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Functional polymorphisms in *CYP2C19* & *CYP3A5* genes associated with decreased susceptibility for paediatric tuberculosis

Wei-Xing Feng, Fang Liu, Yi Gu, Wei-Wei Jiao, Lin Sun, Jing Xiao, Xi-Rong Wu, Qing Miao, Chen Shen, Dan Shen & Adong Shen*

Key Laboratory of Major Diseases in Children & National Key Discipline of Pediatrics (Capital Medical University), Ministry of Education, Beijing Pediatric Research Institute, Beijing Children's Hospital, Capital Medical University, affiliated to Capital Medical University, Beijing, PR China

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***Background & objectives*: Tuberculosis (TB) bacilli ingested by macrophages evade host immune responses by multiple mechanisms including the inhibition of apoptosis. As the cytochrome-P-450 system (CYP) contributes to apoptosis it has been suggested that genetic variation in CYP may be associated with susceptibility to TB infection. This study was carried out to evaluate cytochrome P-450 polymorphisms in Chinese Han children and to investigate the effect of these polymorphisms in paediatric TB.**

***Methods*: Frequencies for the *CYP2C19*, *CYP3A4*, *CYP3A5* and *CYP2E1* mutated alleles and genotypes were compared between 142 Chinese paediatric TB patients and 150 non-infected controls by real time PCR genotyping on peripheral leukocyte DNA.**

***Results*: *CYP2C19* (636 G>A, rs4986893) A allele and AG genotype were associated with decreased susceptibility to TB (*P* = 0.006, OR= 0.33, 95% CI: 0.15-0.76; and *P* = 0.005, OR =0.31, 95% CI: 0.14-0.72 respectively), as were the *CYP3A5* (6986A>G, rs776746) G allele and particularly homozygous GG (recessive mode) genotype (*P* = 0.004, OR=0.61, 95% CI: 0.43-0.85; and *P*=0.002, OR=0.47, 95% CI: 0.29-0.76).**

***Interpretation & conclusions*: The data suggested that *CYP2C19* and *CYP3A5* polymorphisms affect susceptibility to paediatric TB. Further studies are indicated to confirm and elucidate these observations.**

Key words China - *CYP2C19* - *CYP3A5* - pediatric tuberculosis - susceptible

The cytochrome P450 system (CYP) is a multi-gene family of enzymes responsible for multiple metabolic processes. Variations in CYP genotype and phenotype have been associated with various diseases[1]. Bikmaeva *et al*[2] first showed that insertion polymorphism Ins96 of the *CYP2E1* promoter region was associated with risk for pulmonary tuberculosis (TB) in the Bashkortostan population. Since activation of CYP expression leads to accumulation of reactive oxygen species in the cell, it is possible to assume that an increase in free

*Corresponding author

radicals causes oxidative stress, expedites destructive processes, triggers lymphocyte apoptosis, and shifts the immunoregulatory balance towards the Th2 response in pulmonary tuberculosis[1,3-5].

Apoptosis is a key part of innate TB immunity. After phagocytosis by a macrophage, the pathogen in the phagosome should be destroyed by the enzymes in the lysosome. TB bacilli may inhibit phagolysosome fusion and escape from phagosomes to multiply within the cytosol. The appropriate macrophage response to persisting intracellular pathogens is programmed cell death, or apoptosis, which should result in efficient reduction in bacilli, formation of apoptosomes containing the remains of cellular and bacillary structures, enabling phagocytosis by other macrophages resulting in minimal residual debris or inflammation, and immune stimulation by efficient antigen presentation. *Mycobacterium tuberculosis* may successfully inhibit apoptosis, so the delayed death of the macrophage is by necrosis, associated with the release of multiple viable bacilli into an extracellular environment of caseation and inflammation which assists dissemination of the bacilli[6].

The P450 system contributes to apoptosis induction by production of reactive oxygen species (ROS) by autoxidation *in vitro* and *in vivo*. ROS also activates NF-κB through activation of kinases[7]. On activation, NF-κB regulates the expression of almost 400 different genes, which include enzymes such as inducible nitric oxide synthase (iNOS), cytokines such as tumour necrosis factor-α (TNF-α), interleukin-10 (IL-10) and chemokines, and adhesion molecules[8,9]. Oxidative processes enhance the reaction of the adaptive response. It is likely that CYP is part of the normal immune response to TB[10-13].

