RESEARCH ARTICLE

Association of polymorphism in the VEGFA gene 3'-UTR +936T/C with susceptibility to biliary atresia in a Southern Chinese Han population

Fei Liu^{1,2} | Jixiao Zeng² | Deli Zhu² | Ruizhong Zhang² | Xiaogang Xu² | Mengmeng Wang³ | Yan Zhang² | Huimin Xia^{1,2} | Zhichun Feng^{1,4,5,6}

¹Southern Medical University, Guangzhou, Guangdong

²Department of Pediatric Surgery, Guangzhou Institute of Pediatrics, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, Guangzhou, Guangdong

³Department of Anesthesiology, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, Guangzhou, Guangdong

⁴Division of Neonatology, Affiliated BaYi Children's Hospital, Clinical Medical College in PLA Army General Hospital, Southern Medical University, Beijing, China

⁵National Engineering Laboratory for Birth Defects Prevention and Control of Key Technology, Beijing, China

⁶Beijing Key Laboratory of Pediatric Organ Failure, Beijing, China

Correspondence

Zhichun Feng, Division of Neonatology, Affiliated BaYi Children's Hospital, Clinical Medical College in PLA Army General Hospital, Southern Medical University, Beijing, China. Email: bzfcengzc@yahoo.com

Funding information

National Natural Science Foundation of China, Grant/Award Number: 81601423 and 81600399; Science and Technology Project of Guangzhou, Grant/Award Number: 201707010014

Background: Biliary atresia (BA) is a neonatal disease characterized by chronic inflammation of the bile ducts and progressive aggravation of jaundice, but with a poor prognosis and high mortality. The etiology of BA is still uncertain which may be related to gene defect, virus infection, immune disorder, gene polymorphism. As a proinflammatory cytokine, VEGFA gene polymorphism (rs3025039) has been shown to be related to the pathogenesis of BA in Taiwanese population.

Methods: We investigated the association between VEGFA gene polymorphism (rs3025039) and BA susceptibility using the largest case-control cohort, totaling with 506 BA patients and 1473 healthy controls in a Southern Chinese Han population. VEGFA gene polymorphism (rs3025039) was genotyped using the MassARRAY iPLEX Gold system (Sequenom). Odds ratios (OR) and 95% confidence intervals (CIs) were used to access the association between the VEGFA gene polymorphism (rs3025039) and BA risk.

Results: No significant association was found between the VEGFA gene polymorphism (rs3025039) and BA risk in the overall analysis.

Conclusion: These results suggest that VEGFA gene polymorphism (rs3025039) may not be associated with the risk of BA in the Southern Chinese Han population.

KEYWORDS

biliary atresia, GWAS, polymorphism, susceptibility, VEGF

1 | INTRODUCTION

Biliary atresia (BA) is a neonatal disease characterized by fibrotic obliteration of the extrahepatic bile duct and intrahepatic periductal inflammation and injury, eventually resulting in cirrhosis. It is reported that the incidence of BA in Europe and the United States is about 0.6~0.8/10 000 while in Asia country such as Japan is 0.8~1.1/10 000.¹ Kasai is the first choice for the treatment which aims at restoring bile drainage and improving the situation of cholestasis.

However, due to progressive liver fibrosis, cholestatic cirrhosis, and portal hypertension, the majority of BA children eventually need to choose liver transplant as the ultimate treatment,² only less than 18% of BA children can avoid liver transplantation.³

The etiology of BA is still uncertain which may be related to gene defect,⁴ virus infection,⁵ immune disorder,⁶ gene polymorphism,⁷ but each theory can only explain some of the cases of BA. So far, genetic risk factors have been considered to play an important role in the pathogenesis of BA. Genome-wide association

study (GWAS), as the main tool of human genetics research, has been widely used in the study of many complicated diseases⁸ as well as some common traits⁹ and obtains great achievements. With the development of GWAS, the genetic research of BA has made great progress and the related genes including ADD3,¹⁰ GPC1,¹¹ and ARF6¹² have been confirmed. VEGF, as a proinflammatory cytokine, can induce cell adhesion, enhance T-cell differentiation, and promote the transformation of T cell into Th1 cells by increasing interferon- γ (IFN- γ) and decreasing the level of Interleukin-10(IL-10). Enjoji¹³ reported VEGF involved in the pathogenesis of BA. Recently, a new single nucleotide polymorphism (SNP) (rs3025039) in the VEGFA gene was found to be related to the occurrence of BA in Taiwanese population (consisting of 45 cases and 160 controls).¹⁴ The human VEGFA gene is located in chromosome 6p21.3 which contains 14Kb coding region, consisting of 8 exons and 7 introns.¹⁵ The polymorphism of functional gene can affect the regulation of expression, thus leads to the difference in the incidence and severity of disease. Previous study¹⁴ selected three functional VEGF SNPs including rs3025039, rs699947, and rs2010963 to verify the association with BA. Ultimately, SNP rs3025039 especially the C allele was confirmed to be associated with BA (OR = 3.19, 95% CI = 1.48-6.90, $P = .004$).

