

## Genetic association of cytokines polymorphisms with autoimmune hepatitis and primary biliary cirrhosis in the Chinese

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**CONCLUSION:** The polymorphisms of IL-1RN and IL-6 -174G/C appear to be associated with PBC in Chinese patients.

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**Key words:** Autoimmune hepatitis; Primary biliary cirrhosis; Cytokine; Polymorphisms; Gene susceptibility

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### Abstract

**AIM:** To characterize gene polymorphism of several cytokine gene in-patients with AIH and PBC and to analyze the difference of the polymorphism distribution between Chinese patients and healthy controls.

**METHODS:** The study population consisted of 62 patients with AIH, and 77 patients with PBC. The genetic profile of four cytokines was analyzed by restriction fragment length polymorphism after specific PCR amplification (PCR-RFLP) or sequence-specific primers PCR (SSP-PCR). The analyzed gene polymorphism included interleukin-1 (IL-1) (at position +3 953 and IL-1RN intron 2), IL-6 (at position -174), IL-10 promoter (at position -1 082, -819, and -592). The control group consisted of 160 healthy blood donors.

**RESULTS:** The majority of Chinese people including patients and healthy controls exhibited IL-1B 1,1 genotype, and there was no significant difference in AIH, PBC patients and controls. There were highly statistically significant differences in the distribution of the IL-1RN gene polymorphism between the patients with PBC compared with controls. The frequency of IL-1RN 1,1 was significantly higher (90.9% vs 79.4%,  $P = 0.03$ ) and the frequency of IL-1RN 1,2 was significantly lower in PBC patients (6.5% vs 17.5%,  $P = 0.01$ ). No statistical difference was observed between AIH patients and controls. All of the 160 healthy controls and 62 cases of AIH patients exhibited IL-6-174GG genotype, and there were four cases, which expressed IL-6-174GC genotype in 77 cases of PBC patients. The frequency of IL-6-174GC was markedly significantly higher in PBC patients compared with controls (5.2% vs 0%,  $P = 0.004$ ). No statistically significant difference was found in the distribution of IL-10 promoter genotype in AIH and PBC patients compared with controls.

### INTRODUCTION

Autoimmune hepatitis (AIH) and primary biliary cirrhosis (PBC) are two groups of important autoimmune liver diseases. AIH is characterized by elevated serum transaminase levels, hypergammaglobulinemia, serum autoantibodies, and a good response to immunosuppressive therapy. PBC exhibits progressive destruction of the bile ducts leading to fibrosis and cirrhosis. Specific autoantibodies and disorder of liver function also characterize PBC<sup>[1,2]</sup>. Although their etiologies are unknown, genetic factors have been implicated to be involved in the pathogenesis of AIH and PBC<sup>[3-7]</sup>. In previous studies human HLA DRB1\*0301, DRB\*0401 (in Caucasians) and DRB1\*0405 (in the Chinese) have been identified as independent determinants of susceptibility to AIH. In addition, the genetic typing of HLA class II and III alleles revealed a highly significant increase of HLA DR8, DPB1\*0501 and 0301, and C4A\*B2 and C4A\*Q0 complement alleles in patients with PBC compared with controls, and the HLA DRB1\*0801-DQA1\*0401-DQB1\*0402 haplotype was considered to represent a maker of disease progression.

A number of genes outside the MHC locus may play a role in susceptibility to autoimmune liver diseases<sup>[8-11]</sup>. We have recently shown that the polymorphism of cytotoxic T lymphocyte-associated antigen-4 and vitamin D receptor genes have been associated with Chinese patients with AIH and PBC, and there is no association between the polymorphism of tumor necrosis factor (TNF)-alpha promoter and the same group patients with AIH and PBC<sup>[13]</sup>. It has been well established that interleukin-1 (IL-1), IL-6 play important roles in developing chronic inflammation, and IL-10 is an important cytokine with anti-inflammatory, anti-immune, and anti-fibrotic functions. The aim of

this study is to investigate, the relationship between both diseases susceptibility in the Chinese population and the one polymorphic site in IL-6 promoter -174, the three polymorphisms sites in the IL-10 gene promoter -1082, -819, -592), as well as the IL-1 gene family: IL-1B and IL-1RN.