NOS enzymes catalyze the formation of nitric oxide (NO) and the chemistry of these reactions is typical of cytochrome P450 reactions[14]. Microsomal P450s can catalyze the formation of NO and citrulline from N-hydroxyarginine[15]. Other studies have suggested that P450 enzymes participate in the physiological formation of NO during an inflammatory response[16,17]. NO is vital for macrophage function and granuloma formation in the immune response to *M. tuberculosis*, and kills the bacilli *in vitro*. NO is an essential component in the host defense against TB and other intracellular bacteria and an increase of this gaseous signaling molecule may protect against infectious disease[18].

The gene polymorphisms in Cytochrome P450 may contribute to human genetic susceptibility or resistance to TB disease. Therefore, this study was focused on cytochrome P450 family *CYP2C19* *2 (681 G>A, rs4244285),*3 (636 G>A, rs4986893); *CYP3A5* *3 (6986A>G, rs776746); *CYP3A4* *18A (878 T>C, rs28371759); *CYP2E1* *5B (-1053 C>T, rs2031920),*6 (7632 T>A, rs6413432) polymorphisms in Chinese Han children and the influence of the polymorphisms in TB was also investigated.

Material & Methods

Study sample: A total of 142 blood samples were obtained from paediatric patients with TB admitted at the Beijing Children's Hospital (affiliated with Capital Medical University; Beijing, China) between February 2005 and August 2008. Patients came from Beijing city and surrounding provinces in North China. Children admitted consecutively to Beijing Children's hospital with clinical tuberculosis and negative HIV tests were eligible to enter the study. All cases were classified according to the diagnostic standards of the American Thoracic Society (ATS)[19], the Pediatric tuberculosis clinical diagnosis standard in China[20], and WHO guidelines for disease severity classification for non-HIV related TB[21]. Patients were included in the study if they were positive by one or more of the following criteria: Tspot-TB test, acid-fast bacilli stain, culture, imaging (chest X-rays and CT scans for pulmonary patients and military involvement, CT scans for abdominal and skeletal and splenic involvement, MRI for meninges), or definitive clinical response. Primary immunodeficiency (PID) was ruled out by means of careful case history, clinical examination, and laboratory testing for serum immunoglobulin levels (IgG, IgM and IgA) and lymphocyte subpopulations (CD3+ T-cells, CD56+ natural killer (NK) cells, CD19+ B-cells and also CD4+ and CD8+ T-cells measured by flow cytometry). Children born with a height and weight bellow the 50th percentile were not included. None of the patients had a history of HIV infection, malnutrition, pre-conditions affecting immune function, receipt of immunosuppressive therapy or other lung disease. In addition, all cases were new TB cases: none had a history of previous TB or had received previous treatment for TB. All study individuals were of Han Chinese ethnicity. Written informed consent was obtained from all research participants, and the study was approved by the Ethics Committee of the Beijing Children's Hospital.

The control group consisted of 150 paediatric surgical patients admitted to Beijing Children's Hospital

between June 2005 and November 2007. They had no history of TB, HIV or any inflammatory, autoimmune or infectious disease, and had normal radiographic examination findings, and PPD skin test results <5 mm. All controls were reviewed with PPD skin testing 2 years after their initial visit, to ensure that they had no latent TB infection at the time of the study. Thirty randomly selected control individuals were tested by Tspot-TB (Oxford Immunotec, Abingdon, UK).

