+276 G/T single nucleotide polymorphism of the adiponectin gene is associated with the susceptibility to biliary atresia

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Original article

Background: **Biliary atresia (BA) is an intractable neonatal inflammatory and obliterative cholangiopathy, leading to progressive fibrosis and cirrhosis. Adiponectin, an anti-inflammatory adipokine, is known to play a possible role in liver diseases. The objective of our study was to determine the relationship between adiponectin gene polymorphisms and BA susceptibility.**

Methods: **A total of 106 BA patients and 107 healthy controls were included in this study. Two single nucleotide polymorphisms (SNPs) of the adiponectin gene, +45T/G (rs2241766) and +276G/T (rs1501299), were genotyped by polymerase chain reaction-restriction fragment length polymorphism analysis.**

Results: **Genotype distributions of +45 T/G and +276 G/T SNPs were seen in the Hardy-Weinberg equilibrium for both BA patients and controls. The frequency of the G/G genotype at +276G/T was significantly higher in BA patients than in the controls (*P*=0.009). Regarding +45T/G in BA patients, the frequency of the T/T genotype tended to be lower than in the controls, but the difference was not significant. Moreover, the G allele at +276G/T in BA patients was more common than in the controls (*P*=0.0043). In contrast, the frequency of the T allele at +45T/G was not significantly different between BA patients and the controls. None of the haplotypes studied was found to significantly influence the risk of BA.**

Conclusions: **+276G/T SNP is strongly associated with BA, particularly with the G allele. We postulate that the +276G/T adiponectin gene polymorphism confers increased susceptibility to BA.**

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Key words: **adiponectin;**
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Introduction

Biliary atresia (BA) is a devastating disease in neonates characterized by progressive inflammatory cholangiopathy. It results from the fibrosclerotic destruction of the extrahepatic bile duct, leading to obliteration of the biliary tract, liver fibrosis, and portal hypertension. If left untreated, BA patients will develop severe liver injury, biliary cirrhosis, and ultimately die by the age of 2 years. Kasai portoenterostomy remains the first surgical option for BA patients, but this surgery has certain limitations. Failure of the Kasai procedure leaves liver transplantation as the only hope for survival.[1-3] However, the mechanisms underlying the etiology of BA remain uncertain. The pathogenesis of BA can be affected by viral infection, autoimmunity, inflammation, abnormal development and genetics, thus classifying BA as a multifactorial disorder.[4,5] Genetic polymorphisms may lead to susceptibility and development of inflammation and fibrosis in the affected liver. Genes associated with different parts of the inflammatory pathways have variable polymorphism frequencies in patients with BA. Such genes include *CFC1*, CD14 endotoxin receptor genes, and hepcidin antimicrobial peptide genes.[6-8] Other polymorphisms were shown to be associated with inflammation in patients with BA such as *VEGF*, *ICAM-*

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1, *XPNPEP1*, *ADD3* and *IL-18* genes.[9-12] Elucidating the role of genetic polymorphisms might provide therapeutic targets to modify progression of fibrosis and inflammation in the disorder. This hypothesis suggests that investigation of inflammatory substances might shed some light on the pathogenesis of BA.

Adiponectin (ADIPOQ), a protein hormone secreted almost exclusively by adipocytes, exhibits anti-atherogenic and insulin-sensitizing properties.[13] It is a 30-kd anti-inflammatory adipokine found in monomeric and multimeric forms. Adiponectin suppresses production of cytokines, such as tumor necrosis factor-α (TNF-α) produced by macrophages, it also suppresses accumulation of lipids in monocyte-derived macrophages and inhibits transformation of macrophages into foam cells.[14,15] Moreover, adiponectin also decreases expression of adhesion molecules (VCAM-1, ICAM-1 and E-selectin) in endothelial cells in response to inflammatory stimuli such as TNF-α and interleukin-6 (IL-6).[16] Previous experimental investigations have indicated that the inflammatory process may modulate adiponectin secretion. One prior study showed that low serum adiponectin was associated with systemic organ failure in acute pancreatitis and could serve as an inverse marker of systemic inflammatory response to pancreatic injury.[17] In addition, a significant decrease in serum adiponectin levels in non-alcoholic steatohepatitis (NASH) patients has been reported.[18] Adiponectin is associated with liver fibrosis and inflammation,[19] suggesting that it might be associated with the pathogenesis of BA.

