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Research paper

Association of common genetic variants in *VEGFA* with biliary atresia susceptibility in Northwestern Han Chinese

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ABSTRACT

Biliary atresia (BA) is a major neonatal obliterative cholangiopathy, resulting in progressive cirrhosis. The gene *VEGFA* encodes a heparin-binding protein that is a regulator of angiogenesis and a mediator of inflammatory reactions, and accumulating evidence have indicated that *VEGFA* may play a possible role in the pathogenesis of BA. Our study aim was to evaluate the association of common variants within the *VEGFA* gene with BA susceptibility in Northwestern Han Chinese population. Forty tag SNPs within the *VEGFA* gene were selected in the study, and then subsequently genotyped in 1336 Northwestern Han Chinese individuals, consisting of 311 BA patients and 1025 healthy controls. The SNP rs3025039 was found to be strongly associated with BA risk (additive $P = 0.000264$) in our sample, and the CC genotype of rs3025039 had higher prevalence than the other two genotypes, indicating the C allele is a risk allele with an odds ratio (OR) of 1.56 and 95% confidence interval (CI) of 1.23–1.99. Haplotype analyses showed that a LD block containing rs3025039 significantly correlated with BA risk (global $P < 0.001$). Moreover, bioinformatics analysis indicated that hsa-mir-591 and *VEGFA* formed miRNA/SNP target duplexes if the rs3025039 allele was in the T form, suggesting that rs3025039 may alter *VEGFA* expression by affecting hsa-miR-591/single-nucleotide polymorphism target duplexes. Our results indicate additional evidence supporting that there is an important role of the *VEGFA* gene in the increased susceptibility of BA.

Abbreviations:

BA	Biliary atresia
GWAS	genome-wide association studies
SNP	single-nucleotide polymorphism
MAF	minor allele frequencies
HWE	Hardy-Weinberg equilibrium
ORs	Odds ratios
CIs	confidence intervals
LD	linkage disequilibrium
UTR	untranslated region

1. Introduction

Biliary atresia (BA) is known to be a devastating neonatal cholangiopathy characterized by progressive fibroinflammatory involvement of the extrahepatic and intrahepatic biliary tree. If untreated, BA patients would develop progressive cirrhosis and eventually die before two ages (Hartley et al., 2009). The incidence of BA in Caucasians is approximately 1 in 15,000 to 19,000 neonates (Ke et al., 2016), while the incidence in Asians is much higher, ranging from 1 in 5400 to 5800 neonates in China (Hsiao et al., 2007). Kasai surgery is a relatively successful procedure used as an initial strategy towards treating BA, but the surgery needs to be conducted within 3 months of birth to achieve the best therapeutic benefits (Khalil et al., 2010). However, the mechanisms underlying the aetiology of BA are still unknown.

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Previous studies have indicated that BA is a kind of multifactorial disease; multiple hypotheses exist for BA's potential cause, including viral infection, congenital and acquired immune injury (Vijayan and El Tan, 2000; Chuang et al., 2006; Tyler et al., 1998). Recently, genetic risk factors have been considered to play a pivotal role in the pathogenesis of BA (Shneider et al., 2006). With the fast development of high-throughput sequencing technology, genetic association analyses have provided an effective method to elucidate the molecular mechanism of complex diseases, such as schizophrenia (Guan et al., 2013). To date, genetic association studies, including genome-wide association studies (GWAS), have reported many genes associated with BA susceptibility (Ke et al., 2016; Garcia-Barcelo et al., 2010). However, only a small proportion of GWAS could be explained due to the uncertain aetiology of the studied diseases and the lack of systematic biological interpretation (Guan et al., 2016a). Recently, a new single-nucleotide polymorphism (SNP) (rs3025039) in the *VEGFA* gene has been reported to be associated with increased BA risk in Taiwanese individuals (Lee et al., 2010). The *VEGFA* gene is located at 6p12 and encodes a heparin-binding protein that exists as a disulfide-linked homodimer. As a proinflammatory cytokine, VEGFA was also reported to be involved in the pathogenesis of BA (Enjoji et al., 2005). Thus, elucidating the effect of genetic variations within the *VEGFA* gene might provide therapeutic targets to modify inflammation in BA patients.

Significant differences in the contribution of common variations in susceptibility genes among different ethnic populations exist, so replication studies in different populations are necessary and important. Additionally, a previous study examined only three SNPs in the *VEGFA* gene (Lee et al., 2010), and it is unknown if association signals from other SNPs exist or not. Therefore, in the current work, we conducted a case-control study to evaluate the relationship between the *VEGFA* gene with BA susceptibility in Northwestern Han Chinese individuals, including 311 patients and 1025 healthy controls. To the best of our knowledge, this should be the first association study between common variants within the *VEGFA* gene and BA susceptibility in the Northwestern Chinese Han population.