DNA analysis: Blood samples (2 ml) from TB children and controls were collected and stored at -20°C. Genomic DNA was extracted from peripheral blood leukocytes according to standard methods (Qiagen DNA blood mini kit). Primer and probe sequences are listed in Table I. The primers and probes were designed by the software Primer 5.0 and Oligo 6.0 (Molecular Biology Insights, USA). The amplification for *CYP2C19* *2,*3; *CYP3A5* *3; and *CYP3A4* *18A were carried out for a final volume of 20 µl with Taq AllgloTM probe (10 µM) (Chaoshi Bio Technologies, Shanghai) 1.2µl, Csq PCR Master Mix (2×) 10 µl, 1.2 µl of each primer (10 µM) and approximately 1 µl of the genomic DNA mold. The thermal condition of the reaction began with denaturation at 95°C for five min, followed by 40 cycles of denaturation at 95°C for 10 sec, annealing, and extension at 60°C for 45 sec (STRATAGENE-MX3005P). The amplification for *CYP2E1* *5B, *6 was carried out for a final volume of 20 µl with TaqMan probe (20 µM) (Applied Biosystems, America) 0.5 µl, TaqMan GT Master Mix (2×) 10 µl, 0.5 µl of each primer (20 µM) and 40ng the genomic DNA mold. The thermal condition of the reaction began with denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing, and extension at 60°C for 1 min. Following the amplification, the resulting product was submitted to the Melting Curve (MJ Option2). As a quality control of the genotyping, 10 per cent of the total sample genotyped was sequenced. The sequence results are shown in Fig.

Genetic and statistic analysis: Statistical analysis was carried out using the Statistical Package for Social Sciences version 13.0 (SPSS v13.0) (Chicago, USA). Differences between non-contiguous variables, genotype distribution and allele frequency were tested by chi-square analysis and Fisher's exact test as appropriate. Significant differences were indicated by a $P<0.05$. The statistical power was assessed according to disease prevalence, minor allele frequency, significant level and odds ratio[22].

Haplotype analysis: Hardy-Weinberg equilibrium (HWE) in the controls and pair-wise linkage disequilibrium (LD) between various gene polymorphisms were calculated using Haploview 4.2 software[23]. The colour coding of the LD was based on the confidence of the LD values with dark gray indicating strong evidence of LD, light gray being uninformative and white indicating low confidence of LD[24].

Table I. DNA Primers and probes sequences (5'-3')

SNP	Probe Sequence*	Probe	TM (°C)	Primer Sequence
*CYP2C19**2 681G>A	MAR-atttcccGggaaccca-MAR JUP-atttcccCggaaccca-JUP URA-atttcccAggaaccca-URA	Allglo	60	Forward-ATTATTGTTTTCTCTTAGATATGCAATA Reverse-AAAAGCAAGGTTTTTAAGTAATTTG
*CYP2C19**3 636G>A	MAR-agcacccccctgGatcc-MAR JUP-agcacccccctgAatcca-JUP	Allglo	60	Forward-AAAATTGAATGAAAACATCAGGATTG Reverse -GACTGTAAGTGGTTTCTCAGGAAGC
*CYP3A5**3 6986A>G	MAR-agggaagagataCtga-MAR JUP-cagggaagagataTtgaa-JUP	Allglo	60	Forward -ATGATGAAGGGTAATGTGGTCCA Reverse -AACGAATGCTCTACTGTCATTTCTAA
*CYP3A4**18A 878T>C	MAR-acgagctccGgatcgg-MAR JUP-acgagctccAgatcgga-JUP	Allglo	60	Forward- CAAAAATAAAGATAATTGATTGGGC Reverse- GCCCTACATTGATCTGATTTACCT
*CYP2E1**5B -1053C>G	VIC-aggttgcaattttGtacttt-NFQ FAM-aggttgcaattttAtacttt-NFQ	TaqMan	60	Forward-TGACTTTTATTTTCTTCATTTCTCATCATAT TTTCTATTATACAT Reverse-GTTTTTCATTCTGTCTTCTAACTGGCAATAT
*CYP2E1**6 7632 T>A	VIC-cagctgattaaaaattAaaaa-NFQ FAM-cagctgattaaaaattTaaaa-NFQ	TaqMan	60	Forward- GTGCACACCACCACACC Reverse- CACTGTGCCCAGCCAAAATAATT

