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Original article

Association of vitamin D-related genetic variations and the susceptibility among Thai children with biliary atresia

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Background: Vitamin D deficiency is commonly found in children with biliary atresia (BA). Single nucleotide polymorphisms (SNPs) in vitamin D-related genes are linked to circulating vitamin D levels in various chronic liver diseases.

Objective: This study aimed to investigate whether vitamin D related SNPs were associated with disease susceptibility and clinical manifestations in Thai children with BA.

Methods: DNA specimens from 85 Thai children with BA and 90 age and gender matched healthy controls were genotyped for *DHCR7* rs12800438, *CYP2R1* rs10741657 and *GC* rs7041 using TaqMan polymerase chain reactions.

Results: The frequency of the T allele of *GC* rs7041 was significantly higher in children with BA compared with healthy controls (OR = 1.67, 95% CI = 1.06 - 2.64, *P* = 0.028). Similarly, the frequencies of GT+TT genotypes of *GC* rs7041 were significantly higher in the BA group than the control group (OR = 1.88, 95% CI = 1.03 - 3.42, *P* = 0.040). The genotype distributions and allele frequencies of *DHCR7* rs12800438, *CYP2R1* rs10741657 were not different between groups. All these SNPs were not related to baseline clinical parameters including ALT level, jaundice and liver stiffness measured by transient elastography.

Conclusion: The *GC* rs7041 variant had significantly higher prevalence among BA patients than healthy individuals, indicating its potential role that might contribute to the susceptibility of BA. Thus, identification of the SNP genotype might serve as a predictive parameter for assessing the likelihood of BA in children.

Keywords: Biliary atresia, single nucleotide polymorphism, genetic variants, vitamin D, chronic liver diseases.

Biliary atresia (BA), a severe neonatal disease characterized by obstruction of the biliary tract causing bile stasis and progressive cirrhosis, is the most common indication for pediatric liver transplantation.[1] Globally, the incidence of BA varies among populations ranging from 1:8,000 to 1:15,000 newborns, with particularly high rates in East Asia. At presentation, the palliative surgery of choice is Kasai portoenterostomy, although liver transplantation is frequently required later in life. Currently, the etiologic factors of BA are mostly unknown.[2] It has been hypothesized that multifactorial factors may contribute to cholangiocyte injury and the pathogenesis of BA. These factors include perinatal viral infections, toxin exposures, abnormal developmental pathology and aberrant immune mediated disorders.[2] Moreover, increasing evidence has suggested that genetic factors, including single nucleotide polymorphisms (SNPs), may lead to the susceptibility and development of BA. For example, genome-wide association studies (GWAS) have identified that *GPC1* and *ADD3* appear to be genetic variants responsible for BA susceptibility.[3, 4] Recently, it was validated in Thai children that *ADD3* and *ADD3-AS1* variants increased disease susceptibility and might influence the pathogenesis of BA.[5]

In recent years, it has been shown that vitamin D is connected to the pathogenesis of several chronic liver diseases (CLD) in adults such as viral hepatitis,

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alcoholic liver disease, non-alcoholic fatty liver disease and primary biliary cholangitis.[6] In addition to the well-established function as a regulator of calcium and bone homeostasis, vitamin D exhibits immunomodulatory, anti-inflammatory and anti-fibrotic properties.[7] Overall, vitamin D deficiency or insufficiency is frequently found in a variety of CLD. Long-term prospective data also demonstrated that vitamin D deficiency was related to adverse clinical outcomes such as cirrhosis and hepatocellular carcinoma (HCC).[6] Moreover, previous GWAS showed that serum concentrations of vitamin D were linked to genetic variations regulating vitamin D synthesis and transport.[8, 9] These common variants include the *DHCR7* gene (encoding 7-dehydrocholesterol reductase), *CYP2R1* gene (encoding cytochrome P450) and vitamin D binding protein (or group-specific component [GC] globulin). A recent longitudinal study also demonstrated that these common genetic variants were associated with lower serum vitamin D concentrations among healthy children.[10] Interestingly, it was revealed that these SNPs influenced vitamin D levels and might be associated with liver stiffness in adult patients with CLD.[11] For children with BA, vitamin D deficiency or insufficiency is commonly found not only at initial presentation but also post-Kasai operation.[12, 13] As vitamin D deficiency is invariably detected in patients with BA, we hypothesized that variants in vitamin D metabolism-related genes might play an important role in BA development. Thus, we aimed to investigate whether the above SNPs were associated with disease susceptibility and clinical outcomes in Thai patients with BA.

