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Tumour necrosis factor-alpha gene polymorphisms in Iranian patients with biliary atresia

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ABSTRACT

Background: Biliary atresia (BA) is a progressive inflammatory destructive process of the bile ducts. This study evaluated the relationship between single-nucleotide polymorphisms in the promoter region of tumour necrosis factor-alpha (TNF-α) gene and bilaiary atresia. **Materials and Methods:** Genomic deoxyribonucleic acid from 16 patients with established diagnosis of BA and 36 patients with INC was obtained. The genotypes of TNF-α-1031 (T/C) and TNF-α-308 (G/A) were determined using the restriction fragment length polymorphism-polymerase chain reaction and the results were analysis with proper statistic software. **Results:** The frequencies of T/T, T/C in TNF-α-1031 and G/G, G/A in TNF-α-308 were as same as control group. Moreover, we have same deduction for allele frequency and haplotypes analysis (T allele: 84.37%; G allele: 87.5%) in BA patients (T allele: 80.56%; G allele: 86.11%) in controls. In all cases variants of polymorphism did not affect the severity or incidence of BA disease. **Conclusion:** although no significant associations were found between BA and control groups, it seems meaningful that since the nature of BA is multi factorial. Next step will be considering a new target such as downstream modulation of the TNF-α pathway or other cytokines and chemokines which act directly/indirectly.

Key words: Biliary atresia, cholestasis, tumour necrosis factor-alpha polymorphisms

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INTRODUCTION

Cholestasis is a condition in which bile cannot run from the Liver to the duodenum properly.[1] Liver forms the bile to aid the digestion process of fats. This function of liver begins in bile canaliculi which form between two adjacent hepatocytes. The canaliculi join each other and form the larger structures that referred to Canal of Hering, ductules (with an epithelial surface), ducts and the common hepatic duct, respectively.[1,2] Cholestasis has particular symptoms; pruritus is the primary sign of cholestasis which is believed to be due to the interactions between the opioidergic nerves and the serum of bile acids.[3] The second one is jaundice which is not common in intrahepatic cholestasis, but it is prevalent in obstructive cholestasis. Pale stool is the third one that implies the obstructive cholestasis and the last sign is dark urine.[4]

There are several causes of cholestasis and due to these causes it could be categorised into the three comprehensive groups; first, extrahepatic cholestasis which occurs outside the liver. There are various causes for extrahepatic cholestasis, namely; bile duct tumours, stone in common bile duct, cysts, narrowing of bile ducts, pancreatitis and pressure on the bile ducts because of the nearby mass or tumour. Second, intrahepatic cholestasis that occurs inside the liver and it can be caused by amyloidosis, primary biliary cirrhosis, pregnancy, sepsis, viral hepatitis, bacterial abscess in liver and lymphoma. The last group is a certain medications that can also cause cholestasis (antibiotics such as ampicillin, birth control pills, anabolic steroids, prochlorperazine and erythromycin).[4,5]

Different frequencies of cholestasis have been showed in various populations, for instance; approximately 0.02% and 0.01% of live births with cholestasis have been reported in Australia and Norway, respectively.[6]

Neonatal cholestasis syndrome still represents a great challenge for hepatologists and paediatric surgeons. This syndrome comprises a wide range of clinical states varying from congenital malformations of hepatobiliary tree, inborn defects of metabolism and infections to a number of clinical states with genetic preference.[4,7,8] In a nutshell, neonatal cholestasis is divided into two general classes: The extrahepatic class and the intrahepatic class.[1] The majority of the studies have reported the extrahepatic biliary atresia (BA) and idiopathic neonatal hepatitis (INH) as main causes of cholestasis and some studies have reported the intrahepatic cholestasis as the most prevalent form of prenatal cholestasis in some populations.[4,6]

BA is distinguished by inflammatory obliteration of extrahepatic bile ducts within months of birth and progression to liver cirrhosis. Although there is not adequate evidences about the aetiopathogenesis of BA, an interaction of genetic susceptibility and unknown prenatal exposure to infectious and toxic factors have been suggested.[1,2] Approximately 70-80% of cholestasis in infants are caused by extrahepatic BA and INH. Since these two have not still had their own etiopathogenesis, only up to 20% of the cases have their cause determined. On the other hand, subject to the studied populations, there will be significant frequency differences of cholestatic nosology; In the United Kingdom the deficiency of alpha 1-antitrypsin was responsible for 17% of the cholestasis cases in infants whereas in South Africa, congenital syphilis contributed with 22% for causes.

