Epistatic Association of CD14 and NOTCH2 Genetic Polymorphisms with Biliary Atresia in a Southern Chinese Population

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Biliary atresia (BA) is the most common cause of endstage liver disease in infants with poor prognosis and high mortality. The etiology of BA is still unknown, but recent genetic factors have been considered as an important player in BA. We investigated the association of two cis-regulated variants in CD14 (rs2569190) and NOTCH2 (rs835576) with BA susceptibility, using the largest case-control cohort, totaling 506 BA patients and 1,473 healthy controls in a Southern Chinese population. Significant epistatic interaction between the two variants in our samples was observed (p = 8.1E−03; OR = 2.78; 95% CI: 1.32–5.88). The expression of CD14 and NOTCH2 in the BA group was consistently lower than that in the control (CC) group (0.31 ± 0.02 versus 1.00 ± 0.14; p < 0.001), which might be related to the genetic susceptibility of the genes awaiting further validation.

INTRODUCTION
Biliary atresia (BA) is the most common cause of endstage liver disease in infants with poor prognosis and high mortality.1,2 The incidence of BA in Europe and the United States is about 1:15,000–19,000 live births, whereas the Asian incidence is much higher, ranging from 1:5,000 to 1:8,000 live births in China.3–6 The etiology of BA is still unknown, but recent genetic factors have been considered to have an important role in BA.4,7 Recurrent familial cases supported the higher genetic tendency of BA.8 Researchers have identified a number of genes associated with BA, such as the migration inhibitory factor (MIF),9 CD14,10 intercellular adhesion molecule-1 (ICAM-1),11 CFC1,12 and ITGB2 (CD18).13

CD14 is a glycosylphosphatidylinositol-anchored LPS receptor that was first reported as a differentiation marker expressed on the surface of macrophage, neutrophils, and other myeloid-lineage cells.14–17 The T/T homozygote at position 159 (rs2569190) for the CD14 promoter polymorphism is related to the development of BA using 90 cases and 143 controls in Taiwan’s population.18 Recent studies have found that hepatocytes can produce large amounts of CD14 in acute liver injury.19 However, the role of CD14 on the hepatocytes remains unclear in BA.

The Notch signaling pathway plays key roles in the development of the biliary system. NOTCH2, as a receptor on this signaling pathway, has been demonstrated that hepatocytes dedifferentiate into hepatoblasts and further differentiate into biliary epithelial cells (BECs) when the bile duct is injured, which is mainly regulated by the Notch signaling pathway.19 NOTCH2 can keep the normal function of the intrahepatic bile duct (IHB) in the perinatal and postnatal periods, and the low expression of this receptor leads to the abnormality of the IHB.20 NOTCH2 mutations cause Alagille syndrome, which involves biliary developmental defects that are characterized by neonatal jaundice, impaired differentiation of the IHB, and chronic cholestasis.21 CD14 and the Notch signaling receptor NOTCH2 can co-express in hepatocytes of BA, but the interaction between these two genes and their respective impact on the bile duct differentiation process are still unknown, and the association between CD14 and NOTCH2 in BA has not been reported yet.

To explore the association of CD14 (rs2569190) and NOTCH2 (rs835576) in BA, we conducted a case-control study to verify the effects and their interaction in an independent Chinese sample.

RESULTS
Association of CD14 and NOTCH2 with BA in a Southern Chinese Population
CD14 (rs2569190) and NOTCH2 (rs835576) were selected in 506 BA cases and 1,473 controls in a Southern Chinese population. (See Materials and Methods for the detailed selection criteria.) The results

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showed that limited association of both SNPs was observed with BA (Table 1). The SNP rs2569190 in CD14 showed that the frequency of the minor allele (G) was virtually the same between the control group (41.75%) and the BA patients (41.99%), giving an odds ratio (OR) of 1.01 (95% confidence interval [CI]: 0.87–1.17, p = 0.89). A regulatory SNP, rs835576, which was located in the 3’ UTR of NOTCH2, showed a slightly different frequency between the healthy controls (C: 2.4%) and the BA patients (C: 3.1%), with an OR of 1.30 (95% CI: 0.84–2.01, p = 0.23), though it failed to be statistically significant.