## MATERIALS AND METHODS

### Study population

A total of 139 patients who satisfied international criteria for AIH and PBC were obtained from Eastern China, including Shanghai, Zhejiang province and Jiangsu province. Sixty-two patients with AIH fulfilled the following criteria: (1) Absence of epidemiological risk factors and serological markers for hepatitis B and hepatitis C virus infection by immunoassays; (2) Presence of ANA, SMA or LKM-1, anti-soluble liver antigen antibody (SLA); (3) Absence of drug history; (4) Seven patients had concurrent nonhepatic immunologic diseases, including four with chronic ulcerative colitis and three with autoimmune thyroid disease; (5) Thirty patients were seropositive for other defined autoantibodies, including 17 atypical pANCA, 11 parietal cell antibody, 4 thyroglobulin autoantibody or thyromicrosomal autoantibody. The clinical and laboratory features of the 62 patients are shown in Table 1. The diagnosis of AIH was based on the revised criteria defined by the International Autoimmune Hepatitis Group<sup>[1]</sup>.

The PBC group consisted of 77 well-characterized patients, which met the diagnostic criteria for PBC in the practice guideline written by AASLD<sup>[2]</sup>: (1) presence of cholestatic liver disease including jaundice, fatigue and pruritus with abnormally high levels of cholestatic parameters such as serum alkaline phosphatase (ALP), serum bilirubin and gamma glutamyl transpeptidase; (2) Absence of biliary

obstruction as assessed by ultrasound or endoscopic retrograde cholangiopancreatography; (3) Serum positive AMA titer  $\geq 1:1\ 000$ , and type M2 AMA positive; (4) Absence of serological markers for hepatitis B and C virus infection by immunoassays. There were 10 patients who had serum aspartate transaminase 30-50 U/L (normal value 10-40 U/L) underwent liver biopsy in PBC group, and the result showed that the histological change was compatible with PBC. Other patients have elevated serum aspartate transaminase ( $>60$  U/L). There were 41 patients who had ANA positive in their serum (titer  $\geq 1:100$ ). Sixty-eight were females, median age at diagnosis of PBC was 51.3 years (range 32 to 79), and seven were males, median age was 47.0 (range 38 to 60). Controls from the same geographic area, were blood donors.

ANA were sought by indirect immunofluorescence (IIF) on Hep-2 cells and rat liver tissue sections in all patients. SMA was similarly checked by IIF on rat stomach tissue sections in all patients. LKM-1 and AMA were detected on rat stomach and liver tissues sections in all patients. SLA and type M2 AMA were sought by ELISA. The IIF and ELISA kits were bought from Euroimmun Company, Germany.

### DNA extraction

Genomic DNA was isolated from fresh peripheral blood leukocytes using the Qiagen DNA isolation kit (Qiagen, Germany). DNA samples were quantified and subjected to specific PCR reaction as described.

### Cytokines gene polymorphisms

Genomic DNA (100 ng) was amplified in reaction mixtures containing 200  $\mu\text{mol/L}$  each of dATP, 1 pmol each primer, 1.5  $\mu\text{mol/L}$  MgCl<sub>2</sub>, 2 U Taq polymerase (MBI), 1  $\times$ PCR buffer with NH<sub>2</sub>SO<sub>4</sub> (MBI) on a MJ PTC-200 Peltier Thermal Cycler, according to the following protocols (Table 2).

### IL-1B +3953 C/T polymorphism

For detection of IL-1B +3 953 C/T polymorphism, we used PCR-restriction fragment length polymorphism (PCR-RFLP). Amplification was performed at 94 °C for 2 min followed by 40 cycles at 94 °C for 30 s, 50 °C 45 s, and 72 °C for 40 s, and a final extension at 72 °C for 5 min. The 249-bp product was digested with Taq I and electrophoreses on 3% agarose. The presence of a base-exchange substitution at position +3 953 creates the Taq I restriction site in allele 1, but not allele 2. Therefore, Taq I digestion of the 249-bp IL-1B amplicon results in fragments of 114- and 135-bp (allele 1) and/or intact amplicon (allele 2).