Over all, it is estimated that prevalence of BA is nearby 1.5% of all neonatal cholestasis and is 0.007-0.012% among the live births.

In addition, all types of neonatal cholestasis have similar clinical features such as is jaundice, Pale stool, splenomegaly and dark urine therefore liver biopsy plays an important role to screen the different classes. And the point is that, drawing a distinction between the extrahepatic and intrahepatic neonatal cholestasis to manage the progress of disease.[4]

Cholestasis frequently occurs as a complication in patients with sepsis, extrahepatic bacterial infections, endotoxin (lipopolysaccharide [LPS]) and LPS-induced pro-inflammatory cytokines like necrosis factor (tumour necrosis factor-alpha [TNF-α]), interlukin-1 (IL-1) and IL-6 which are secreted mainly by macrophages and Kupffer cells.[2,5,9]

Several studies have reported a significant association between the mutated jagged1 gene and increased production of TNF-α in some severe cases of BA.[5,6,10] Moreover a genome–wide expression analysis indicated genetic susceptibility to pro-TH1 immunity in BA patients. These results and other findings show that the cytokine family productions have considerable effects in pathogenesis of BA and are parts genetically determined. TNF-α is a pro-inflammatory cytokine which has many effector functions, including directly stimulating of production of nitric oxide by macrophages and induction of apoptosis through direct interactions with TNF-Receptor p55 or, indirectly, by enhancing Fas expression in the Fas/Fas ligand apoptotic pathway.[11,12] Plus, the TNF-α is encoded in the class III region of HLA complex (6p21.3). It is revealed that the-308G/A promoter polymorphism have a role in development of primary sclerosing cholangitis. It is supposed that gene polymorphisms might influence the transcription and expression levels and the TNF-α-1031T/C and TNF-α-308G/A polymorphisms were related to TNF-α production.[5,10]

A number of single nucleotide polymorphisms in TNF-α promoter region have been identified, of which we have selected the two genotypes, TNF-α-1031T/C and TNF-α-308G/A,. It has been suggested that the -1031C and the -308G alleles cause an increase in TNF-α production.[5,10]

The objective of current study was to characterise association of TNF-α polymorphisms (TNF-α-1031T/C and TNF-α-308G/A) and susceptibility to cholestasis among the Iranian Azeri Turkish patients.

MATERIALS AND METHODS

A total of 52 sequential unrelated cases who have received a clinical and pathological diagnosis of cholestasis were screened in this study (16 patients with BA and 36 cases with neonatal cholestasis and others as control group). This study was performed according to approvals of the Ethics committee of Children Hospital, Tabriz University of Medical Sciences. For each patient, an ample pre-designed questionnaire filled out to rule out other causes. All families were informed about the study and written informed consent was obtained. A detailed history and clinical examination was done for all patients including parameters such as; age, sex and demographic analysis after surgery (for children with BA), presence and absence of jaundice, assessment of weight, size and consistency of liver, size of spleen below costal margin and presence of ascites.

The following laboratory investigations were carried out: Cell blood Count with differential count, liver function tests (total and direct bilirubin, international normalized ratio and albumin) and liver enzymes (alanine transaminase, aspartate transaminase, gamma glutamyl transpeptidase). Abdominal ultrasonographies were done for all. Moreover, liver biopsies performed at the time of diagnosis were re-evaluated for hepatic architecture, degree of fibrosis and inflammatory cellular filtrate. All BA patients received post-operative antibiotic prophylaxis for cholangitis and all the cholestatic children were on ursodeoxycholic acid and fat soluble vitamins.

Molecular analysis of TNF-α gene

Genomic deoxyribonucleic acid was extracted from peripheral leukocytes in whole-blood samples using the standard laboratory protocols. Noted region of TNF-α gene at position (-1031T/C) and (-308G/A) were investigated by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) using appropriate primers which were described by Bonyadi *et al*.[13] The 270 bp amplicon of TNF-α gene, encompassing the -1031T/C polymorphism, was amplified using the primers N1031F: 5'-GGGGA GAACAAAAGGATAAG-3' and N1031R: 5-CCCCATA CTCGACTTTCATA-3'. "Hot-start" PCR was subjected to initial denaturation for 5 min at 95°C, followed by 35 cycles of amplification (30 s at 95°C, 30 s at 55°C and 30 s at 72°C and 5 min at 72°C as final extension). The PCR is followed by an overnight digestion with the restriction enzyme BbsI (or BpiI) at 37°C. (T allele 270 bp, C allele 159 bp and 111 bp).