*The capital letters are the SNPs; TM, melting temperature

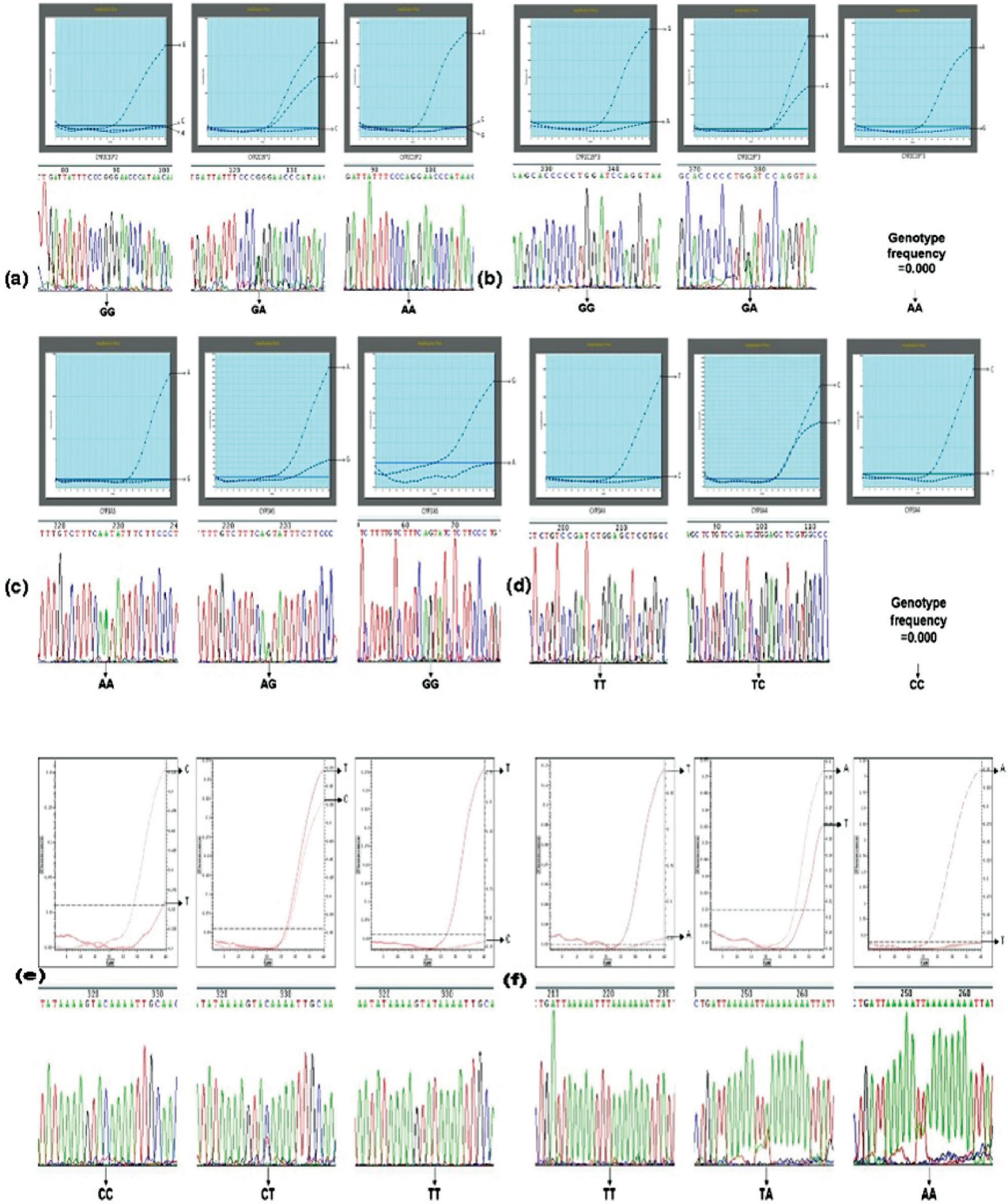

Fig. 1. Realtime-PCR and DNA sequencing of CYP450 polymorphisms tested. **(a)** *CYP2C19* *2, **(b)** *CYP2C19* *3, **(c)** *CYP3A5* *3, **(d)** *CYP3A4* *18A, **(e)** *CYP2E1** 5B, **(f)** *CYP2E1** 6.

Results

The patient group (n=142) included 45 (31.7%) cases of active pulmonary TB (PTB), 97 (68.3%) cases of extra-pulmonary TB (EPTB) (Table II). The cases of PTB had involvement of lung parenchyma exclusively (pulmonary; pulmonary with hilar lymph nodes or pulmonary with pleural effusions). The WHO severity classification for non-HIV-related EPTB was adopted, with most severe and less severe defined as follows: most severe EPTB1 (n= 92; 64.8%) had involvement of two or more sites with meningeal, spinal, intestinal, splenic or miliary pericardial sites as the primary focus with or without lung involvement. Less severe EPTB2 (n= 5; 3.5%) was defined as TB in extra-pulmonary peripheral sites restricted to pleural effusions without involvement of lung parenchyma.