The human adiponectin gene (*APM1*) is located on chromosome 3q27. Single nucleotide polymorphisms (SNPs), including +45T/G (rs2241766) in exon 2 and +276G/T (rs1501299) in intron 2, have been demonstrated to be associated with diabetes, insulin resistance and atherosclerosis,[20,21] and the G allele at the +276G/T (rs1501299) locus has been proven to be associated with lower serum adiponectin levels.[22] However, information on adiponectin genetic polymorphisms pertaining to the risk of BA has not been documented. The aim of this study was to investigate whether common adiponectin gene, SNPs, +45T/G (rs2241766) and +276G/T (rs1501299), are associated with BA. We analyzed the genetic association between these two polymorphisms and BA, which may provide a better understanding of the adiponectin gene variation's contribution to the development of BA.

Methods

Study population

This study was approved by the Institutional Review Board on Human Research of the Faculty of Medicine, Chulalongkorn University and was conducted in agreement with the *Declaration of Helsinki*. All parents or legal guardians of the recruited children with BA and of healthy controls were informed of the study's purpose. Written informed consent was obtained from the parents prior to the children entering the study.

Peripheral blood samples were obtained from 106 patients (mean age±SD, 8.04±5.44 years) with an established diagnosis of BA (44 males and 62 females) who were included in the course of a routine follow-up at King Chulalongkorn Memorial Hospital, Bangkok, Thailand. A single sample of whole blood was collected in a standard EDTA tube for DNA extraction. A group of 107 (42 males and 65 females) anonymous, unrelated, healthy Thai children served as a control group (13.12±2.18 years). All the controls had no history of BA, autoimmune, or liver diseases. The case-control study was performed on these 106 children with BA and 107 controls. "Thai" was defined as the parents (father and mother) of the BA and healthy control subjects having been born in Thailand.

Genotyping of adiponectin polymorphisms

Genomic DNA was extracted from peripheral blood leukocytes of BA children and healthy controls using Illustra Blood Genomic Prep Midi Flow Kit (GE Healthcare, Buckinghamshire, UK). Genomic DNA samples were stored at -20°C for further analysis. The adiponectin +45T/G (rs2241766) and +276G/T (rs1501299) genotypes were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. The +45T/G SNP in the adiponectin gene was genotyped by amplification of genomic DNA using the following primers:[23] forward, 5'-TCCTTTGTAGGTCCCAACT-3' and reverse, 5'GCAGCAAAGCCAAAGTCTTC-3'. The amplification conditions were as follows: 95°C for 15 minutes, followed by 35 amplification cycles at 95°C for 30 secconds, 56°C for 30 seconds, and 72°C for 60 seconds, and a final extension at 72°C for 7 minutes. The amplified PCR product was 503 bp in length. The PCR product was subsequently digested with the enzyme *BspH1* (New England Biolabs, Beverly, MA), which yielded 375- and 128-bp fragments (T allele of +45T/G). The +276G/T SNP in the adiponectin gene was genotyped by amplification of genomic DNA using another pair of primers:[23] forward, 5'-ACACTGATATAAACGCCATGAA-3' and reverse, 5'-GCAGCAAAGCCAAAGTTTC-3'. The amplification conditions were as follows: 95°C for 10 minutes, followed by 40 amplification cycles at 95°C for 30 seconds, 50°C for 30 seconds and 72°C for 60

seconds, and a final extension at 72°C for 7 minutes. The amplified PCR product was 168 bp in length. The polymorphism was typed using the enzyme *Bgl1* (New England Biolabs), which yielded 147- and 21-bp fragments (G allele of +276G/T). In the genotyping experiments for both SNPs, the digestion fragments were subjected to electrophoresis on 2.5% agarose gel and 12% polyacrylamide gel, respectively, containing ethidium bromide and visualized on an ultraviolet transilluminator.

