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*Original Article*

# Association of two toll-like receptor 4 single nucleotide polymorphisms with biliary atresia in Chinese patients

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**Abstract:** Biliary atresia (BA) is a disease of the liver characterized by progressive fibro-inflammatory obliteration of the biliary tree in neonates. Toll-like receptor 4 (TLR4) is expressed in human biliary epithelial cells and mediates innate and adaptive immune responses. To evaluate the potential association between *TLR4* gene polymorphisms and BA in the Chinese population, a case-control study was conducted with 113 patients with BA and 133 healthy controls. The rs10759930 and rs2149356 SNPs in the *TLR4* gene were selected for genotyping by the Sequenom MassARRAY platform (Sequenom; San Diego, CA, USA). There was no significant differences between BA and controls in allele distribution (rs10759930, $P = 0.369$, OR = 1.180, 95% CI = 0.822-1.694; rs2149356, $P = 0.416$, OR = 0.861, 95% CI = 0.599-1.236), and similar results were found in genotype and haplotype frequencies of these *TLR4* gene polymorphisms. Our results indicated that, for the first time, the *TLR4* gene polymorphisms analyzed do not appear to play a major role in the development of BA in Chinese children.

**Keywords:** Biliary atresia, polymorphism, toll-like receptor 4

## Introduction

Biliary atresia (BA) is a progressive fibro-inflammatory obliteration of the extrahepatic biliary tree that leads to cirrhosis and liver failure in neonates [1, 2]. Without treatment, such as the Kasai hepatoportoenterostomy to establish bile drainage, the condition is fatal before patients reach 2 years age [3]. BA incidence is higher in Asia than in western countries, affecting 1.7, 1.04, 0.58 and 0.52 per 10,000 live births in Taiwan, Japan, the United Kingdom and Canada, respectively [4].

Numerous factors have been proposed to be responsible for the etiology of BA, including genetic factors, abnormal morphogenesis, environmental toxins, viral infections, and immune-mediated bile duct injury [5]. Of these, immune dysregulation is considered to be a central part of BA pathogenesis, which in response to viral infection or other foreign antigens leads to biliary obstruction and hepatic fibrosis [6, 7]. Moreover, gene-expression analyses on BA bile duct or liver tissues indicated that genetic factors may play an important role in the pathogenesis of BA [8, 9]. Multiple studies of single nucleotide polymorphisms (SNPs) have identified a number of BA-susceptible genes, including intercellular adhesion molecule-1 (*ICAM-1*), adiponectin (*APM1*) and integrin, beta 2 (*CD18*) [10-12]. In particular, recent multinational studies revealed a significant association between common genetic variants in the adducing 3 (*ADD3*) gene and susceptibility to developing BA [13-16].

Toll-like receptors (TLRs) are a family of pattern-recognition receptors that play a pivotal role in innate and adaptive immunity. Activation of TLRs can trigger inflammatory and antimicrobial responses by recognizing pathogen-associated molecular patterns derived mainly from bacteria, viruses and other microorganisms [17, 18]. Among the TLRs family members, TLR4 recognizes microbial lipopolysaccharides to activate intracellular signaling pathways, and then induce production of pro-inflammatory cytokines [19]. TLR4 has been reported to be expressed in human biliary epithelial cells,

**Table 1.** Oligonucleotide sequences used for genotyping

| SNP | Primers | Sequences |
|---|---|---|
| rs10759930 | First | 5'-ACGTTGGATGTGGAGCCAAGAGAATACCCT-3' |
| | Second | 5'-ACGTTGGATGCATGGACCAATGCTCTTGTG-3' |
| | Extension | 5'-GAGAATACCCTTTATGCCTTTG-3' |
| rs2149356 | First | 5'-ACGTTGGATGTCTAGCTGTCATGTAAGCAC-3' |
| | Second | 5'-ACGTTGGATGGGTAGCCAAGATAAATGACTG-3' |
| | Extension | 5'-CCTAGTATCTGTGACACTTATGTGTAAT-3' |

**Table 2.** Allele frequencies of the two SNPs in the *TL*R4 gene of patients with BA and control group

| | Allele | Case, n (%) | Control, n (%) | *P* value | OR (95% CI) |
|---|---|---|---|---|---|
| rs10759930 | T | 139 (61.5) | 153 (57.5) | 0.369 | 1.180 (0.822-1.694) |
| | C | 87 (38.5) | 113 (42.5) | | |
| rs2149356 | T | 87 (38.5) | 112 (42.1) | 0.416 | 0.861 (0.599-1.236) |
| | G | 139 (61.5) | 154 (57.9) | | |

OR, odds ratio; CI, confidence interval.

mediating innate immune system functions [20, 21]. Many SNPs in *TLR4* genes have been associated with genetic susceptibility to various infectious and inflammatory diseases [22-24]. However, this is the first study to investigate the potential association between *TLR4* SNPs and BA.

## Subjects and methods

### *Study subjects*

One hundred and thirteen unrelated Chinese children (70 boys, 43 girls) were diagnosed with BA by exploratory laparotomy with operative cholangiography at the Children's Hospital of Fudan University (Shanghai, China). All patients underwent a Roux-en-Y hepatic portoenterostomy (Kasai operation) successfully between August 2014 and July 2015. The mean age of these patients was 68.1 ± 20.7 days (mean ± standard deviation) (range 23-163) at the time of the operation.

A control group was formed from 133 unrelated healthy Chinese children (85 boys, 48 girls) recruited randomly from the Department of Pediatrics. None had a history of BA or liver disease. The study was approved by the ethics committee of the Children's Hospital of Fudan University. Blood samples from the children were collected after written informed consent had been obtained from their parents or legal guardians.