Because of the difference of gene polymorphism among different races, so the replication studies are necessary. In this study, we used different genetic models to verify the VEGFA gene polymorphism (rs3025039) in BA. To the best of our knowledge, this should be the first association study to explore the relationship between VEGFA gene polymorphism and the incidence of BA in a southern Chinese Han population (consisting of 506 cases and 1473 controls).

2 | MATERIALS AND METHODS

2.1 | Study subjects

A total of 1979 Chinese subjects were included in the present study. All subjects were recruited at the Guangzhou Women and Children's Medical Center between January 2005 and April 2016. Among them, 506 patients (292 males, 214 females) were diagnosed with BA. The diagnosis of BA is based on clinical manifestations, laboratory tests, imaging examinations, and ultimately confirmed by cholangiography. The mean age of the patients at cholangiography was 2.088 ± 1.934 months (range: 1~7 months). None of the patients had any associated congenital malformations.

During the same period, a group of 1473 health, unrelated subjects (967 males, 506 females) were randomly selected as a control group with matched gender and age. None had any history of liver or autoimmune disease, or underwent liver transplantation. The study was approved by the Institutional Review Board of Guangzhou Women and Children's Medical Center. Medical histories were obtained during the first routine visit. Informed written consent was obtained from the parents or guardians of all subjects who participated in the study.

2.2 | Polymorphism analysis

Genomic DNA was mainly extracted from 2 mL of peripheral blood sample using the TIANamp Blood DNA Kit (TianGen Biotech Co. Ltd., Beijing, China) following the manufacturer's instructions. DNA concentration and purity were determined by spectrophotometric measurement of absorbance at 260 and 280 nm by a UV spectrophotometer (Nano Drop Technologies, Inc., Wilmington, DE).

Briefly, qualified DNA samples were diluted to 10 ng/ μ L and loaded in 96-well plates. Then, the VEGFA gene SNP (rs3025039 T/C) was successfully designed on a customized multiplex genotyping panel using the MassARRAY iPLEX Gold system (Sequenom). Significant deviation from Hardy-Weinberg equilibrium in controls and all passed the test with P -value $>.05$ ($P = .874$). To ensure the accuracy of genotyping results, four positive controls and four negative controls were included in each of the 384-well plates. The assays were repeated for 5% of the samples, and the results were 100% concordant.

2.3 | Statistical analysis

We used χ^2 test to evaluate the differences in the frequency distributions of the demographics and genotypes between the BA cases and the controls. PLINK software was used to verify the relationship between the VEGFA gene polymorphism (rs3025039) and BA risk. Odds ratios (ORs) and 95% confidence intervals (CIs) were used to assess the correlations between the VEGFA gene SNP (rs3025039 T/C) and BA susceptibility. P value $<.05$ was considered as statistically significant. Linkage disequilibrium (LD) patterns and values were obtained using Haploview.¹⁶ Association of the SNPs with disease risk was also corrected by logistic regression using age and sex as covariates and the associations found in this study remain significant after all the corrections. Association with subphenotype was analyzed by comparing cases with a certain subphenotype, cases without the subphenotype with controls and healthy controls. Heterogeneity test of three ORs was compared among the cases with and without the subphenotypes.

3 | RESULTS

3.1 | Population characteristics

The demographic characteristics of participants are reviewed in Table S1. Regarding subphenotype of BA, 44(8.7%) were cystic biliary atresia (CBA) patients and 462(91.3%) were non-CBA subjects.

3.2 | The replication results of SNP rs3025039 in VEGFA in a southern Chinese population

In this study, 506 cases and 1473 controls were successfully genotype. The replication results of VEGFA gene SNP (rs3025039) is shown in Table 1. We found that there is no significant association between rs3025039 T/C polymorphism and BA susceptibility ($P = .62$).

