Analysis of Connective Tissue Growth Factor Promoter Polymorphism in Thai Children with Biliary Atresia

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Objective: *Connective tissue growth factor (CTGF) has been proposed to play a key role in the pathogenesis of hepatic fibrosis in biliary atresia (BA). The aim of the present study was to determine the single nucleotide polymorphism (SNP) in the promoter region of CTGF gene in a Thai population, and to investigate the possible role of CTGF promoter polymorphism in the susceptibility of BA.*
Material and Method: *Genomic DNA was obtained from 84 patients with BA and 142 healthy controls. The -447G/C and -132C/G in CTGF promoter were amplified and examined by amplification-refractory mutation system (ARMs) and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis, respectively. The test of Hardy-Weinberg equilibrium (HWE) was performed using HWE program of SNPAnalyzer. Statistical analysis was carried out with SPSS and Epi Info.*
Result: *According to the previous experiment, there were two SNPs, which were at position -447 and -132 on the promoter. However, there was only one SNP at the position -447 in the Thai population. No significant differences in genotype and allele frequency were observed between BA and controls or with BA subgroups.*
Conclusion: *The present study demonstrated that CTGF polymorphism at -447G/C was not associated with BA and the jaundice status of the postoperative BA patients.*

Keywords: *Biliary atresia, CTGF, SNP, Polymorphism*

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Biliary atresia or BA is a cholestatic disease of infancy characterized by the complete obliteration of the lumen of the biliary tree[1-3]. BA is the most common cause of chronic cholestatic jaundice in neonates. Both intrahepatic and extrahepatic bile ducts demonstrate evidence of a progressive inflammatory fibrosclerosing cholangiopathy, which results in biliary cirrhosis and portal hypertension[4]. If left untreated, BA leads to death from end-stage liver disease within the first few years of life[5]. Despite the long-term survival of liver transplantation, Kasai portoenterostomy remains the standard of treatment to reestablish bile flow in infants with BA[6]. Although the etiology and pathogenesis of this disorder remains unknown, several possible mechanisms have been proposed to be involved including infectious, toxic, genetic, and immunological factor.

Fibrogenesis, characterized by cell proliferation and accumulation of extracellular matrix components requires numerous mediators. Various cytokines and growth factors have been implicated in the pathogenesis of fibrosis and wound healing[7] as well as hepatic fibrosis. Previous studies from the authors'

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laboratory have shown that a number of pro-inflammatory cytokines and growth factors, such as procollagen-III-peptide[8], endothelin-1[9], hyaluronan[10], hepatocyte growth factor[11], interleukin-8[12], and inter-cellular adhesion molecule-1[13], have been implicated in the pathogenesis of hepatic fibrosis and were elevated in the serum of children with BA. These cytokines could play a variety of potential roles in the regulation of diverse functions of inflammatory cells and influence the regulation of fibrotic process by autocrine and/or paracrine mechanisms[7].

Connective tissue growth factor (CTGF) has currently received much attention as a possible key determinant of progressive fibrosis, excessive scarring, and wound repair[14]. CTGF is a highly profibrogenic molecule which is over-expressed in many fibrotic lesions of major organs and tissues, including those of the liver[15]. There are many experimental studies on CTGF and its expression. Previous studies have demonstrated that serum CTGF levels and CTGF mRNA expression correlated significantly with progression and severity of hepatic fibrosis in BA[16-18], suggesting that CTGF might serve as a potential indicator for monitoring the progress of fibrosis in this disease. The CTGF gene is located on chromosome 6q23.1. Recently, Blom et al searched for polymorphisms in promoter of *CTGF* gene and identified two single nucleotide polymorphisms[19]. The first is a G→C substitution at position -447 and the other is a C→G substitution at position -132. The gene polymorphisms in the promoter region at the -447G/C and -132C/G probably influence transcription activity and gene expression levels. The position -447 is close to a binding site for MZF1 or myeloid zinc Finger 1, which acts as a bifunctional transcription regulator depending on the cellular environment. When CTGF was transfected into NIH3T3 cells, these cells were shown to increase growth rate. Moreover, these MZF1 transfected cells were able to develop into fibrosarcomas when injected into athymic mice[19]. The -132C/G lies adjacent to a Pbx-1 homeo domain box. Heterodimers between Pbx and Hox genes regulate the expression of a gene that is involved in anterior-posterior patterning and development[19]. However, previous studies were documented in different populations and these resulted in conflicting information. Nevertheless, the polymorphisms in *CTGF* gene among children with biliary atresia have not been studied. There may be some links between the pathophysiology of BA and polymorphisms of promoter in the *CTGF* gene. The authors' hypothesis of the present study is that polymorphisms in *CTGF* promoter are associated with BA.