### *Genotyping*

Genomic DNA was extracted from whole blood using the TIANamp Blood DNA Kit (Tiangen, Beijing, China). The two observed SNPs (rs10759930 and rs2149356) were located in the 5'-untranslated region and intron of the *TLR4* gene, respectively. SNPs were selected from among previous reports [25], with minor allele frequencies >5% according to the National Center for Biotechnology Information SNP Database (dbSNP) (http://www.ncbi.nlm.nih.bov/SNP/). Primers for PCR and single-base extension were designed using the Assay Designers software, version 3.0 (Sequenom, San Diego, CA, USA), and synthesized by Benegene Biotech (Shanghai, China; **Table 1**). Genotyping was performed by MassARRAY on a matrix-assisted laser desorption ionization-time of flight mass spectrometry platform and analyzed using the MassARRAY Typer software, version 3.4 (Sequenom).

### *Statistical analysis*

Hardy-Weinberg equilibrium testing was performed for each SNP for the case and control groups. Differences in allele and genotype frequencies between the BA and control subjects were evaluated using the $\chi^2$ test. A $p$ value of $< 0.05$ was considered statistically significant. The odds ratio (OR) and 95% confidence intervals (CI) were calculated. Statistical analysis was performed using the SPSS 18.0 program (SPSS Inc., Chicago, IL, USA). The haplotype frequencies of *TLR4* were estimated using the Haploview 4.2 program (http://www.broad.mit.edu/mpg/haploview/).

## Results

A total of 246 subjects (113 patients with BA, 133 controls) were successfully genotyped for two polymorphisms in the *TLR4* gene. All SNPs for patients and controls were found to follow Hardy-Weinberg equilibrium, and the minor allele frequencies of the two SNPs were > 5%.

**Table 3.** Genotype frequencies of the two SNPs in the *TLR4* gene of patients with BA and control group

| | Genotype | Case, n (%) | Control, n (%) | *P* value | OR (95% CI) | *P* value for HWE |
|---|---|---|---|---|---|---|
| rs10759930 | CC | 19 (16.8) | 23 (17.3) | | | 0.721 |
| | TT | 45 (39.8) | 43 (32.3) | | | |
| | CT | 49 (43.4) | 67 (50.4) | 0.448 | | |
| | TT+CT | 94 (83.2) | 110 (82.7) | 0.920 | 0.967 (0.496-1.884) | |
| rs2149356 | TT | 19 (16.8) | 23 (17.3) | | | 0.642 |
| | GG | 45 (39.8) | 44 (33.1) | | | |
| | GT | 49 (43.4) | 66 (49.6) | 0.525 | | |
| | GG+GT | 94 (83.2) | 110 (82.7) | 0.920 | 0.967 (0.496-1.884) | |

OR, odds ratio; CI, confidence interval; HWE, Hardy-Weinberg equilibrium.

**Table 4.** *TLR4* haplotypes in patients with BA and control group

| Haplotype | Case, n (%) | Control, n (%) | *P* value |
|---|---|---|---|
| TG | 139 (61.5) | 153 (57.5) | 0.369 |
| CT | 87 (38.5) | 113 (42.5) | 0.416 |

Allele and genotype frequencies of the two SNPs are listed in **Tables 2** and **3**. Statistical analysis revealed no significant differences between patients with BA and controls (rs10759930, $P = 0.369$, OR = 1.180, 95% CI = 0.822-1.694; rs2149356, $P = 0.416$, OR = 0.861, 95% CI = 0.599-1.236). Possible haplotypes were also constructed for rs10759930 and rs2149356 (**Table 4**), and no significant differences were found in the distribution of haplotypes between BA and controls.

## Discussion

In this study, we investigated whether two *TLR4* gene polymorphisms affect the development of BA in Chinese patients. We demonstrated no association for the SNPs analyzed.

The precise etiology and pathogenesis of BA, a multifactorial disease, remains to be elucidated. Furthermore, with the ongoing identification of multiple BA-susceptible genes, it has become clear that BA is not a simple inherited disorder sparking interest in potential correlations between genetic variations and BA. A genome-wide association study of 324 Chinese patients revealed a strong association between BA and the SNP rs17095355 on chromosome 10q24, located between the *ADD3* and *XPNPEP1* (X-prolyl aminopeptidase 1) genes [15]. This association was subsequently found to be replicated in Caucasian and Thai populations [14, 16]. Interestingly, most BA-susceptible genes identified to date, including *ICAM-1*, *CD18* and *ADD3* [11-13], play a role in inflammatory and immune responses.

The *TLR4* gene, which is expressed in the biliary epithelial cells, is localized on chromosome 9q33.1 and participates in the induction of inflammatory responses against microorganisms, leading to the transcription of various genes including cytokines, such as tumor necrosis factor-α, interferon-γ and interleukins (IL-6, IL-8 and IL-12) [20]. These pro-inflammatory cytokines, which perpetuate liver injury and amplify the inflammatory cascade, are involved in the progression of BA [26]. Furthermore, *TLR4* gene polymorphisms have been reported to be associated with genetic susceptibility to several immune diseases [22-24], and were therefore proposed to potentially play a role in BA. However, our findings showed otherwise.

These negative results may be due to the relatively small sample size of the present study. Ideally, a secondary investigation with a larger sample size, and thus greater statistical power, should be performed. Our findings may also be due to the limited selection of SNPs investigated, which did not widely cover the gene.

In conclusion, our findings indicate, for the first time, the lack of association between the *TLR4* gene polymorphisms (SNPs rs10759930 and rs2149356) and BA in Chinese children. Future studies using a larger dataset, and incorporating different ethnicities, are necessary to investigate potential associations between BA and a wider range of *TLR4* genetic polymorphisms.

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## Disclosure of conflict of interest

None.

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