TABLE 1 The replication results of VEGFA gene SNP (rs3025039)

SNP	Gene	CHR	BP	A1/A2	F-A	F-U	P	OR (95% CI)	P-adj
rs3025039	VEGFA	6	43784799	T/C	0.18	0.17	.62	1.05 (0.87~1.27)	.79

OR, odds ratio; CI, confidence interval; A1/A2, Minor allele/Major allele; F-A means of the minor allele (T) frequency in BA patients; F-U means the minor allele (T) frequency in normal control subjects; P-adj means P value after adjustments of age and gender.

TABLE 2 The detailed replication results following different genetic models

CHR	SNP	A1/A2	TEST	F-A	F-U	P	OR (95% CI)
6	rs3025039	T/C	GENO	16/142/332	43/411/1002	.88	-
6	rs3025039	T/C	ADD	174/806	497/2415	.62	1.05 (0.87-1.27)
6	rs3025039	T/C	DOM	158/332	454/1002	.66	1.05 (0.84-1.31)
6	rs3025039	T/C	REC	16/474	43/1413	.73	1.11 (0.62-1.99)

OR, odds ratio; CI, confidence interval; F-A means the genotypic and allelic distribution of BA group; F-U means the genotypic and allelic distribution of normal control group. The genotypic (GENO) model provides a general test of association in the 2-by-3 table of disease-by-genotype. The dominant (DOM) and recessive (REC) models are tests for the minor allele. The genotypic or allelic counts are given for cases and controls separately. For recessive and dominant tests, the counts represent the genotypes, with two of the classes pooled.

We utilized different genetic models to verify the feasibility of the result. The detailed replication results are shown in Table 2. There is no significant association between VEGFA gene SNP (rs3025039) and BA susceptibility in GENO model ($P = .88$). Similar results were found in TREND model, DOM model and REC model ($P = .62$, $P = .66$, $P = .73$, respectively).

3.3 | The results of VEGFA gene SNP (rs3025039) and BA subphenotype

Based on the results of previous studies, the cystic biliary atresia (CBA) has a better clinical outcome after a hepaticojunostomy.¹⁷ In this context, we explore the relationship between VEGFA gene SNP (rs3025039) and BA subphenotype (including 44 cases of CBA and 462 non-CBA subjects). All CBA patients were confirmed by cholangiography. However, we found no significant association between the polymorphism of VEGFA gene and BA subphenotype. The detailed results are shown in Table 3.

4 | DISCUSSION

In this study, we investigated whether VEGFA gene SNP (rs3025039) was associated with the risk of BA in southern Chinese Han children. To our disappointment, we found no significant association between

rs3025039 T/C polymorphism and BA susceptibility. These findings suggest the VEGFA gene +936 SNP does not play an important role in BA predisposition in southern Chinese Han children.

Although the etiology and pathogenesis of BA is still not clear, induction and maintenance of systemic and local inflammatory responses are considered to be an important factor.¹⁸ Cytokines play an important role in the pathogenesis of cholangitis and hepatic fibrosis in BA. Interleukin-18 (IL-18) as a proinflammatory cytokine can induce the production of IFN- γ , enhance the expression of intercellular adhesion molecule-1 (ICAM-1) and Fas ligand, thereby induce the injury and apoptosis of hepatic cells. Urushihara¹⁹ found that the plasma level of IL-18 was elevated in BA, suggesting IL-18 may play an important role in progressive inflammation and fibrosis of BA. Activation of hepatic stellate cells (HSCs) and hepatic portal fibroblasts is a major cellular factor to enhance collagen deposition in liver fibrosis. Platelet-derived growth factor- β (PDGF- β) is a potent mitogen and chemokine for activation of HSCs and myofibroblasts. Yoshida²⁰ reported that PDGF- β could activate HSC in MDR2-null mice and promote the development of liver fibrosis. TGF- β (transforming growth factor- β) are potent regulators of profibrogenic and can regulate extracellular matrix formation, degradation and remodeling in some chronic liver diseases.²¹ Lee²² identified that TGF- β 2 is the main product of TGF- β gene expression in the process of fibrosis in BA. The upregulation of TGF- β 2 expression may be involved in the occurrence of BA. As a proinflammatory cytokine, VEGF also involved in the pathogenesis of BA. Compared with