Material and Method

Study population

The present study was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University. All parents or legal guardians of the recruited children with BA, healthy children and adults were informed of the purpose of the present study. Written informed consent was obtained from each of them.

Eighty-four BA patients (44 girls and 40 boys; mean age $\pm$ SD = 6.12 $\pm$ 4.79 years) from the Thai population were included during routine follow-up at the Department of Pediatrics, King Chulalongkorn Memorial Hospital, Bangkok, Thailand. In total, the 142 controls consisted of two groups: (1) healthy children control (63 girls and 47 boys; mean age $\pm$ SD = 13.30 $\pm$ 3.01 years) who attended the Well Baby Clinic at the King Chulalongkorn Memorial Hospital for immunization with normal physical examinations and no underlying disease; and, (2) 32 pregnant women coming for antenatal care at the hospital (mean age $\pm$ SD = 34.69 $\pm$ 5.84 years). To compare the treatment outcomes among BA patients, they were classified according to their serum levels of total bilirubin (TB) into two groups: patients without jaundice (TB < 2.0 mg/dl, n = 47) and patients with jaundice (TB $\geq$ 2.0 mg/dl, n = 37). No subject in the present study received liver transplantation or exhibited symptoms and signs of fever or ascending cholangitis or clotting abnormalities at the time of blood sampling.

DNA preparation

Blood samples were collected in EDTA tubes. Genomic DNA of BA and healthy control was extracted from buffy-coat leukocytes by proteinase-K treatment and the standard phenol/chloroform extraction method[20].

Polymorphism analysis

The two SNPs, *CTGF* -447G/C and -132C/G, used in the present study were from a previously published report by Blom et al[19] and were determined by amplification refractory mutation system (ARMs) and restriction fragment length polymorphism (RFLP), respectively. Before the *CTGF* promoter polymorphisms were analyzed, the region between position -559 and 39 relative to the promoter were amplified by polymerase chain reaction (PCR) by using the forward primer (CTGF-F) 5'-CAGGTAGGCATCTTGAG-3' and a re-

verse primer (CTGF-R) 5'-CACTGGCTGTCTCCT-3' from previously published information[19]. Using a programmable thermal cycler gradient PCR system (Eppendorf AG, Hamburg, Germany), samples were subjected to initial denaturation for 3 min at 94 C, followed by 40 cycles of 94 C for 30 s, for denaturing, 30 s at 59 C for annealing and 1 min at 72 C for extension, followed by a final 7 min extension at 72 C. The primary PCR reaction mixture comprised 100 ng/ml of genomic DNA, 0.5 μM CTGF-F, 0.5 μM CTGF-R, 10 μl of 2.5X MasterMix (Eppendorf, Germany) and sterile water to a final volume of 25 μl A 598 bp fragment containing -447G/C and -132C/G polymorphism in promoter of *CTGF* were amplified.

To analyze the -447G/C promoter polymorphism, the polymorphic site was determined by ARMs. The 2 μl of 1:1000 dilution of primary PCR product were combined with a reaction mixture containing 10 μl of 2.5X MasterMix (Eppendorf, Germany), 0.5 μM CTGF/G (5'-GCAAATCATTGCTAAAGGGTTG-3') or CTGF/C (5'- GCAAATCATTGCTAAAGGG TTC-3'), 0.5 μM CTGF-R and sterile water to 25 μl. PCR was performed under the following conditions: after an initial 3 min denaturation step at 94 C, 40 cycles of amplification were performed, each including 30 s denaturation at 94 C, 30 s annealing at 63 C and 1 min extension at 72 C, followed by a final 7 min extension at 72 C.