Statistical analysis

All statistical analyses were performed with SPSS statistical package, version 17.0 (SPSS Inc., Chicago, IL). Data were expressed as mean ± standard deviation. Genotype and allele frequencies of the adiponectin +45T/G and +276G/T SNPs were determined by direct counting. The Hardy-Weinberg equilibrium was assessed using the Chi-square test. The frequencies of adiponectin haplotypes and linkage disequilibrium between paired SNPs in BA patients and the controls were estimated by using the Haploview 4.0 program. Distribution of alleles and genotypes of adiponectin in BA and control groups was determined by the Chi-square test (3 × 2, 2 × 2 contingency table) with Yates's correction for continuity. Student's *t* test was used for comparison of the two groups where appropriate. The data were presented as odds ratio (OR) with 95% confidence intervals (CIs) for the association between adiponectin genetic variants and risk of BA. *P* values <0.05 were considered statistically significant.

Results

The genotype and allele frequencies of adiponectin gene SNPs in BA patients and healthy controls are demonstrated in Table 1. The genotype distributions of +45 T/G and +276 G/T SNPs were in the Hardy-Weinberg equilibrium for both BA patients and the controls (P>0.05). The genotype distributions in BA group were as follows: 47 (44.3%) T/T, 41 (38.7%) T/G, 18 (17.0%) G/G at +45 T/G, and 71 (67.0%) G/G, 30 (28.3%) G/T, 5 (4.7%) T/T at +276 G/T. The frequencies of T and G alleles at +45 T/G were 135 (63.7%) and 77 (36.3%), respectively, and those of G and T alleles at +276 G/T were 172 (81.1%) and 40 (18.9%), respectively.

As shown in Table 1, the frequency of the G/G genotype at +276G/T was significantly higher in BA patients than in the controls (P=0.009). As for +45T/G in BA patients, the frequency of the T/T genotype tended to be lower than in the controls, but the difference was not significant. Moreover, the G allele at +276G/T in BA patients was significantly more common than in the controls (P=0.0043). In contrast, the frequency of the T allele at +45T/G was not significantly different between BA patients and the controls.

The frequency of SNPs between females and males is shown in Table 2. The frequency of G/G genotype at +276G/T in BA females was significantly higher than in female controls (P=0.002). However, the frequency of G/T genotype in BA females was significantly lower than in female controls (P=0.0003). In contrast, BA males had no statistically significant differences in the frequencies of +276G/T genotype. The frequencies of +45T/G genotype were not different when comparing BA females and males with the controls. Moreover, the frequency of the G allele at +276G/T in BA females was significantly higher than in the controls (P=0.003), whereas that of the G allele in males was not different between BA patients and the controls. Regarding +45T/G, the frequency of the T allele in both females and males was not different between BA patients and controls.

Table 3 shows the association between cholestasis and adiponectin SNPs. We observed no statistically significant difference in allele frequencies and genotype distributions of +45T/G SNP when comparing BA patients with jaundice and those without jaundice. Concerning the +276G/T SNP, the frequency of G allele was significantly higher in BA patients with jaundice

Table 1. Genotypes and allele frequencies of the adiponectin gene in biliary atresia (BA) patients and the controls