Table II. Diagnostic modality used for confirmation of TB

Investigations or diagnostic procedures	Type of tuberculosis (Positive out of number of patients on procedure)*	
	PTB (n=45)	EPTB (n=97)
PPD	42/45	76/97
Tspot-TB test	2/2	9/9
Acid-fast bacilli stain	3/45	5/97
Culture	1/45	6/97
Imaging	45/45	78/97

PTB, pulmonary tuberculosis; EPTB, extra-pulmonary TB

The control group included 150 paediatric surgical patients without TB infection. The mean age was 5.1 ± 4.6 yr (range 2 months-15 yr) in the TB patients group and 4.9 ± 4.1 yr (range 3 months-16 yr) in the control group. The proportion of male patients was 61.3 per cent in the patient group and 60.7 per cent in the control group. The control cases were matched with the TB cases by age, sex and ethnicity.

Distribution of cytochrome P450 gene polymorphisms in the study groups: Allelic and genotypic frequencies of each SNP were compared between the case patients

Table III. Alleles and genotypes frequencies of cytochorome P450 gene polymorphism in TB patients and controls

Polymorphism	Alleles	Cases (%)	Control (%)	Genotypes	Cases (%)	Control (%)
*CYP2C19**2 681G>A	G	186 (65.5)	197 (65.7)	G G	61 (43.0)	64 (42.7)
	A	98 (34.5)	103 (34.3)	A G	64 (45.0)	69 (46.0)
	OR 95% CI	1.01 (0.72-1.42)		A A	17 (12.0)	17 (11.3)
	P value	0.964		*P* value	0.980	
***CYP2C19**3 636G>A**	G	276 (97.2)	276 (92.0)	G G	134 (94.4)	126 (84.0)
	A	8 (2.8)	24 (8.0)	A G	8 (5.6)	24 (16.0)
	OR 95% CI	0.33 (0.15-0.76)		OR 95% CI	0.31(0.14-0.72)	
	P value	**0.006**		*P* value	**0.005**	
***CYP3A5**3 6986A>G**	A	122 (43.0)	94 (31.3)	A A	27 (19.0)	17 (11.3)
	G	162 (57.0)	206 (68.7)	A G	78 (47.9)	60 (40.0)
	OR 95% CI	0.61 (0.43-0.85)		G G	47 (33.1)	73 (48.7)
	P value	**0.004**		*P* value	**0.017**	
*CYP3A4**18A 878T>C	T	281 (97.9)	297 (98.0)	T T	139 (97.9)	147 (98.0)
	C	3 (2.1)	3 (2.0)	C T	3 (2.1)	3 (2.0)
	OR 95% CI	1.06 (0.21-5.33)		OR 95% CI	1.06 (0.21-5.32)	
	P value	0.946		*P* value	0.946	
*CYP2E1**5B -1053 C>T	C	225 (79.2)	234 (78.0)	C C	88 (62.0)	90 (60.0)
	T	59 (20.8)	66 (22.0)	C T	49 (34.5)	54 (36.0)
	OR 95% CI	1.08 (0.72-1.60)		T T	5 (3.5)	6 (4.0)
	P value	0.718		*P* value	0.933	
*CYP2E1**6 7632 T>A	T	216 (76.1)	232 (77.3)	T T	81 (57.0)	89 (59.3)
	A	68 (23.9)	68 (22.7)	A T	54 (38.0)	54 (36.0)
	OR 95% CI	1.07 (0.73-1.58)		A A	7 (5.0)	7 (4.7)
	P value	0.715		*P* value	0.924	