Genotyping of the -132C/G polymorphism was performed by digestion of primary PCR product with the each restriction enzyme *Bst*UI (New England Biolabs, MA). After PCR, 10 μl of the restriction mixture was digested with 1 U *Bst*UI in 10X NE buffer 2 (New England Biolabs, MA) for 4 h at 60 C. The digested mixture was resolved on a 2.0% agarose gel stained with ethidium bromide. DNA from individuals with the homozygous C genotype (CC) produced three bands at 427, 88 and 83 bp; the homozygous G genotype (GG) produced three bands at 515, 88 and 83 bp; and the heterozygous genotype (CG) produced four bands at 515, 427, 88 and 83 bp. The result was confirmed by *Hpa*II (New England Biolabs, MA) at 37 C for 4 h. *Hpa*II digestion gave fragments of 427, 97 and 74 bp for the CCGG base arrangement. Fragment digested with *Hpa*II were subjected to electrophoresis and visualized under UV light.

Statistical analysis

Allele and genotype frequencies were estimated by gene counting method. With 2x2 table, the odd ratios were calculated together with 95% confidence intervals by χ^2 from Epi Info version 6, (CDC Atlanta, GA). Hardy-Weinberg equilibrium was evaluated by SNPAnalyzer program (www.istech21.com/bionics/consulting_6.htm). All statistical tests were performed using SPSS software for Windows, Version 13 (SPSS Inc Chicago, IL). Values of $p < 0.05$ were considered to indicate statistical significance.

Results

Samples from 84 cases of BA, 142 control subjects were genotyped for -447 and -132 *CTGF* promoter polymorphisms. Genotype distributions of these polymorphisms were all in Hardy-Weinberg equilibrium. Sequence of *CTGF* promoter polymorphisms (-447G/C and -132C/G) were shown in Fig. 1. At -132C/G position, *BstU*I digestion shows the results of both populations having homozygous GG (Fig. 1c). Sequence analysis to confirm RFLP revealed that all samples from both populations were not homozygous GG, but had an arrangement of bases different from a study reported by Blom et al[19] (Fig. 1f). *BstU*I digestion designed for the polymorphism in other populations cannot digest at this position. Therefore, the result was initially misinterpreted as homozygous GG. In addition, *Hpa*II designed for the polymorphism in the authors' populations to confirm sequence analysis can digest all samples from both populations (Fig. 1e). These sequences have been submitted to Genbank with the accession number DQ337193-4. The gel electrophoresis of ARMs is shown in Figure 1a and sequence chromatograms of homozygous GG and heterozygous GC are shown in Fig. 1b and 1d, respectively. Allele and genotype frequencies for polymorphisms are shown in Table 1. Genotype frequencies of -447G/C among the 84 cases and 142 controls are as follows: G/G 83.33% and 92.25%, G/C 16.67% and 7.75%, C/C 0% and 0%, respectively.

The G and C allele frequencies were 91.67% and 96.13% (BA vs controls), and 8.33% and 3.87%, respectively. There was a significant difference in the genotype distributions ($p = 0.04$) and there was no significant difference in the allele frequencies of the *CTGF* -447 promoter polymorphism between the cases and the controls ($p = 0.05$). Further analysis showed no association with relative risk from polymorphism of genotype and allele with BA, OR (95% CI) = 2.38 (0.96-5.98) and 1.33 (0.37-4.84), respectively. Genotype frequencies for the *CTGF* -447G/C polymorphism were further analyzed based on the status of jaundice by using serum total bilirubin cut-off at 2 mg/dl, as shown in Table 2. There were no significant differences in the frequencies of G/G, G/C and C/C genotypes that were