Materials and Methods

Study population

This cross-sectional study included 85 Thai children with an established diagnosis of BA, who regularly followed-up at King Chulalongkorn Memorial Hospital, Bangkok, Thailand. The mean age of patients was 10.1 ± 6.2 years, with female gender predominant (40 males and 45 females). The healthy control group consisted of 90 age and gender-matched Thai children with no apparent liver disease (10.5 ± 4.5 years). This study was performed in accordance with the Declaration of Helsinki for the participation of human subjects. All parents or legal guardians of the recruited subjects were informed about the protocol and procedure in this study. Written informed consents were obtained from the parents prior to the subject's recruitment.

Biochemical parameters of liver function tests including serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT), serum total bilirubin (TB), serum direct bilirubin (DB), and serum alkaline phosphatase (ALP) were performed by a Hitachi 912 Chemistry Analyzer at the central laboratory of King Chulalongkorn Memorial Hospital.

DNA extraction and SNPs genotyping

DNA specimens were collected from patients with BA prior to undergoing Kasai portoenterostomy. Genomic DNA was extracted from 100 µl peripheral blood mononuclear cells (PBMCs) using a phenol-chloroform-isoamyl alcohol isolation method as previously described.[14] The quality of DNA was measured using a spectrophotometer (NanoDrop 2000c, Thermo Scientific). The respective SNPs (*DHCR7* rs12800438, *CYP2R1* rs10741657 and *GC* rs7041) were genotyped using a real-time PCR protocol based on TaqMan SNP Genotyping Assays. The reaction performed included 4 µl of 2.5X master mix (5 PRIME, Germany), 0.25 µl of 40X primers and probes mixture (TaqMan SNP Genotyping Assay (assay ID:C_7241_10), Applied Biosystems, USA), 50 - 100 ng of genomic DNA and nuclease-free water to the final volume of 10 µl. The real-time polymerase chain reaction (PCR) was performed in StepOne Plus Real-time PCR system (Applied Biosystems, USA) according to the manufacturer's protocol. Briefly, initial denaturation was held at 95°C for 10 min, followed by 50 cycles of amplification including denaturation at 92°C for 10 sec, and annealing/ extension at 60°C for 1 min. Fluorescent signals (FAM and VIC) were acquired at the end of each cycle. Positive and negative controls were included in each experiment in order to ensure appropriate data interpretation. The allelic discrimination plots was analyzed using StepOne TM software (version 2.2, Applied Biosystems).

Liver stiffness measurement

Liver stiffness was measured by transient elastography (FibroScan, Echosens, Paris, France) after fasting for at least 2 hours. The subject was requested to be lain in a supine position with maximum right arm abduction then the transducer probe of FibroScan was placed on the right intercostal space. The targeted location was the right lobe of the liver

where its thickness was at least 6 cm without any major vascular structure. The measurement was performed to obtain 10 validated results. The median of the 10 validated results was used as an elastic modulus and recorded in kilopascals (kPa).[15]

Statistical analysis

Statistical analysis was performed using the SPSS software for Windows 22.0 (SPSS Inc., Chicago, IL). Comparisons between groups were calculated by the Chi-square test or Fisher's exact test for categorical variables, and by the Mann–Whitney U-test or Student's *t*-test for quantitative variables. Hardy-Weinberg Equilibrium (HWE) was determined using Pearson's Chi-square as implemented in online software. Associations of different genetic models were assessed under genotype and allele frequency models. Odds ratio (OR) with 95% confidence intervals (CI) between each group were determined using MedCalc statistical software version 13.3.3 (http://www.medcalc.org/calc/odds_ratio.php). *P* - values of less than 0.05 were considered to be statistically significant.