### The Genetic Epistasis between CD14 and NOTCH2 Was Associated with the Risk of BA Development

The results suggested a strong effect from the epistatic interaction between the two variants in our samples (p = 8.1E–03; OR = 2.78; 95% CI: 1.32–5.88) (Table 2). Pairwise multifactor dimensionality reduction (MDR) analysis was adopted to cross-validate the epistatic interaction between these two variants. The results of cross-validation consistency (CVC) and balanced accuracy were obtained from MDR analysis of the two-locus model (Table 3). The combinations of low- and high-risk groups were classified in the model (Figure 1). The dark-shaded cells indicate the high risk of BA with genotype combination (GG:TT). In agreement with the results obtained by logistic regression from PLINK, a significant synergistic epistatic interaction (p = 0.0026) was observed, giving an OR of 1.44 (95% CI: 1.14–1.84) for this model.

### The cis Effect of eQTL of Two Interacting SNP Pairs with CD14 and NOTCH2

The expression quantitative trait loci (eQTL) associations between SNPs and the potential regulated genes were closely examined. We assessed these two candidate SNPs in the Genotype-Tissue Expression (GTEx) project containing genome-wide-based, tissue-specific, regulatory eQTL variants (http://gtexportal.org). The genotypes of SNP rs2569190 and SNP rs835576 correlated with the expression of CD14 in fibroblast and of NOTCH2 in liver, respectively (Table 4). The results also further demonstrated that G, the minor allele of SNP rs2569190, is correlated with lower expression of CD14 in fibroblast. C, the minor allele of SNP rs835576, is correlated with lower expression of NOTCH2 in liver. In order to further confirm the expression level of these two genes in BA patients, real-time PCR results showed that the expression level of CD14 in the BA group (n = 28) was significantly lower (0.31 ± 0.02 versus 1.00 ± 0.14, p < 0.001) than that in the choledochal cyst (CC) group (n = 20); and the expression of NOTCH2 in BA was also decreased (0.59 ± 0.07 versus 1.00 ± 0.19, p = 0.0262) (Figure 2). This result had been further verified by western blot, which showed that the gene-related protein expression of CD14 was significantly decreased (209.9 ± 12.66 versus 546.3 ± 112.3, p = 0.0408) and that the expression level of NOTCH2 also obviously declined (101.1 ± 58.55 versus 425.1 ± 72.89, p = 0.0257) in BA (Figure 3).

### DISCUSSION

In the present study, we investigated the association of CD14 (rs2569190) and NOTCH2 (rs835576) with BA. We found that these two SNPs had a genetic interaction to increase the risk to BA, and both SNPs showed cis-regulatory roles in gene expression. This result suggested that CD14 (rs2569190) could interact with NOTCH2 (rs835576) and, together, downregulate the expression of the NOTCH2. The expression of CD14 and NOTCH2 was significantly decreased in BA patients, which could be related to the genetic susceptibility locus of these two genes.

### Table 1. The Association of Two SNPs Located in CD14 and NOTCH2 with Biliary Atresia

| SNP     | Gene     | Function | CHR | BP     | A1/A2 | F_A | F_U | p     | OR    | CI, 0.95 | p_adj |
|---------|----------|----------|-----|--------|-------|-----|-----|-------|-------|---------|-------|
| rs835576| NOTCH2   | 3’ UTR   | 1   | 119912963 | C/T   | 0.03| 0.02| 0.23  | 1.30  | 0.84–2.01| 0.59  |
| rs2569190| CD14     | intronic | 5   | 140633331 | G/A   | 0.42| 0.42| 0.89  | 1.01  | 0.87–1.17| 0.57  |

Function refers to the function role of SNP in the gene. CHR, chromosome; BP, base pair of where the SNP is located; A1/A2, minor allele/major allele; F_A, the minor allele (T) frequency in BA patients; F_U, the minor allele (T) frequency; OR, odds ratio; CI: confidence interval; p < 0.05 was considered statistically significant; p_adj, p value adjusted by gender.

### Table 2. Logistic Regression Showing Interactive Effect of Two Regulatory SNPs Located in CD14 and NOTCH2

| SNP 1   | Gene 1 | SNP 2  | Gene 2 | OR   | CI, 0.95 | p Value |
|---------|--------|--------|--------|------|----------|---------|
| rs2569190| CD14   | rs835576| NOTCH2 | 2.78 | 1.32–5.88 | 8.1E–03 |

The p value indicates the significance based on different genetic models. OR, odds ratio; CI, confidence interval; p < 0.05 was considered statistically significant.
the sample size limit or potential subpopulation stratification. Therefore, in our subjects, we enlarged the sample sizes to gain enough convincing evidence. Another study reported that the plasma-soluble CD14 level might serve as a biological marker and was significantly reduced in patients with the T/T and T/C genotypes when the disease progressed to liver cirrhosis, while there was no significant change in patients with the C/C genotype. This finding was supported by Ming-Huei Chou’s research, which demonstrated that the hepatic CD14 mRNA and soluble CD14 plasma levels of patients with early-stage BA were considerably higher than those with late-stage BA. Additionally, Ahmed et al. also indicated that the expression of CD14 in BA had a time-related change with overexpression in the early stage and decreased expression in the late stage. However, there may be several potential limitations in these previous studies. First, the small sample size may have reduced the statistical power of the study. Second, the individual differences in genetic susceptibility genes may lead to variable degrees of hepatic fibrosis and surgical prognosis in BA patients. Therefore, the liver tissue of BA patients should be in strict accordance with the criteria of clinical pathological staining to determine the degree and stage of hepatic fibrosis (including early and late stages), rather than simply judging by the time of Kasai surgery or liver transplantation.