TABLE 3 The results of VEGFA gene SNP (rs3025039) and BA subphenotype

CHR	SNP	A1/A2	F-A	F-U	P	OR	0.95 CI
CBA-non-CBA							
6	rs3025039	T/C	0.1905	0.1727	.68	1.127	(0.64~2.00)
CBA-Control							
6	rs3025039	T/C	0.1905	0.1707	.63	1.143	(0.66~1.99)

OR, odds ratio; CI, confidence interval; F-A means of the minor allele (T) frequency in BA subphenotype (CBA or non-CBA) group; F-U means the minor allele (T) frequency in normal control group.

the control group, the serum VEGF level in acute cholangitis stage was significantly increased, indicating that VEGF was involved in the occurrence of BA.¹⁴

Gene polymorphisms can contribute to the occurrence and the development of disease by regulating the gene expression. Previous studies have reported a correlation between VEGF gene polymorphisms and the occurrence of autoimmune inflammation disease, such as ankylosing spondylitis(AS), rheumatoid arthritis(RA), and Behcet's disease.²³ Wang²⁴ reported that the average concentration of plasma VEGF was significantly higher than that control group, also found VEGF SNP (rs2010963 G/C) was associated with a decreased risk of AS.

Although VEGF gene polymorphisms are closely related to the occurrence of autoimmune inflammatory diseases, there have been fewer investigations about the relationship between genetic polymorphisms of VEGF and BA. SNPs in VEGFA including -2578C/A (rs699947) in the promoter region, -634G/C (rs2010963) in the 5'-untranslated region and +936C/T (rs3025039) in the 3'-untranslated region were investigated in the study showing rs699947 and rs2010963 did not play an important role in BA susceptibility.¹⁴ We found mild linkage disequilibrium (LD, r^2) between VEGFA gene SNPs (rs699947 and rs2010963) in different populations ($r^2 = .28$, Asian population; $r^2 = .40$, European population) through Linkage disequilibrium analysis (Figure S1), so this study only explore the relationship between target SNP (rs3025039) and the occurrence of BA. In the end, we found no significant difference of VEGFA gene +936T/CSNP (rs3025039) in patients with BA compared with normal control. Lee¹⁴ holded different view from us and considered the polymorphism of VEGFA +936 C/T, especially C allele, is significantly associated with the onset of BA, possibly conferring increased susceptibility to the disease. We consider the reasons for these differences as follows:(1) this study contained only 506 patients, the relatively small sample size may reduce the statistical power;(2) ethnicity difference may contribute to the different results;(3) clinical data are not complete, limiting our stratification analysis, may also lead to differences in results.

In conclusion, our study shows VEGFA gene +936T/C SNP (rs3025039) has no significant correlation with BA in the Southern Chinese Han population. VEGFA gene polymorphism (rs3025039) is not an important risk factor for BA predisposition. We may use larger sample size with subjects involving different ethnicities to explore the relationship between VEGF gene polymorphism and BA susceptibility in further study.

ACKNOWLEDGMENTS

All the individuals involved this study gave informed consent for research publication. The study was approved by the institutional review board. All the data involved in the study can be supplied upon request. Our work was supported by the grant of National Natural Science Foundation of China (Grant No. 81601423) and the National Natural Science Foundation of China (Grant No. 81600399), Also supported by the Science and Technology Project of Guangzhou (Grant No.201707010014). We thank Yanlu TONG and Hezhen WANG for their assistance in DNA extraction and medical histories information collection.

CONFLICTS OF INTEREST

The authors confirm that there are no conflicts of interest.