Results

Association of polymorphisms and BA risk

The genotype frequencies of each studied SNP in the whole cohort did not deviate from Hardy-Weinberg Equilibrium ($P > 0.05$). The genotype distributions and allele frequencies of *DHCR7* rs12800438 are presented in Table 1. The frequencies of the TT, TG and GG genotypes in patients with BA were 54.1%, 37.7% and 9.2%, respectively, while the corresponding genotypes were 54.4%, 36.7%, and 8.9% in the control group. The genotype distributions and allele frequencies of this SNP were not different between the groups.

For, *CYP2R1* rs10741657, the genotyping assay could not be determined in 7 samples (6 samples in the BA group). The genotype distributions and allele frequencies of this SNP are shown in Table 2. In the BA group, the frequencies of the GG, GA and AA genotypes were 3.8%, 49.4% and 46.8%, respectively. The corresponding genotypes in the control group were 3.4%, 40.4% and 56.2%, respectively. The genotype distributions and allele frequencies of this SNP were not significantly different between groups.

The genotype distributions and allele frequencies of *GC* rs7041 are shown in Table 3. In the BA group, the frequencies of GG, GT and TT genotypes were 40.0%, 47.5% and 12.9%, respectively, while their distributions in the control group were 55.5%, 37.8% and 6.7%, respectively. The frequency of the T allele was significantly higher in patients with BA compared with healthy controls (OR = 1.67, 95% CI = 1.06 - 2.64, $P = 0.028$). Likewise, the frequencies of GT+TT genotypes were significantly higher in the BA group than the control group (OR = 1.88, 95%CI = 1.03 - 3.42, $P = 0.040$).

Table 1. Genotype and allele frequencies of SNP rs12800438 in BA patients and controls.

DHCR7	**BA (n = 85)**	**Controls (n = 90)**	**BA vs. controls OR (95% CI)**	***P - value***
Allelic model				
Major (T)	124	131	1	-
Minor (G)	46	49	0.99 (0.62 - 1.59)	0.973
Additive model				
TT	46	49	1	-
TG	32	33	1.03 (0.55 - 1.94)	0.919
GG	7	8	0.93 (0.31 - 2.77)	0.899
Dominant model				
TT	46	49	1	-
TG + GG	39	41	1.01 (0.56 - 1.84)	0.965
Recessive model				
TT+TG	78	82	1	-
GG	7	8	0.92 (0.32 - 2.66)	0.877

Table 2. Genotype and allele frequencies of SNP rs10741657 in BA patients and controls.

CYP2R1	BA (n = 79)	Controls (n = 89)	BA vs. controls OR (95%CI)	*P - value*
Allelic model				
Major (G)	45	42	1	-
Minor (A)	113	136	0.78 (0.48 - 1.26)	0.308
Additive model				
GG	3	3	1	-
GA	39	36	1.08 (0.20 - 5.72)	0.925
AA	37	50	0.74 (0.14 - 3.88)	0.722
Dominant model				
GG	3	3	1	-
GA + AA	76	86	0.88 (0.17 - 4.51)	0.882
Recessive model				
GG+GA	42	39	1	-
AA	37	50	0.69 (0.37 - 1.26)	0.227

Table 3. Genotype and allele frequencies of SNP rs7041 in BA patients and controls.

GC	BA (n = 85)	Controls (n = 90)	BA vs. controls OR (95%CI)	*P - value*
Allelic model				
Major (G)	108	134	1	-
Minor (T)	62	46	1.67 (1.06 - 2.64)	0.028*
Additive model				
GG	34	50	1	-
GT	40	34	1.73 (0.920 - 3.25)	0.089
TT	11	6	2.69 (0.91 - 7.99)	0.073
Dominant model				
GG	34	50	1	-
GT + TT	51	40	1.88 (1.03 - 3.42)	0.04*
Recessive model				
GG+GT	74	84	1	-
TT	11	6	2.08 (0.73 - 5.90)	0.168

