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Original Article

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 the 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.[10] Recent studies have found that hepatocytes can produce large amounts of *CD14* in acute liver injury.[18] 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 (IHBD) in the perinatal and postnatal periods, and the low expression of this receptor leads to the abnormality of the IHBD.[20] *NOTCH2* mutations cause Alagille syndrome, which involves biliary developmental defects that are characterized by neonatal jaundice, impaired differentiation of the IHBD, 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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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.

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 synergistical 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.

A previous study showed that a T/T homozygote at position 159 of the *CD14* promoter polymorphism was associated with the development of BA in 90 BA patients (A allele: 61.7%, G allele: 38.3%) and 143 healthy controls (A: 52.1%, G: 47.9%), giving an OR of 1.09 (95% CI: 1.01–2.16, p = 0.043) in a southern Taiwan population.[10] However, in our study, the frequency of the minor allele (G) was almost the same between 1,473 healthy controls (41.75%) and 506 BA patients (41.99%), giving an OR of 1.01 (95% CI: 0.87–1.17, p = 0.89). The allele frequency was slightly different between the two studies. Checking 1000G data from an East Asian population (EAS), the minor allele (G) frequency was found to be 42.76%, showing an allele frequency similar to that of the Southern Chinese population. The differences between the Southern Chinese population and previously reported Taiwanese individuals could be due to

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.

Table 3. MDR Validation Showing the Interactive Effect of the Two SNPs

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.

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.[10] This finding was supported by Ming-Huei Chou's research,[22] 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.[23] 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.[24,25] Mutations in Jagged1 (Jag1) and *NOTCH2* result in Alagille syndrome (AGS) in humans, which is characterized by biliary dysplasia and cholestasis,[26,27] and similar findings were observed in mice,[28] 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.[20] 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

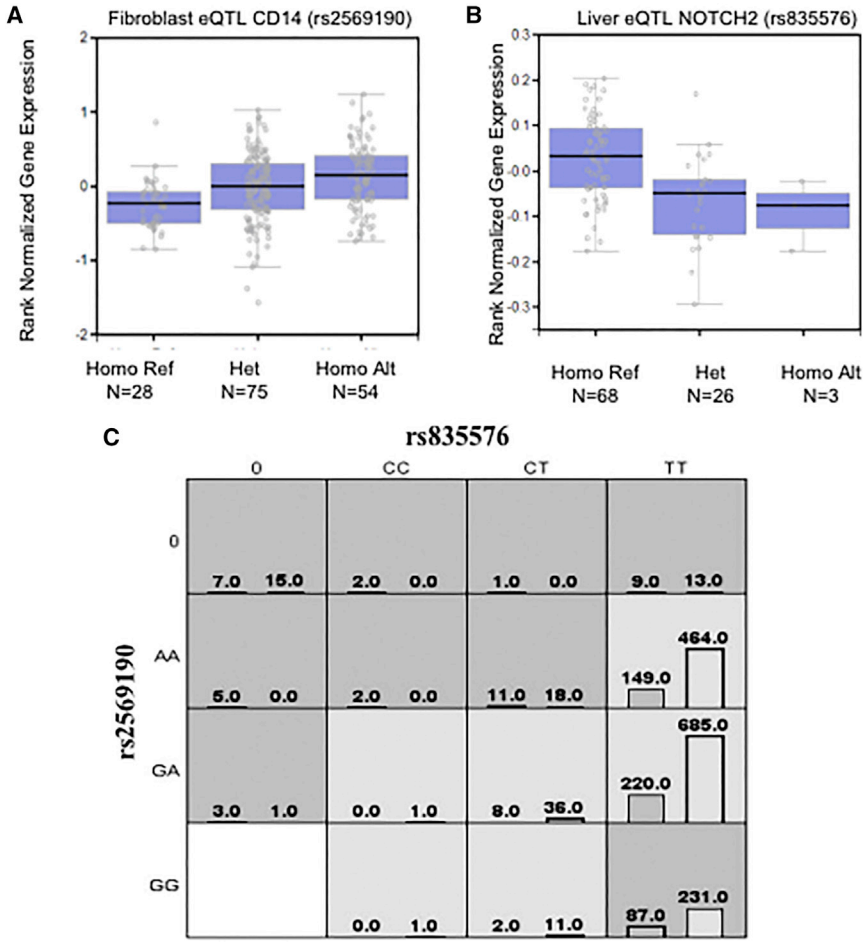

Figure 1. CC and GG Regulate the Lower Expression of Regulated Gene, which Is Associated with the Biliary Atresia

(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.