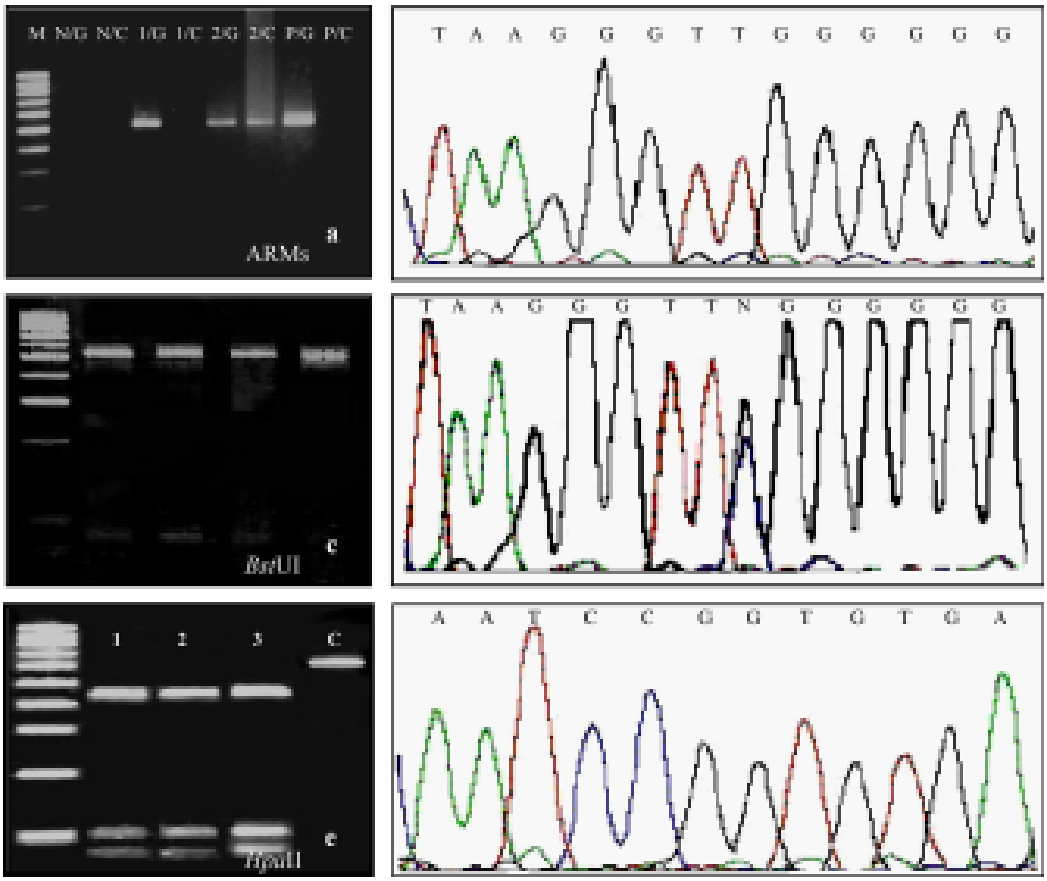

Fig. 1 2% agarose gel electrophoresis of ARMs and RFLP together with sequencing results of the samples(a) - ARMs result for -447G/C position: M 100 bp marker, N Negative control, 1 Homozygous GG, 2 Heterozygous GC, P Positive control GG; (b) and (d) - The two different genotypes (homozygous GG and heterozygous GC) are confirmed by sequence analysis; (c) - RFLP result for -132C/G position: *Bst*UI digestion produced three bands (515, 88 and 83 bp) as homozygous GG; (e) - *Hpa*II digestion for confirm sequence analysis (427, 97 and 74 bp) 1, 2 and 3 are samples, C is the control tube of distilled water instead of enzyme. (f) - Sequencing results of all samples that giving homozygous GG indicated that all samples cannot be digested by *Bst*UI

observed between BA patients with jaundice and those without jaundice.