+45 T/G (rs2241766) genotypes	Genotypes			OR (95% CI)	*P* value	Allele		OR (95% CI)	*P* value
	T/T	T/G	G/G			T allele	G allele		
Controls (*n*=107)	50 (46.7%)	45 (42.1%)	12 (11.2%)	1		145 (67.8%)	69 (32.3%)	1	
BA patients (*n*=106)	47 (44.3%)	41 (38.7%)	18 (17.0%)	0.91 (0.51-1.61)	NS	135 (63.7%)	77 (36.3%)	1.20 (0.79-1.83)	NS
+276 G/T (rs1501299) genotypes	Genotypes			OR (95% CI)	*P* value	Allele		OR (95% CI)	*P* value
	G/G	G/T	T/T			G allele	T allele		
Controls (*n*=107)	53 (49.5%)	42 (39.3%)	12 (11.2%)	1		148 (69.2%)	66 (30.8%)	1	
BA patients (*n*=106)	71 (67.0%)	30 (28.3%)	5 (4.7%)	2.07 (1.14-3.74)	0.009*	172*(81.1%)	40 (18.9%)	1.92 (1.19-3.08)	0.0043*

BA: biliary atresia; OR: odds ratio; CI: confidence interval; NS: not significant. *: P<0.05 by χ^2 test, *P* value for difference in distribution of genotypes and alleles between controls and BA patients.

compared to those without jaundice (P=0.014). The frequency of GG genotype was also found to be higher in BA patients with jaundice (79.5%) than in those without jaundice (59.7%); however, the difference was not significant. Noteworthy, no TT genotype at the +276G/T was found in BA patients with jaundice.

We examined the linkage of these two SNPs in BA patients and the controls. Linkage disequilibrium analysis revealed low linkage disequilibrium between +45T/G and +276G/T in both BA patients and the controls (D'=0.214, r^2=0.008). Haplotype analysis was conducted to evaluate the combined effect of the two polymorphisms on BA risk. Four haplotypes were reconstructed from the two SNPs in BA patients and the controls (Table 4). The most common haplotype was +45 T and +276 G (TG), found in 49.1% of the BA patients and 46.3% of the controls. Haplotype frequency revealed a significant difference between BA patients and the controls with regard to the +45 G and +276 G (GG) combination (P=0.029) and the +45 G and +276 T (GT) combination (P=0.027), but the difference for each individual haplotype was not significant after 100 000 permutations for single markers.

Discussion

BA is an inflammatory obstructive cholangiopathy of unknown etiology, leading to progressive fibrosis and cirrhosis.[24] Regardless of the triggering insult, the close association between inflammatory cells and

Table 2. Genotype and allele frequencies of the adiponectin gene in female and male patients

+45 T/G (rs2241766) genotypes	Genotypes			OR (95% CI)	*P* value	Allele		OR (95% CI)	*P* value
	T/T	T/G	G/G			T allele	G allele		
Male control (*n*=42)	21 (50.0%)	15 (35.7%)	6 (14.3%)	1	NS	57 (67.9%)	27 (32.1%)	1	NS
Male BA (*n*=44)	20 (45.5%)	16 (36.4%)	8 (18.2%)	0.83 (0.33-2.12)		56 (63.6%)	32 (36.4%)	0.83 (0.42-1.63)	
Female control (*n*=65)	29 (44.6%)	29 (44.6%)	7 (10.8%)	1	NS	87 (66.9%)	43 (33.1%)	1	NS
Female BA (*n*=62)	27 (43.5%)	25 (40.3%)	10 (16.2%)	0.96 (0.45-2.05)		79 (63.7%)	45 (36.3%)	0.87 (0.50-1.50)	
+276 G/T (rs1501299) genotypes	Genotypes			OR (95% CI)	*P* value	Allele		OR (95% CI)	*P* value
	G/G	G/T	T/T			G allele	T allele		
Male control (*n*=42)	23 (54.8%)	13 (30.9%)	6 (14.3%)	1		59 (70.2%)	25 (29.8%)	1	
Male BA (*n*=44)	26 (59.1%)	16 (36.4%)	2 (4.5%)	1.19 (0.47-3.07)	NS	68 (77.3%)	20 (22.7%)	1.19 (0.47-3.07)	NS
Female control (*n*=65)	30 (46.2%)	29‡(44.6%)	6 (9.2%)	2.76 (1.20-6.43)	0.008†	89 (68.5%)	41 (31.5%)	1	
Female BA (*n*=62)	45*(72.6%)	14 (22.6%)	3 (4.8%)	3.09 (1.38-6.95)	0.002*	104‡ (83.9%)	20 (16.1%)	2.40 (1.26-4.59)	0.004‡