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	transformed fibroblasts
			0.79	liver
rs835576	*NOTCH2*	*cis*	4.90E−06	liver

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

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

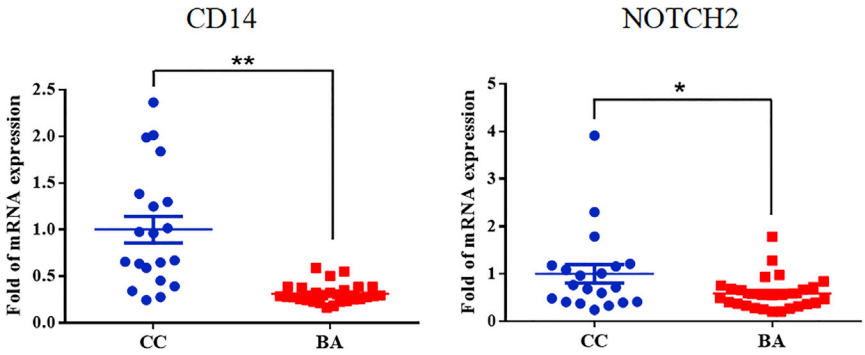

Figure 2. Quantitative Expression of *CD14* and *NOTCH2* in the Liver

Each group of liver mRNA was detected by real-time PCR. The total expression of *CD14* and *NOTCH2* in biliary atresia (BA; n = 28) was significantly lower than that in choledochal cyst (CC; n = 20). *p < 0.05; **p < 0.01.

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.

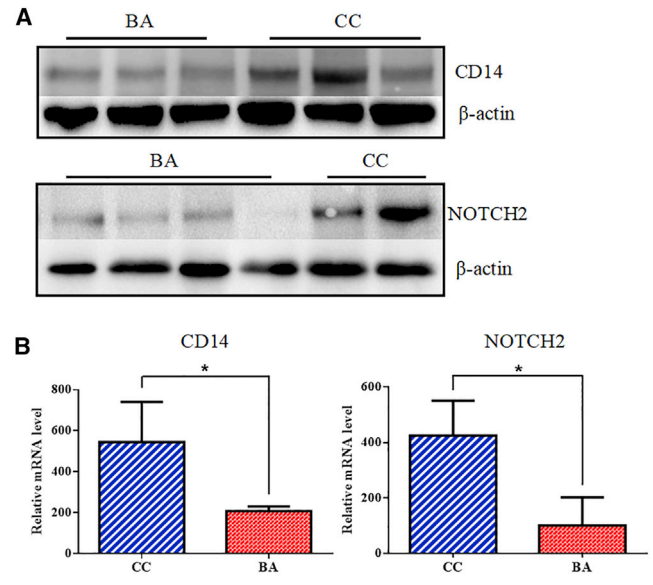

Figure 3. Expression of *CD14* and *NOTCH2* in Western Blot

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.

Statistical Analysis

The allele frequency distribution included variants for the BA patients, and the controls were compared by χ^2 test. $p < 0.05$ was considered to be statistically significant. ORs and corresponding 95% CIs were calculated from the logistic regression model. Epistatic interaction between *CD14* (rs2569190) and *NOTCH2* (rs835576) was examined using PLINK[33] (based on logistic regression analysis) on 506 cases and 1,473 controls. Higher order genetic interactions were detected and characterized by pairwise multifactor dimensionality reduction (MDR).

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.

AUTHOR CONTRIBUTIONS

Z.L., H.L., Y.Z., and R.Z. designed and performed the study and wrote the manuscript. M.F., L.S., Y.T., X.X., H.C., H.W., and J.Z. collected the samples and information. Z.L., X.X., and Y.Z. participated in analyzing data. R.Z. and H.X. coordinated the study over the entire time. All authors reviewed the final manuscript.

CONFLICTS OF INTEREST

The authors declare no competing financial interests.

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This study was approved by the Institutional Review Board of the Guangzhou Women and Children's Medical Center. All the data involved in the study can be supplied upon request. This study was supported by the National Natural Science Foundation of China (grant nos. 81770510, 81771629, and 81671498) and the Science and Technology Planning Project of Guangdong Province 2017 (grant no. 2017A020214017). The authors would like to thank the Department of Pathology and Biobank, Guangzhou Women and Children's Medical Center, for providing the clinical samples.

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