In our experiments, the expression of CD14 was significantly decreased in BA patients, similar to the findings in noted studies, but we failed to replicate the previous reported variant on CD14 in our cohort. We suggested that the genetic epistatic effect of this previously reported variant with SNP on NOTCH2 might boost the risk to disease by using a case-control study. The Notch signaling pathway plays a key role in the development and differentiation of hepatic stem cells into BECs in order to establish the biliary system. Mutations in Jagged1 (Jag1) and NOTCH2 result in Alagille syndrome (AGS) in humans, which is characterized by biliary dysplasia and cholestasis, and similar findings were observed in mice, suggesting functional conservation. In a previous study, we also found that lower expression of NOTCH2 could lead to malformation of the IHBD in the perinatal and postnatal periods. However, the specific mechanism for downregulating the NOTCH2 expression in BA is not clear.

A regulatory SNP rs835576 located in the 3’ UTR of NOTCH2 was previously identified to validate the susceptibility with BA. However, we found different minor alleles between healthy controls (C: 2.4%) and BA patients (C: 3.1%) with an OR of 1.30 (95% CI: 0.84–2.01, p = 0.23), which we observed similarly with the allele frequency of the SNP for Han Chinese in Beijing (HCB; C: 2.3%) reflecting the data reliability. The NOTCH2 (rs835576) SNP showed limited evidence of associations with BA, which may be due to the relative rare minor allele frequency for the SNP and the sample size limit of the replication cohort. It is also possible that these two genes are associated with certain disease subphenotypes but not with overall susceptibility. An analysis of our patient samples did not show significant differences for this locus with any subphenotypes, although this could be due to the reduced sample sizes for each subgroup based on certain phenotypes.

Hepatocytes dedifferentiate into hepatoblasts and further differentiate into BECs when the bile duct is injured, which is mainly regulated by the Notch signaling pathway. Our experiment found that these two SNPs had a definite interaction within a haplotype to influence the risk of BA. This result suggests that CD14 (rs2569190) may interact with NOTCH2 (rs835576) and, together, downregulate the NOTCH2 gene’s expression. Our previous study showed that the maturation of BECs and the expression of Notch may play a role in the pathogenesis of BA, as well as the increased levels of immature BECs in patients with BA. However, newly formed immature bile ducts or ductules have no bile transport function, and bile may accumulate to form bile plugs. Together, these results suggest that CD14 (rs2569190) may interact with NOTCH2 (rs835576) and, together, downregulate NOTCH2 gene’s expression, which could result in immature and malfunction of BECs. The real-time PCR and western blot results showed that the expression of CD14 and NOTCH2 was significantly lower in BA patients compared to the control group. This further hinted that the genetic susceptibility locus of these two genes was associated with the risk of BA. Although the locations of CD14 and NOTCH2 in the BA liver were different, it might be related to hepatoblasts differentiated into BECs or hepatocytes in the Notch signaling pathway. These results were similar to the previous result of an epistasis test using PLINK.

However, several limits should be noted in our study. BA is a complex trait resulting from both environmental and genetic factors. Environmental factors and rare genetic variations associated with BA have not yet been identified, which limited further investigation of the gene-environment interactions. Epistasis power of the present study was examined through the Epistasis Power Calculator (https://gump.qimr.edu.au/general/manuelf/epistasis/epipower4i.html); based on the present sample size with the incidence rate of 1 per 10,000 infants, though the present sample size is large enough (0.98 for the case-only study, 0.97 for the case-control study), further replication in an independent cohort was still required. Replication studies from other cohorts with a larger sample size are encouraged to confirm the association. Also, the different location of CD14 and NOTCH2 in BA liver

| SNP 1   | Gene 1 | SNP 2   | Gene 2 | Balanced Accuracy | OR    | CI 0.95    | p Value |
|---------|--------|---------|--------|------------------|-------|------------|---------|
| rs2569190 | CD14  | rs835576 | NOTCH2 | 0.53             | 1.44  | 1.14–1.84  | 2.6E−03 |