Association of polymorphisms and clinical data in patients with BA

The correlation between the studied SNPs and clinical data of patients with BA at baseline was further examined. It was noted that all of these SNPs (*DHCR7* rs12800438, *CYP2R1* rs10741657 and *GC* rs7041) were not associated with hepatocellular injury (serum ALT) or the severity of jaundice (serum TB). In addition, we did not observe any association between vitamin D metabolism-related polymorphisms and liver stiffness measured by transient elastography (Table 4). However, liver stiffness positively correlated with AST (r = 0.744, $P < 0.001$), ALT (r = 0.627, $P < 0.001$), TB (r = 0.626, $P < 0.001$) and alkaline phosphatase (r = 0.542, $P < 0.001$), whereas it was inversely correlated with patient's age (r = - 0.456, $P < 0.001$).

Table 4. Association between clinical outcomes of BA patients and SNP genotypes.

	***DHCR7* rs12800438**			***CYP2RI rs 10741657***			***GC* rs 7041**		
	TT (n = 46)	**TG+GG (n = 38)**	***P***	**AA (n = 36)**	**GA+GG (n = 42)**	***P***	**GG (n = 34)**	**GT+TT (n = 50)**	***P***
Age (years)	9.6 ± 6.1	10.6 ± 6.3	0.492	10.4 ± 6.2	9.7 ± 6.1	0.600	9.2 ± 5.7	10.7 ± 6.5	0.264
Sex (%)			0.967			0.972			0.719
Male	47.8%	47.4%		47.2%	47.6%		50%	46%	
Female	52.2%	52.6%		52.8%	52.4%		50%	54%	
AST (IU/L)	146.5 ± 106.2	134.5 ± 123.3	0.634	145.6 ± 113.5	137.9 ± 121.1	0.774	146.6 ± 102.3	137.1 ± 122.1	0.707
ALT (IU/L)	150.2 ± 125.6	121.2 ± 108.9	0.266	140.7 ± 112.6	139.4 ± 130.9	0.962	158.8 ± 129.3	121.7 ± 109.1	0.159
TB (mg/dL)	2.3 ± 3.5	3.1 ± 4.1	0.327	2.7 ± 3.4	2.7 ± 4.3	0.962	2.5 ± 3.5	2.8 ± 3.9	0.676
DB (mg/dL)	1.8 ± 3.2	2.7 ± 3.8	0.275	2.3 ± 3.3	2.3 ± 4.0	0.981	2.1 ± 3.3	2.4 ± 3.7	0.699
ALP (mg/L)	407.6 ± 188.0	456.3 ± 424.1	0.52	462.6 ± 402.9	388.4 ± 218.2	0.318	416.1 ± 221.8	438.6 ± 367.7	0.754
Liver stiffness (kPa)	28.5 ± 26.1	28.8 ± 24.8	0.961	28.4 ± 25.3	29.9 ± 26.7	0.800	31.7 ± 26.6	26.6 ± 24.5	0.370

Discussion

Vitamin D, a potent immune-modulator, has recently been linked to the pathogenesis of several CLD including BA.[6, 16] In general, CLD is characterized by the accumulation of extracellular matrix as a consequence of chronic liver injury, which leads to the development of liver fibrosis and cirrhosis. Several pro-fibrogenic cytokines, including transforming growth factor-β (TGF-β), are involved in inappropriate wound-healing response, progressive fibrogenesis and ultimately developing cirrhosis.[17] Previous data have shown that vitamin D represses fibrogenic TGF-β signaling in human hepatic stellate cells, suggesting an anti-fibrotic property of vitamin D.[18] Cumulative data have also suggested that genetic variations in vitamin D-related genes play an important role in natural history and outcome of patients with CLD.[11, 16] To our knowledge, this is the first report that evaluates the role of vitamin D-related genes on the risk of BA development. Compared with matched-age and gender healthy controls, the results of our Thai cohort demonstrated that *DHCR7* rs12800438 and *CYP2R1* rs10741657 did not affect the susceptibility of BA. In contrast, our data showed that *GC* rs7041 was associated with a significantly higher prevalence of BA in the Thai population. Specifically, children with GT+TT genotypes had approximately a twofold increased BA prevalence in comparison with those harboring the GG genotype. These data indicate the potential role of the SNP in vitamin D-related genes that might contribute to disease susceptibility of BA.

