present (%)	Absent (%)	OR (95% CI)	*P* value
either the C/C genotype at rs2070401 in *BACH1* or the G/A or A/A genotype at rs4720833 in *MAFK*	16 (88.9)	37 (45.1)	9.730 (2.100 – 45.08)	0.0006
other genotypes	2 (11.1)	45 (54.9)		
Total number of patients	18	82		

*The factors were statistically analyzed by Fisher's exact test.

Abbreviations: ATDH, anti-tuberculosis drug-induced hepatotoxicity; OR, odds ratio; CI, confidence interval.

Figure Legend

Figure 1: Activator and repressor arms in the antioxidant pathway. Schematic representation indicates the location and translocation of relevant genes involved in activator arm (Nrf2/small Mafs/Xpo1) and repressor arm (Bach1/small Mafs/Keap1) in the antioxidant pathway as well as transcriptional regulation of antioxidant enzymes (NQO1/HO1) against oxidative stress in hepatocytes.

Nrf2: nuclear factor erythroid 2-related factor 2; Keap1: Kelch-like ECH-associated protein 1; Bach1: BTB and CNC homology 1; Xpo1: exportin 1; ARE: antioxidant-responsive element; GST: glutathione *S*-transferase; NQO1: NAD(P)H dehydrogenase quinone 1; HO1: heme oxygenase 1; ROS: reactive oxygen species

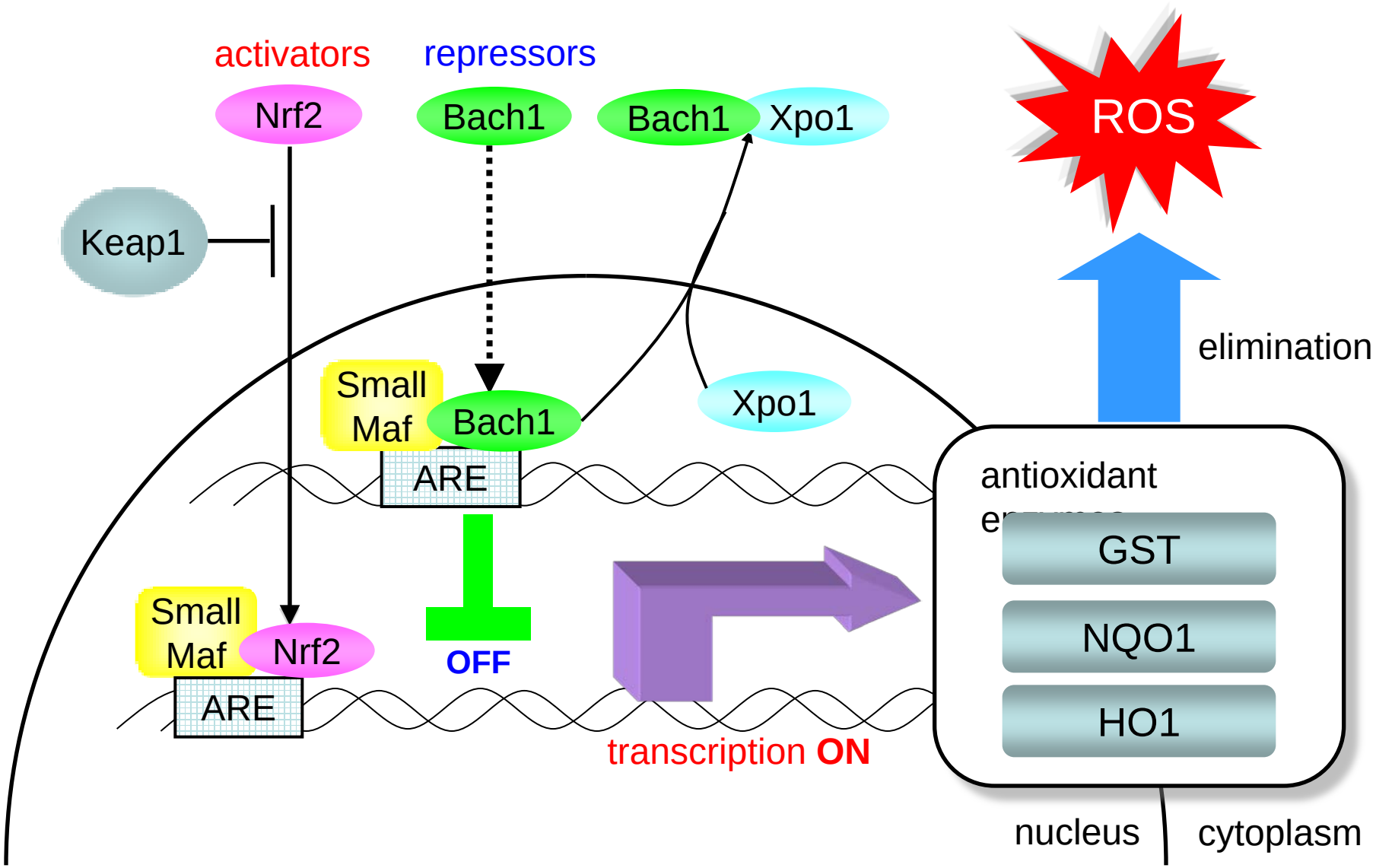

Figure 1: Activator and repressor arms in the antioxidant pathway