ORCID

Fei Liu <http://orcid.org/0000-0001-7632-3066>

REFERENCES

- Zhan J, Feng J, Chen Y, Liu J, Wang B. Incidence of biliary atresia associated congenital malformations: a retrospective multicenter study in China. *Asian J Surg*. 2016;40:429-433.
- Keplinger KM, Bloomston M. Anatomy and embryology of the biliary tract. *Surg Clin North Am*. 2014;94:203-217.
- Caruso S, Miraglia R, Milazzo M, et al. Multidetector computed tomography hepatic findings in children with end-stage biliary atresia. *Eur Radiol*. 2010;20:1468-1475.
- Cui S, Leyva-Vega M, Tsai EA, et al. Evidence from human and zebrafish that GPC1 is a biliary atresia susceptibility gene. *Gastroenterology*. 2013;144:1107-1115. e1103.
- Zani A, Quaglia A, Hadzić N, Zuckerman M, Davenport M. Cytomegalovirus-associated biliary atresia: an aetiological and prognostic subgroup. *J Pediatr Surg*. 2015;50:1739-1745.
- Mack CL. What causes biliary atresia? unique aspects of the neonatal immune system provide clues to disease pathogenesis. *Cell Mol Gastroenterol Hepatol*. 2015;1:267-274.
- Verkade HJ, Bezerra JA, Davenport M, et al. Biliary atresia and other cholestatic childhood diseases: advances and future challenges. *J Hepatol*. 2016;65:631-642.
- Hu Z, Liu Y, Zhai X, et al. New loci associated with chronic hepatitis B virus infection in Han Chinese. *Nat Genet*. 2013;45:1499-1503.
- Hao Y, Liu X, Lu X, et al. Genome-wide association study in Han Chinese identifies three novel loci for human height. *Hum Genet*. 2013;132:681-689.
- Cheng G, Tang CS, Wong EH, et al. Common genetic variants regulating ADD3 gene expression alter biliary atresia risk. *J Hepatol*. 2013;59:1285-1291.
- Smith K. Biliary tract: GPC1 genetic risk further links Hedgehog signalling with pathogenesis of biliary atresia. *Nat Rev Gastroenterol Hepatol*. 2013;10:127.
- Ningappa M, So J, Glessner J, et al. The role of ARF6 in biliary atresia. *PLoS ONE*. 2015;10:e0138381.
- Enjoji M, Nakamuta M, Yamaguchi K, et al. Clinical significance of serum levels of vascular endothelial growth factor and its receptor in biliary disease and carcinoma. *World J Gastroenterol*. 2005;11:1167-1171.
- Lee HC, Chang TY, Yeung CY, et al. Genetic variation in the vascular endothelial growth factor gene is associated with biliary atresia. *J Clin Gastroenterol*. 2010;44:135-139.
- Makni L, Stayoussef M, Ghazouani E, Mezlini A, Almawi WY, BesmaYacoubi L. Distinct association of VEGF-A polymorphisms with laryngeal and nasopharyngeal cancer. *Meta Gene*. 2016;10:90-94.
- Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics*. 2005;21:263-265.
- Caponcelli E, Knisely AS, Davenport M. Cystic biliary atresia: an etiological and prognostic subgroup. *J Pediatr Surg*. 2008;43:1619-1624.
- Dong R, Zheng S. Interleukin-8: a critical chemokine in biliary atresia. *J Gastroenterol Hepatol*. 2015;30:970-976.
- Urushihara N, Iwagaki H, Yagi T, et al. Elevation of serum interleukin-18 levels and activation of Kupffer cells in biliary atresia. *J Pediatr Surg*. 2000;35:446-449.
- Yoshida S, Ikenaga N, Liu SB, et al. Extrahepatic platelet-derived growth factor-beta, delivered by platelets, promotes activation of

- hepatic stellate cells and biliary fibrosis in mice. *Gastroenterology*. 2014;147:1378-1392.
21. Iordanskaia T, Hubal MJ, Koeck E, Rossi C, Schwarz K, Nadler EP. Dysregulation of upstream and downstream transforming growth factor-beta transcripts in livers of children with biliary atresia and fibrogenic gene signatures. *J Pediatr Surg*. 2013;48:2047-2053.
 22. Lee SY, Chuang JH, Huang CC, et al. Identification of transforming growth factors actively transcribed during the progress of liver fibrosis in biliary atresia. *J Pediatr Surg*. 2004;39:702-708.
 23. Naykoo NA, Dil A, Rasool R, et al. Single nucleotide polymorphisms, haplotype association and tumour expression of the vascular endothelial growth factor (VEGF) gene with lung carcinoma. *Gene*. 2017;608:95-102.
 24. Wang M, Zhou X, Zhang H, Liu R, Xu N. Associations of the VEGF level, VEGF rs2010963 G/C gene polymorphism and ankylosing spondylitis risk in a Chinese Han population. *Immunol Lett*. 2016;179:56-60.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Liu F, Zeng J, Zhu D, et al. Association of polymorphism in the VEGFA gene 3'-UTR +936T/C with susceptibility to biliary atresia in a Southern Chinese Han population. *J Clin Lab Anal*. 2018;32:e22342. <https://doi.org/10.1002/jcla.22342>