2. Materials and methods

2.1. Subjects

The study contained 311 unrelated patients (133 males and 178 females) who were diagnosed with BA by laparoscopic cholangiography and biopsy of the liver and extrahepatic biliary tree. The patients with other associated congenital malformations were excluded from the study. For the controls, 1025 unrelated healthy individuals (433 males and 592 females) without diagnosis of BA, congenital disease, autoimmune, or liver disease were enrolled. All participants were unrelated Northwestern Han Chinese individuals and were collected between 2006 and 2014 from Xi'an Children's Hospital and the Second Affiliated Hospital of Xi'an Jiaotong University. Written informed consent to take blood samples from the subjects was obtained from each subject or their legal guardians. Peripheral blood samples were collected in a standard EDTA tube for DNA extraction. This study was performed in line with the Declaration of Helsinki (version 2002) and was approved by the Ethics Committee of the Xi'an Children's Hospital.

2.2. SNPs genotyping

First, all common SNPs with minor allele frequencies (MAF) ≥ 0.01 for the *VEGFA* gene were searched in the 1000 genomes CHB dataset. Then, pair-wise tagging and $r^2 \geq 0.8$ were considered as the threshold criteria of tag SNPs selections. Finally, a total of 40 tag SNPs within the *VEGFA* gene were selected in our study (Fig. 1). Peripheral blood samples were collected from all participants, and genomic DNA was isolated from leukocytes with the commercial DNA extraction kit according to the manufacturer's protocol (Tiangen Biotech Co. Ltd., Beijing, China). The genotyping of selected tag SNPs were conducted at the Sequenom MassARRAY platform (Sequenom, San Diego, CA, US) based on the manufacturer's instructions (Gabriel et al., 2009), and the genotype results generated from the samples were processed using Sequenom Typer 4.0 software (Guan et al., 2016b). Throughout the process, data entry and statistical analyses were independently reviewed. Five percent of the samples were randomly selected for repeat genotyping with a concordance of 100%. The final genotype call rate of each SNP was > 99%, ensuring the reliability of further statistical analysis.

2.3. Statistical analysis

Hardy-Weinberg equilibrium (HWE) of each SNP in both groups was tested with Haploview v4.2. Single SNP association analyses were conducted with Plink v1.9 in a logistic model to detect the association signals between all SNPs and BA risk. Odds ratios (ORs) and corresponding 95% confidence intervals (CIs) were calculated to indicate BA risk from the logistic regression model. The linkage disequilibrium (LD) of the candidate SNPs was analysed by using Haploview v4.2, and further haplotype analyses were carried out for the common haplotypes with GENECOUNTING v2.2 (Guan et al., 2012a). In addition, Haplo Stats package v1.6.11 was implemented to avoid a possible statistical bias resulted from the estimated haplotype frequencies (Guan et al., 2014). To develop a potential explanation of the biological possibilities underlying associated SNPs with BA risk, we conducted bioinformatics analysis using an online database (http://genome.ucsc.edu/). In addition, RegulomeDB (http://www.regulomedb.org) was utilized to evaluate the potential functional significance for associated SNPs, and an online tool of protein-protein interaction (STRING, http://string-db.org) was also used to explore the feature of gene-gene networks surrounded *VEGFA*. In all statistical tests, $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Population characteristic

In the study, 311 BA patients and 1025 normal controls were genotyped successfully. The characteristics of the BA patients and healthy controls were shown in Table 1. The female to male ratio in the case group was 1.34 (178/133), and the ratio in the control group was 1.37 (592/433). We found no significant difference in the distribution of gender between both groups ($P = 0.87$, Pearson $\chi^2 = 0.027$). Furthermore, there were no significant differences regarding weight, pregnancy weeks and Neonatal score between cases and controls (Table 1).

3.2. Association of VEGFA polymorphisms with BA risk

Forty selected tag SNPs were successfully genotyped in the study. We found that there were no polymorphisms for 3 SNPs (rs4392721, rs536764187 and rs535119313) in our samples, and they were excluded from the subsequent analyses. The results of HWE test and single SNP-based association analyses of other 37 tag SNPs were summarized in Table 2 and Supplemental Table S1. The genotype distributions of all SNPs were in the HWE for both groups ($P > 0.05$) (Table 2 and Supplemental Table S1). As shown in Table 2, we observed that rs3025039 was significantly associated with BA, and the C allele at rs3025039 was more common in BA patients than in the healthy controls. Using a logistic regression model adjusted for sex, individuals with C alleles had an increased risk of BA ($P = 0.000264$, OR = 1.56, 95% CI = 1.23–1.99) compared with those with T alleles. For genotype distributions, the frequency of the C/C genotype at rs3025039 was obviously higher in case group than in control group, confirming that the C allele increased BA risk susceptibility in the co-dominant and recessive models. In contrast, we found that there were not any