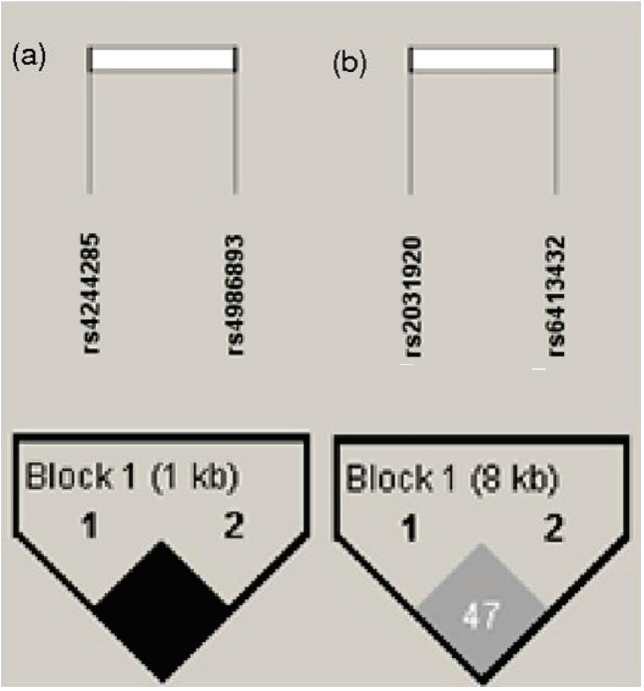

Fig. 2. LD patterns of *CYP2C19* and *CYP2E1*. (a) D' based on *CYP2C19**3 (rs4986893)with*2(rs4244285) among 142 individuals in cases; (b) D' based on *CYP2E1**-1053(rs2031920) with *7632 (rs6413432) among 142 individuals in cases.

and control subjects (Table III). All the polymorphisms were in Hardy-Weinberg equilibrium in the control group. *CYP2C19* (636 G>A) A allele and AG genotype (*P*=0.006, OR=0.33, 95% CI: 0.15-0.76; and *P*=0.005, OR=0.31, 95% CI: 0.14-0.72, respectively) was associated with decreased tuberculosis risk. *CYP3A5* (6986A>G, rs776746) G allele and particularly homozygous GG genotype were associated with decreased susceptibility (*P*=0.004, OR=0.61, 95% CI: 0.43-0.85; and *P*=0.002, OR=0.47, 95% CI: 0.29-0.76), indicating a recessive mode effect. No significant differences were observed in *CYP2C19* *2 (681G>A); *CYP2E1* *5B (-1053 C>T),*6 (7632 T>A); *CYP3A4* *18A(878 T>C).

LD pattern among P450 gene polymorphisms and frequencies of haplotypes: The analysis of pair-wise linkage disequilibrium revealed LD patterns in cases between the variants *CYP2C19* 636 G>A (rs4986893) with 681 G>A(rs4244285) (D'=1) and *CYP2E1* -1053 C>T (rs2031920) with 7632 T>A (rs6413432) (D'=0.47) (Fig. 2). The haplotype comprising *CYP2C19* 681G /636A showed a significantly decreased tuberculosis risk (OR 0.33, 95% CI: 0.15-0.76; *P*=0.006) (Table IV).

Discussion

In this study *CYP2C19**3 showed a decreased risk of tuberculosis. The wild type (*CYP2C19**1) was defined by exclusion of the mutant alleles *CYP2C19**2 (rs4244285) and *3 (rs4986893); *2 shows a single-base mutation (G>A) in exon 5 which produces an aberrant splice site. *CYP2C19* *3 consists of a premature stop codon (G>A) in exon 4 and is reported in Oriental populations including both Japanese and Chinese but is rare in Caucasians[25,26]. These alleles result in the loss of enzyme activity[27]. Significant protective association was observed for *3 and heterozygous *CYP2C19* AG (*1/*3) genotype. This may be due to the reduction of intracellular *M. tuberculosis* by successful apoptosis influenced by ROS generated by this CYP genotype. Klose *et al*[28] reported that *CYP2C19* was expressed in liver and duodenum. *CYP2C19* has been associated with the activation of lung cancer[29]. CYP2C19 expression may affect lung disease such as PTB. The two SNP haplotype (GA based on rs4244285 and rs4986893) were also associated with protection from tuberculosis.