The p value indicates the significance based on different genetic models: OR, odds ratio; CI, confidence interval; p < 0.05 was considered statistically significant.
might be related to the Notch signal pathway and the differentiation and proliferation of hepatoblasts, but the association was not clear. Without functional experiments, it is difficult to determine whether these two SNPs are causally related to BA. Hence, cells and animal model experiments are needed to further explore the genetic function of associated interacting pairs as reported in a previous study.29–31

In conclusion, the results of our study in a Chinese population verified that CD14 (rs2569190) may interact with NOTCH2 (rs835576) and, together, downregulate NOTCH2 (rs835576) expression, which results in immature and malfunction of BECs in BA.

Table 4. The eQTL Effect of Two SNPs in CD14 and NOTCH2 with Biliary Atresia

| SNP       | Gene     | Regulatory Pattern | p Value  | Tissue              |
|-----------|----------|--------------------|----------|---------------------|
| rs2569190 | CD14     | cis                | 6.40E−07 | liver (transformed fibroblasts) |
| rs835576  | NOTCH2   | cis                | 4.90E−06 | liver               |

The p value indicates the significance based on different genetic models.

Figure 1. CC and GG Regulate the Lower Expression of Regulated Gene, which Is Associated with the Biliary Atresia

(A and B) Fibroblast samples of minor alleles C from rs2569190 and G from rs835576 showed relatively lower expression of (A) CD14 and (B) NOTCH2, respectively. (C) CC and GG showed a genetic epistatic effect tested by MDR.

MATERIALS AND METHODS

Study Subjects

We enrolled 506 BA cases and 1,473 controls in this hospital-based case-control study as we described previously.32 The candidate SNPs were selected upon the following criteria: SNPs rs2569190 and rs835578 were selected by the potential regulatory roles according to the GTEx portal database (https://gtexportal.org/home/). Furthermore, SNP rs2569190 is located on the promoter region of CD14 and showed suggestive evidence as associated with BA.10 All subjects were recruited at the Guangzhou Women and Children’s Medical Center, and the BA cases were confirmed by clinical diagnosis and pathology. 1,473 healthy children were randomly selected as the controls who had received a routine physical examination in the same hospital and matched to cases on age and gender. The exclusion criteria of the control were as follows: other types of liver diseases or autoimmune disease or children who received a liver transplantation. In addition, the liver tissues of 28 BA patients and 20 congenital CC patients (all less than 1 year old) were selected for further real-time PCR and western blot detection and comparison. This study was approved by the Institutional Review Board of the Guangzhou Women and Children’s Medical Center, and the experimental process strictly abided by medical ethics. All participants have signed informed consent.

Polymorphism Analysis

Total genomic DNA was isolated from tissue and peripheral blood samples using the TIANamp Blood DNA Kit (TianGen Biotech, Beijing, China) according to the manufacturer’s protocol. SNPs (rs2569190, G/A; and rs835576, C/T) were successfully designed using the MassARRAY iPLEX Gold System (Sequenom). Moreover, 5% of samples were selected randomly for repeated assays, and the results were 100% concordant.

Real-Time qPCR

The mRNA expression levels of CD14 and NOTCH2 were performed by real-time PCR using the SYBR Green Supermix (Bio-Rad, 172-5124, Hercules, CA, USA). Data were collected and quantitatively...
analyzed on a Quant Studio 6 Flex System (Applied Biosystems, Foster City, CA, USA). The β-actin gene was used as an endogenous control to normalize for differences in total RNA in each sample. The primers were used as listed in Table S1.

Western Blot Analysis
Protein samples of patients’ liver extracts were dissolved on 8% SDS-PAGE gels and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% BSA and incubated with CD14 (Abcam, ab106285, Cambridge, UK) and NOTCH2 (GeneTex, GTX101593, Irvine, CA, USA) antibodies at 4°C overnight. After three washes, the membranes were incubated with secondary antibodies at room temperature for 1 hr. After another three washes, the membranes were incubated with ECL Western Blotting Substrate for 8 min, and then the protein bands were visualized on X-ray films.

Figure 3. Expression of CD14 and NOTCH2 in Western Blot
Liver tissue lysates were immunoblotted with anti-CD14 and anti-NOTCH2 antibodies. β-actin was selected as the parameter of the corresponding indicator (A). Each group included 3 samples; *p < 0.05, unpaired t tests (B). BA, biliary atresia; CC, choledochal cyst.

Supplemental Information
Supplemental Information includes one table and can be found with this article online at https://doi.org/10.1016/j.omtn.2018.10.006.
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