Plausible biological mechanisms by which the *GC* rs7041 variant influences the higher BA prevalence in our cohort remain unclear but might be in part related to vitamin D activity. In this context, several crucial steps are involved in the complex process of vitamin D homeostasis. According to previous GWAS data, variants in several genes involved in vitamin D metabolism, such as *DHCR7, CYP2R1* and *GC*, are associated with serum vitamin D levels. [8, 9] DHCR7 is the enzyme that converts 7-dehydrocholesterol to cholesterol, while CYP2R1 is responsible for the hydroxylation of vitamin D to 25(OH) vitamin D in the liver. Vitamin D-binding protein (DBP), encoded by the *GC* gene, is the major carrier of 25(OH) vitamin D and plays an important role in vitamin D transport.[7] The GC gene, located on chromosome 4q11-q13, contains 13 exons that incorporate a 42.5 kb nucleotide length encoding the protein. The amino acid sequence is arranged in three major polymorphic forms coded by several non-synonymous SNPs. Among these SNPs, rs7041 has been shown to influence serum DBP levels, and consequently affects the circulating vitamin D status.[19] In patients with chronic hepatitis C virus (HCV) infection, for example, individuals carrying the minor allele of *GC* rs7041 were found to have significantly lower vitamin D levels compared with those harboring the major allele.[20, 21]

Besides transporting vitamin D metabolites, DBP also plays an alternative role in the inflammatory cascade and innate immune regulation.[22] For instance, GC globulin exerts stimulating effects on macrophages and enhances chemotactic activity of the complements. In addition, the protein can transport several lipids, such as arachidonic acid and endotoxin, responsible for immune-mediated inflammatory processes. Given the various immunomodulatory roles of DBP, it is implicated that its functional SNPs might predict adverse outcomes in several chronic inflammatory disorders. For instance, *GC* rs7041 was recently shown to be linked with susceptibility of HCV infection in a high-risk Chinese population.[23] Furthermore, the polymorphism could influence the clinical outcomes of patients with chronic HCV infections receiving antiviral therapy.[21] Regarding the pathogenesis of BA, several lines of evidence support immune dysregulation as an essential mechanistic factor of disease susceptibility and development.[24] Taken together, it is speculated that the *GC* rs7041 variant might affect DBP and vitamin D activity, thereby altering its immunological properties and modulating disease pathogenesis in BA. In light of this association, the functional roles of this SNP in the development of BA need to be clarified in further studies.

Of note, the minor allele frequency of rs7041 in our cohort (approximately 0.31) was comparable to those found in Asian genome database (0.29) (http://browser.1000 genomes.org/) and was also similar to previous reports among Thai adult individuals (approximately 0.31-0.32).[25, 26] In this study, we also investigated whether vitamin D-related genes could affect the clinical severity of BA in Thai individuals. Our data showed that, among patients with BA, all these SNPs (*DHCR7* rs12800438, *CYP2R1* rs10741657 and *GC* rs7041) were not associated with hepatocellular injury, severity of jaundice and liver stiffness. Previous data demonstrated that *DHCR7*

rs12800438, but not *CYP2R1* rs10741657 and *GC* rs7041, was associated with liver stiffness in adult patients with CLD.[11] For *GC* rs7041, the lack of an association with clinical parameters was in line with a recent report, which showed that the SNP was not related to liver fibrosis in patients with chronic HCV infection.[20]

Our report had some limitations including its retrospective design, which disallowed us to measure baseline serum vitamin D concentrations. Nonetheless, it should be noted that the interpretation of vitamin D levels must be taken into account of multiple potential confounding factors such as the individual's age, dietary and supplemental intake, sunlight exposure and seasonal variability.[27] In spite of the limitation, the key finding of this study was the association between *GC* rs7041 variation and the higher BA prevalence in Thai population. Thus, the determination of GC rs7041 might serve as a genetic marker for assessing an increased likelihood of BA in Thai children. Additional studies are, however, needed to verify such correlation in other racial-ethnic populations and geographical areas.

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Conflict of interest

The authors, hereby, declare no conflict of interest.

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