An intronic single nucleotide polymorphism (SNP) in the *CYP3A5* gene (*CYP3A5**3; SNP rs776746) which affects RNA splicing and enzymatic activity, has been identified as protective for TB[30]. The CYP3A enzyme subfamily, especially CYP3A4 and CYP3A5 are among the most versatile biotransformation systems involved in the elimination from the body of a wide variety of endogenous and exogenous compounds. CYP3A5 protein is present in the liver and some extrahepatic tissues, such as the gut wall, kidney, adrenal gland, prostate

Table IV. Haplotypes distribution of the single nucleotide polymorphisms *CYP2C19**2 and *CYP2C19**3 in tuberculosis patients and healthy control subjects

Haplotypes[a]	Patients (%)	Control (%)	χ^{2} [b]	P value	OR (95%CI)
A G	98 (34.5)	103 (34.2)	0.002	0.965	1.23 (0.89-1.71)
G A	8 (2.8)	24 (7.8)	7.567	**0.006**	0.33 (0.15-0.76)
G G	178 (62.7)	173 (58.0)	1.527	0.217	1.01 (0.72-1.42)

[a]Haplotypes were estimated with the expectation maximization algorithm; [b]Genotype χ^{2} value for comparison of case and control haplotype frequencies

and many cell types in the lung. In the lung, the highest amounts of CYP3A5 protein are present in bronchial and alveolar epithelial cells, bronchial glands and alveolar macrophages[31]. Since the CYP3A4/5 enzymes mediate the metabolism of many exogenous and endogenous compounds with direct relevance to pulmonary physiology and pathology, the functions of these enzymes and factors controlling these should be elucidated.

In the cytochrome P450 family, the *CYP2E1* Ins 96(-/-) polymorphism which induces reduced metabolic activity, was associated with decreased susceptibility to pulmonary tuberculosis in the Bashkortostan population[2,32]. Presumably, a drop in cytochrome P450 activity in experimental inflammation or infection is a protective response to oxidative stress[4]. T helper type 1(Th1) cells produce IFN- γ to activate infected macrophages and promote the formation of granulomas around infected macrophages which participate in the protective responses against *M. tuberculosis* growth. On the contrary, Th2 cells interfere with these protective immune responses[33].

In the control group the minor allele frequency (MAF) of *CYP2C19* *2 (681 G>A, rs4244285, 0.34); *CYP3A5* *3 (6986A>G, rs776746, 0.31); *CYP3A4* *18A (878 T>C, rs28371759 0.002); *CYP2E1* *5B (-1053 C>T, rs2031920, 0.22) polymorphisms was analysed in Chinese Han children. The data were close to the MAF of *CYP2C19* *2 (681 G>A, rs4244285 0.26); *CYP3A5* *3 (6986A>G, rs776746, 0.29); *CYP3A4* *18A (878 T>C, rs28371759 0.007); *CYP2E1* *5B (-1053 C>T, rs2031920, 0.25) in Han Chinese Beijing(CHB) of HapMap database (*http://hapmap.ncbi.nlm.nih.gov/*). The data of *CYP2C19**3 (636 G>A, rs4986893 and *CYP2E1**6 (7632 T>A, rs6413432) were absent in the HapMap database.

Our study had several limitations. We did not have detailed information for comparison between patients and controls, such as children's family history, habitat, family income, parents' educational level, or family size. However, although associated illnesses, medical treatments and environmental factors may be associated with TB, these conditions account for only a small proportion of TB cases, highlighting the importance of host genetic factors as determinants of the inter-individual and inter-population differences in susceptibility to disease. Further, there was limited sample in this study. Considering the difficulties in recruiting children and the relatively smaller size of the total paediatric TB population (compared to adult TB), the design of our study may be considered appropriate. In practice, case-control studies are much more susceptible to various forms of bias. It will not detect rare events and directly measure the risk and there were inconsistent results among studies[34].

In conclusion, our results lend support to the hypothesis that the gene polymorphisms in cytochrome P450 contribute to human genetic susceptibility or resistance to TB disease. Variants of *CYP2C19**3 and *CYP3A5* *3 contribute to protection from a paediatric TB in China. These findings need to be confirmed in a variety of ethnic populations and larger studies are needed to elucidate the true role of the cytochrome P450 molecule in the pathogenesis of TB. Further research into cytochrome P450 may provide more insights that will aid in the development of immunotherapy and immunoprophylaxis for TB disease.

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Reprint requests: Dr Adong Shen, Beijing Pediatric Research Institute, Beijing Children's Hospital affiliated to Capital Medical University, Beijing, 56 Nanlishi Road, Xicheng District, Beijing 100045, PR China
e-mail: shenad16@hotmail.com