Discussion

Connective tissue growth factor (CTGF), a member of the recently described CCN gene family, is a cysteine-rich pro-adhesive matricellular polypeptide that plays a major role in the formation of blood vessels, bone, and connective tissue. CTGF is produced by a variety of cell types in the liver (such as fibroblast, myoid cells, hepatocyte, bile duct epithelial cell, mononuclear cell, and endothelial cell) in response to diverse stimuli, including those associated with fibrosis and/or hepatic stellate cell (HSC) activation. CTGF is bound to both low density lipoprotein receptor-related protein (LRP) and integrins on the HSC surface. This results in the activation of intracellular signaling cascades that promote proliferation, adhesion, motility, and collagen synthesis. CTGF may also be involved in direct synergistic binding interactions with other fibrogenic molecules such as TGF-β[21].

In the past few years, there have been many studies supporting the notion that CTGF mRNA and protein levels are associated with the progression of hepatic fibrosis such as primary biliary cirrhosis and chronic hepatitis[22,23]. Especially, CTGF expression is increased in BA and correlated with the degree of fibrosis in the liver of the patients. Tamatani et al

Table 1. Genotype and allele frequencies (%) of *CTGF* -447G/C polymorphism between BA patients and controls

	BA n = 84 (%)	Controls n = 142 (%)	Odds ratio (95%CI)	*p*
Age (yr)	6.12 ± 4.79	17.39 ± 9.21		<0.0005
Male:female	40:44	47:95		0.03
Genotype				
GG	70 (83.33)	131 (92.25)	Ref	0.04
GC	14 (16.67)	11 (7.75)	2.38 (0.96-5.98)	
CC	0 (0)	0 (0)		
Alleles				
G	154 (91.67)	273 (96.13)	Ref	0.05
C	14 (8.33)	11 (3.87)	2.26 (0.94-5.47)	

Ref. = Reference category

Table 2. Genotype frequencies (%) of *CTGF* -447 polymorphism in BA patients (n = 84) based on the status of jaundice

	No Jaundice n = 47 (%)	Jaundice n = 37 (%)	Odds ratio (95%CI)	*p*
Age (yr)	6.70 ± 4.35	5.38 ± 5.26		0.210
Male:Female	24:23	16:21		0.476
Genotype				
GG	40 (85.11)	30 (81.08)	Ref	0.623
GC	7 (14.89)	7 (18.92)	1.33 (0.37-4.84)	
CC	0 (0)	0 (0)		

Ref. = Reference category

reported that CTGF was specifically induced in human fibroblasts by TGF-β and showed that the serum levels of CTGF were significantly correlated with the progression of hepatic fibrosis in BA[16]. By using in situ hybridization of liver biopsies, Narkewicz et al observed high levels of CTGF expression in 9 of 11 BA, with localization of biliary epithelial cells and vascular endothelial cells, and CTGF mRNA was correlated with fibrosis in BA and all livers[17]. Additionally, Kobayashi et al evaluated the expression of CTGF mRNA of liver biopsy specimens and revealed that CTGF was strongly expressed in BA, especially in HSC and hepatocytes. These suggest that HSC and hepatocytes may be a source of CTGF synthesis and the strong correlation of CTGF with collagen type IV indicates that CTGF plays an essential role in the pathogenesis of progressive fibrosis in BA[18].

Blom et al studied human connective tissue growth factor promoter polymorphisms in 45 healthy controls and 32 patients with ischemic heart disease using M13 tailed sequence specific CTGF primers for amplification of 600 bp fragment upstream of the transcription start site. Amplicons were bi-directionally sequenced with a dye primer M13 forward and reverse sequencing kit[19]. At -132 position of *CTGF* promoter, they found a C to G polymorphism in one patient with IHD. Moreover, in five of the 32 patients with IHD and in six of the 45 healthy controls, a G to C polymorphism was found at position -447. The present study examined the promoter region along with the previous work on *CTGF* polymorphisms (-447G/C and -132C/G)[19]. We hypothesized that these polymorphisms might be associated with the susceptibility of BA.