The 107 bp PCR product for-308G/A polymorphism were amplified using N308F:5'-AGGCAATA GGTTTTGAGGGCCAT-3' and N308R: 5'-TCCTCCCTGCTCCG ATT CCG-3' primers. PCR conditions were 5 min for initial denaturation at 95°C; 35 cycles at 95°C for 1 min for denaturation, 30 s at 65°C for annealing and 30 s at 72°C for extension, followed by 5 min at 72°C for final extension. After that, the PCR products were digested (at 37°C) by restriction enzyme NcoI (A allele, 107 bp, G allele 87 and 20 bp).

The results were evaluated after electrophoresis of RFLP products in 15% acrylamide gel and visualized by ethidium bromide and/or silver staining.

Statistical analysis

Comparison of alleles and genotype frequencies between patients and controls was carried out using Chi-square test with Yates' correction or Fisher's exact test, where appropriate. Differences in haplotypes frequencies of TNF-α promoter region between patients and controls were also analysed using Chi-square test. Hardy-Weinberg equilibrium for the genotype frequencies was also verified by the Chi-square test. $P \leq 0.05$ were regarded as statistically significant. The odds ratio (OR) and confidence intervals (CI) at 95% significant level were estimated for all data.

RESULTS

The age of patient and control groups varied from less than 1 month (15 days) to 4 months (mean: 2 months) and 1 months to 3 months (mean: 2 months) at the time of admission respectively. Moreover the consanguinity was discovered in about 40% of the families in both. The male-to-female ratio was estimated 0.78 (males: 7, 44%; females: 9, 56%) in patients and 1.8 (males: 23, 64%; females: 13, 36%) in controls.

All patients were diagnosed according to the diagnostic criteria proposed by the international study group for BA.[14] The frequency of each symptom in BA patients was: 75% (12/16) jaundice, 50% (8/16) dark urine, 75% (12/16) hepatomegaly, 41% (7/16) splenomegaly and 69% (11/16) pale stool respectively.

Comparison of the allele and genotype frequencies of TNF-α-1031T/C and TNF-α-308G/A showed no significant between BA patients and controls in this ethnic [Table 1]. The frequencies of the TNF-α-1031 T/T, T/C and C/C genotypes were 0.6875, 0.3125 and 0 in BD patients; and 0.6112, 0.3888 and 0 in controls, respectively, ($P = 0.15$ by Fisher's exact test and 0.2 by χ^2) and the allele frequencies of TNF-α-1031T were 0.8437 in BA patients and 0.8056 in controls ($P = 0.22$, OR = 1.39; 95% CI = 0.59-2.88).

On the other hand, the frequencies of the TNF-α-308 G/G, G/A and A/A genotypes were 0.875, 0.125 and 0 in BD patients; and 0.8611, 0.1389 and 0 in controls, respectively, ($P = 0.39$ by Fisher's exact test and 0.77 by χ^2) and the allele frequencies of TNF-α-308G were 0.9375 in BA patients and 0.9305 in controls ($P = 0.52$, OR = 1.12; 95% CI = 0.32-3.88).

We also analysed the data according to the presence of genital ulcer, ocular involvements and skin lesions, of TNF-α-1031T/C and TNF-α-308G/A polymorphisms (data not shown) and haplotypes in patients with these clinical characters were not significantly different [Table 2].

Table 1: Genotypic and allelic distribution of TNF-α-1031 and TNF-α-308 polymorphisms between the BA patient and the control groups

Genotypes and alleles (TNF-α)	BA group (n = 16)		Control group (n = 36)		OR (95% CI)	P value	
	n	F	n	F		Fisher	χ^2
T/T (TNF-α-1031)	11	0.6875	22	0.6112	1.39 (0.74-2.61)	0.15	0.2
T/C (TNF-α-1031)	5	0.3125	14	0.3888	0.71 (0.38-1.33)	0.14	0.2
C/C (TNF-α-1031)	0	0.00	0	0.00	–	–	–
T (TNF-α-1031)	27	0.8437	58	0.8056	1.30 (0.59-2.88)	0.22	0.47
C (TNF-α-1031)	5	0.1563	14	0.1944	0.76 (0.34-1.69)	0.22	0.47
G/G (TNF-α-308)	14	0.8750	31	0.8611	1.12 (0.46-2.76)	0.39	0.77
G/A (TNF-α-308)	2	0.125	5	0.1389	0.88 (0.36-2.16)	0.35	0.77
A/A (TNF-α-308)	0	0.00	0	0.00	–	–	–
G (TNF-α-308)	30	0.9375	67	0.9305	1.12 (0.32-3.88)	0.52	0.84
A (TNF-α-308)	2	0.0625	5	0.0695	0.89 (0.25-3.07)	0.47	0.84