BA: biliary atresia; OR: odds ratio; CI: confidence interval; NS: not significant. *: P<0.05 by χ^2 test; ‡: P<0.05 by χ^2 test.

Table 3. Genotype frequencies of the adiponectin gene in biliary atresia (BA) patients with jaundice and without jaundice

+45 T/G (rs2241766) genotypes	Genotype			OR (95% CI)	*P* value	Allele		OR (95% CI)	*P* value
	T/T	T/G	G/G			T allele	G allele		
Non-jaundice (*n*=67)	30 (44.8%)	25 (37.3%)	12 (17.9%)	1		85 (63.4%)	49 (36.6%)	1	
Jaundice (*n*=39)	17 (43.6%)	16 (41.0%)	6 (15.4%)	0.95 (0.40-2.28)	NS	50 (64.1%)	28 (35.9%)	1.03 (0.55-1.92)	NS
+276 G/T (rs1501299) genotypes	Genotype			OR (95% CI)	*P* value	Allele		OR (95% CI)	*P* value
	G/G	G/T	T/T			G allele	T allele		
Non-jaundice (*n*=67)	40 (59.7%)	22 (32.8%)	5 (7.5%)	1		102 (76.1%)	32 (23.9%)	1	
Jaundice (*n*=39)	31 (79.5%)	8 (20.5%)	0 (0.0%)	2.62 (0.96-7.28)	NS	70 (89.7%)	8 (10.3%)	2.75 (1.13-6.90)	0.014*

OR: odd ratio; CI: confidence interval; NS: not significant. *: P<0.05 by χ^2 test, P value for difference in distribution of genotypes between BA patients with jaundice and without jaundice.

Table 4. Haplotype frequencies for adiponectin variations in BA patients and the controls

Haplotypes	BA patients (2N=212), *n* (%)	Controls (2N=214), *n* (%)	χ^2	*P* value*	Empirical *P* value†
TG	104 (49.1)	99 (46.3)	0.274	NS	NS
GG	68 (32.1)	50 (23.4)	4.752	0.029*	NS
TT	32 (15.1)	45 (21.0)	3.129	NS	NS
GT	8 (3.7)	20 (9.3)	4.897	0.027*	NS

BA: biliary atresia; NS: not significant. *: P<0.05 by χ^2 test using haploview 4.0, for comparison of haplotypes between BA patients and the controls; †: Empirical P value: after 100 000 permutations for single markers.

injured bile ducts in patients with BA suggests that inflammatory cells may play a major role in disease pathogenesis.[25,26] Inflammation is an important feature of BA, and recent studies suggest that it occurs in a genetically susceptible host. Adiponectin is now recognized as an anti-inflammatory adipokine and is the only adipokine that has been shown to be down-regulated in obese subjects. Although the mechanism governing regulation of adiponectin is not entirely understood, TNF-α has been demonstrated to down-regulate the expression of adiponectin. Adiponectin leads to down-regulation of pro-inflammatory cytokines such as TNF-α and IL-6, while inducing anti-inflammatory cytokines such as IL-10 and IL-1 receptor antagonist.[27,28] Adiponectin can further attenuate the effect of inflammation by decreasing expression of certain adhesion molecules, including intracellular adhesion molecule-1.[27] Thus, adiponectin may be a key intermediary in cell-mediated inflammatory diseases such as BA.