The present study clearly demonstrated that there was no *CTGF* -132C/G polymorphism in the Thai population. The difference in the arrangement of base obtained in the other two studies mentioned above may reflect the geographical and racial differences in the population. In addition, the *CTGF* -447G/C polymorphism did not show any difference in the genotype frequencies between the controls and the BA patients. However, a trend towards an increased genotype frequency of the heterozygous combination (G/C) of -447 polymorphism was seen in BA patients, more than the

control subjects (p = 0.04; OR: 2.38; CI: 0.96-5.98, Table 1). Furthermore, the variant genotype frequencies of -447G/C did not differ between BA patients without jaundice and those with persistent jaundice. These suggest that *CTGF* promoter polymorphisms are unlikely to be associated with the risk and susceptibility to BA patients.

Although there are significant differences in age and gender between BA patients and healthy controls in the present study, these discrepancies are not confounding factors. The explanation for these is likely that BA is a unique disorder occurring exclusively during the neonatal period. The fetal/perinatal form is evident within the first two weeks of life, whereas the postnatal type presents in infants aged 2-8 weeks. Furthermore, human beings have the same genome throughout one's life and the incidence of BA between male and female is similar. Therefore, differences in age and gender between BA patients and controls in the present study are unlikely to affect the present results.

The negative findings found in the present study have various explanations. Firstly, the findings are real. Secondly, variation in *CTGF* promoter polymorphisms may contribute to the susceptibility to BA, but the effect is small. A much larger sample size is probably required to detect any difference. Thirdly, the present study only sequenced in the promoter region and cannot exclude a chance variant elsewhere in the gene. However, CTGF is still required for progress of liver fibrosis. Finally, sequence variation in other genes in this pathway may affect the risk and susceptibility to BA patients.

In conclusion, -447G/C CTGF promoter gene polymorphism is not associated with BA and the jaundice status of the postoperative BA patients. Thus, the increase in hepatic CTGF expression in BA patients reported by other groups[17,18] is unlikely to be related to -447G/C CTGF promoter gene polymorphism.

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การศึกษา polymorphism ในส่วน promoter ของจีน connective tissue growth factor ในผู้ป่วยเด็กโรคท่อน้ำดีตีบตันที่พบในประเทศไทย

ภัทรธิดา สงวนหมู่, ไพศาล เวชชพิพัฒน์, วรนุช จงศรีสวัสดิ์, จินตนา จิรถาวร, สิทธิศักดิ์ หรรษาเวก, อภิรดี เทียมบุญเลิศ, ยง ภู่วรวรรณ

connective tissue growth factor (CTGF) มีบทบาทสำคัญต่อพยาธิกำเนิดของการเกิดเยื่อพังผืดที่ตับในโรคท่อน้ำดีตีบตัน วัตถุประสงค์ของการวิจัยนี้เพื่อศึกษา polymorphism ในส่วน promoter ของจีน CTGF ในกลุ่มตัวอย่างประเทศไทย และศึกษาความสัมพันธ์ของ polymorphism ดังกล่าวกับโรคท่อน้ำดีตีบตัน โดยศึกษาที่ตำแหน่ง -447G/C และ -132C/G จากกลุ่มผู้ป่วยโรคท่อน้ำดีตีบตันจำนวน 84 คน และกลุ่มควบคุมจำนวน 144 คน ด้วยวิธี amplification-refractory mutation system (ARMs) และ polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) ตามลำดับ จากนั้นทำการวิเคราะห์ด้วยโปรแกรมทางพันธุศาสตร์และทางสถิติ

จากการศึกษาพบว่าในกลุ่มตัวอย่างประชากรไทยที่ใช้ศึกษาครั้งนี้พบ polymorphism ตำแหน่ง -447G/C แต่ไม่พบในตำแหน่ง -132C/G นอกจากนี้พบว่าความถี่ของจีโนไทป์และอัลลีลระหว่างผู้ป่วยและกลุ่มควบคุมไม่มีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ อีกทั้งยังไม่พบความแตกต่างกันระหว่างกลุ่มผู้ป่วยที่มีภาวะเหลืองและไม่มีภาวะเหลือง การศึกษาครั้งนี้สรุปได้ว่า CTGF promoter polymorphism ที่ตำแหน่ง -447 นั้นไม่มีความสัมพันธ์กับโรคท่อน้ำดีตีบตันรวมทั้งสภาวะเหลืองในผู้ป่วยดังกล่าว