*P < 0.05. TNF-α: Tumour necrosis factor alpha; BA: Biliary atresia; OR: Odds ratio; CI: Confidence interval

Table 2: Haplotype analysis of two-SNP TNF-α haplotype in BA patient and the control groups

TNF-α haplotypes	BA group (n = 32)		Control group (n = 70)		OR (95% CI)	P value	
	n	F	n	F		Fisher	χ^2
TG	25	0.7812	53	0.7571	1.14 (0.56-2.33)	0.31	0.68
TA	2	0.0625	4	0.0571	1.1 (0.3-4.05)	0.39	0.87
CG	5	0.1563	13	0.1858	1 (0.43-2.3)	0.47	0.99
CA	0	0.0	0	0.0	–	–	–

*P < 0.05. TNF-α: Tumour necrosis factor alpha; BA: Biliary atresia; OR: Odds ratio; CI: Confidence interval; SNP: Single nucleotide polymorphisms

DISCUSSION

BA is a progressive, inflammatory cholangiopathy of infancy that leads to fibrosis and obliteration of the extrahepatic and intrahepatic bile ducts.[1] At the time of diagnosis, the extrahepatic bile ducts are partly or entirely destroyed with residual inflammatory cells present within duct remnants.[13] The intrahepatic ducts have an on-going inflammatory response with lymphocytes surrounding and invading the ducts.[2,5] Without intervention, children with BA die from biliary cirrhosis in the first 2 years of life and this disease is the indication for >50% of paediatric liver transplants.[14,15]

The aetiology of BA is unknown and theories of pathogenesis involve viral infection, immune mediated duct destruction and abnormalities in duct development.[1-5,13,15]

The pathogenesis of BA may involve both an initial viral infection and a subsequent auto-reactive immune response. Several studies have investigated the role of the immune system in pathogenesis of BA.[2,5,6,16,17]

The portal tract infiltrating cells secrete the Th1 inflammatory cytokines such as TNF-α, IL-2 and IL-12, which further induce Th1-cell mediated ductal destruction.[5] TNF-α is a pleiotropic and pro-inflammatory cytokine that takes part in regulation of the immune responses which are in charge of the recurrent inflammatory reactions.[2,5,6] Recently, some studies have expressed that increased expression of TNF-α at the time of diagnosis in human BA and increased expression of hepatic TNF-α and inducible nitric oxide synthase (iNOS).[16,18,19]

Our result which showed there is no association between BA and TNF-α-308G/A is in agreement with other investigation in Taiwan.[4] The allelic distribution of TNF-α-1031T/C is similar in both of the BA and control groups. Moreover the frequency of T allele (-1031) and G allele (-308) are very similar in both of groups (84:80)(93:93) respectively, but it should be consider that Interestingly, we did not observe any homozygote genotype in patient and control groups for this locus (homozygous C/C for TNF-α-1031 and homozygous A/A for TNF-α-308). In addition, Haplotypes analysis of the TNF-α promoter in this cohort showed that there was not significant association between patient and control groups (P = 0.6, OR = 1.14; 95% CI = 0.56-2.33). Altogether these results prove the deductions of Tucker *et al*.[5] who stated when we elevate expression of TNF-α (promoter), this cytokine does not perform an obligate play in BA progression and despite the clinical observations and their studies have shown that there is not any improvement in the progression of BA with TNF-α blockade. Based on these studies,

TNF-α neutralising agent should not be utilised in human BA until more is known about this cytokine in human disease.

In addition, the progressive nature of BA is multi-factorial and many other cytokines may be contributing, independent of TNF-α, it has been shown that interferon-gamma plays a critical role in BA[20] and there is increased expression of iNOS.[18] Despite TNF-α blockade, either of these two potent molecules may have an effect on downstream modulation of the TNF-α pathway. Future studies will investigate other cytokines and chemokines, both individually as well as combinatorialy. By blocking more than one pathway, one might be able to prevent progressive biliary destruction.

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