In this study, we investigated whether specific SNPs or haplotypes of the adiponectin gene were associated with a risk of BA in Thai patients. Our results suggested that adiponectin SNP is associated with progression of liver fibrosis and inflammation. Adiponectin is known to be a key adipokine in several chronic inflammatory disorders.[29,30] Out of two SNPs studied, we found that the frequency of G/G and G alleles at +276G/T SNP was significantly higher in BA patients than in the controls. The G allele at +45T/G SNP has been reported to be associated with decreased adiponectin expression,[31] whereas others have found that the T allele is associated with increased expression.[32,33] An association between the G/G genotype and G allele of + 276G/T SNP and increased risk of diabetes in Japanese subjects has been reported by another study.[34] In agreement with these studies, we also found that the G/G genotype and G allele of +276G/T SNP was more frequent in BA patients.

BA is a rare condition by which females are affected slightly more often than males.[25] The survey data suggested that genomic background might have more influence on BA females. Another finding in our study showed that the frequencies of the adiponectin SNP sites differed significantly between BA patients and the controls only among females. With regard to the genotype distribution of +276G/T SNP in only females, we found the G/G genotype in BA patients was significantly higher than in the controls; however, the G/T genotype in BA patients was significantly lower than in the controls. The association between adiponectin SNPs and the pathogenesis of disease, especially in women has also been demonstrated. Based on the reported association between the G/G genotype of + 276G/T SNP and lower serum adiponectin levels, an increased risk of diabetes has been emphasized in women with polycystic ovary syndrome.[32,33] Moreover, Tokushige et al[35] revealed that in female non-alcoholic fatty liver disease patients, the frequency of G/G at +276G/T SNP was significantly higher than in the controls. Therefore, our findings suggest that the G/G genotype and G allele increase the susceptibility to BA and the G/T genotype in female patients may confer protection against BA. However, our results indicate that while the adiponectin +276G/T SNP may influence the susceptibility of BA, +45T/G SNP does not seem to play a major role in the susceptibility of BA.

Furthermore, Tacke et al[36] reported that adiponectin was associated with biochemical markers of cholestasis and was significantly elevated in patients with biliary liver diseases. These findings suggested that the adiponectin elevation in cholestatic liver diseases was mediated by reduced biliary excretion. Nevertheless, our current investigation demonstrated that the +45T/G and +276G/T variants did not significantly affect the risk of BA patients with jaundice or without jaundice. It is noteworthy that the T/T genotype at +276G/T was not found in BA patients with jaundice. Since our study was limited by the small numbers of BA patients with regard to the subgroup of jaundice status, the potential connection between adiponectin gene polymorphisms and susceptibility to cholestatic liver diseases should be investigated in a larger group in order to reach an unequivocal conclusion.

For case-control studies, a methodological approach based on haplotypes is more beneficial than single-locus analysis to provide insight into disease development. Previous publications showed that the haplotypes at the adiponectin locus were associated with adiponectin gene expression and metabolic parameters in Caucasian subjects,[37] Swedish subjects[38] and Japanese subjects.[23] Although no significant association between the haplotypes of adiponectin SNPs and BA was found in this study, the possibility of an unidentified BA-related adiponectin haplotype could not be excluded. Therefore, ethnic differences might be responsible for the discrepancies in haplotypes. It could be speculated that +276G/T SNP may participate in several different haplotypes. If that was the case, a combination of other unrecognized functional variants located nearby or in strong linkage disequilibrium might be required to elicit predisposition to BA.

In summary, adiponectin and other adipokines are known to modulate the inflammatory response and liver injury.[39] Our study demonstrates that the G/G genotype and G allele of this adiponectin +276G/T SNP are more frequent in Thai BA patients and may serve as an inverse marker of the systemic inflammatory response to BA since

adiponectin mitigates the pro-inflammatory effects of other adipokines. Eventually, we hope that genetic analysis including that of the adiponectin genes will clarify the pathogenesis and progression of BA, and this particular SNP might be a useful marker for the risk of BA.

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