### IL-1 receptor antagonist gene 86-bp mini-satellite

Genotyping for IL-1 receptor antagonist gene (IL-1RN) was performed by PCR. There is a penta-allelic polymorphic site containing variable numbers of an 86-bp tandem repeat sequence in intron 2 of IL-1RN. Briefly, the conditions for amplification were as follows: 94 °C for 2 min, 40 cycles of 94 °C for 30 s, 67 °C 45 s, and 72 °C for 40 s, and a final extension at 72 °C for 5 min. Following amplification, the amplicon was visualized on a 2% agarose gel electrophoresis. Five alleles were assigned based on amplicon size: allele 1 four repeats (410 bp), allele 2 two repeats (240 bp), allele 3

**Table 1** Clinical features in patients with AIH

| Clinical features                       | Female<br>(n = 44) | Male<br>(n = 18) |
|---|--------------------|------------------|
| Age (yr)                                | 50 (16-79)         | 47 (21-62)       |
| Duration symptoms (mo)                  | 36.4 (6-134)       | 38.8 (18-72)     |
| Concurrent immunologic diseases         | 5                  | 2                |
| ALP:AST <1.5                            | 44                 | 11               |
| 1.5-3.0                                 | 5                  | 2                |
| Bilirubin (3.4-17.1 $\mu\text{mol/L}$ ) | 62.4 (40-84.7)     | 63.5 (42-79.6)   |
| Immunoglobulin G (7.6-16.6 g/L)         | 30.9 (18.1-34)     | 32.6 (18-33.4)   |
| ANA $\geq 1:100$                        | 30                 | 11               |
| SMA $\geq 1:100$                        | 3                  | 1                |
| ANA and SMA $\geq 1:100$                | 8                  | 3                |
| LKM-1 $\geq 1:100$                      | 1                  | 1                |
| SLA $\geq 1:100$                        | 2                  | 2                |
| Average alcohol intake                  | <25 g/d            | <25 g/d          |
| Other autoantibodies                    |                    |                  |
| atypical pANCA $\geq 1:32$              | 13                 | 4                |
| PCA                                     | 7                  | 4                |
| TG or TM                                | 4                  | 0                |
| AIH Score <sup>1</sup>                  | >15                | >15              |

TG, thyroglobulin autoantibody; TM, thyromicrosomal autoantibody; PCA, parietal cell antibody. <sup>1</sup>AIH score: revised scoring system for diagnosis of AIH.

three repeats (325 bp), allele 4 five repeats (500 bp) and allele 5 six repeats (595 bp).

### IL-6 promoter-174 G/C polymorphism

IL-6 promoter-174 genotype was determined in an allele-specific PCR as previously described<sup>[14]</sup>. The DNA template was denatured at 95 °C for 2 min. A total of 40 cycles of PCR were performed, consisting of a denaturation step for 45 s at 94 °C, an annealing step for 45 s at 59.5 °C, and an extension reaction for 1 min at 72 °C. A final extension step at 72 °C for 2 min was added after the last PCR cycle. PCR products were resolved by agarose gel electrophoresis (2%) and visualized by ethidium bromide.

### Polymorphisms of IL-10-1082G/A, -819C/T and -592A/C

Polymorphisms at two positions of the IL-10 promoter gene sequence -1 082 and -592 were determined by PCR-RFLP. Briefly, a 139-bp fragment (for -1 082C/T) and a 412-bp fragment (for -592G/A) were separated and amplified. PCR conditions: 94 °C for 2 min, 40 cycles of 94 °C for 30 s, annealing step for 45 s at 58 °C (61.8 °C, for -592G/A), and 72 °C for 40 s, and a final extension at 72 °C for 5 min. The presence of nucleotides G at position -1 082 creates the Mn1 I restriction site, therefore, Mn1 I digestion of the 139 bp IL-10 amplicon result in fragments of 106 and 33 bp. The presence of nucleotides A at position -592 creates the Afa I restriction site, Afa I digestion of the 412-bp IL-10 amplicon result in fragments of 176 and 236 bp. After the digestion reactions, products were resolved by agarose gel electrophoresis (3%) and visualized by ethidium bromide.

### Statistical analysis

Statistical analyses (two-tailed Fisher's exact test, odds ratios, confidence intervals) was calculated using the NOSA software package.

**Table 2** PCR primer pairs for genotyping in cytokines gene

| Gene     | Position                    | Primers                          | Product size (bp) |
|----------|-----------------------------|----------------------------------|-------------------|
| IL-1B    | +3 953                      | 5'-GTGTGCATCAGACTTTGACC-3'       | 249               |
|          |                             | 5'-TTCAGTTCATATGCACCAGA-3'       |                   |
| IL-1RN   | exon2                       | 5'-GCCCTCAGCAACACTCCTAT-3'       | 1                 |
|          |                             | 5'-CATCTTCTGGTCTGCAGC-3'         |                   |
| IL-6     | -174G/C                     | 5'-CCCTAGTTGTGCTTGCC-3'          | 190               |
|          |                             | 5'-CCCTAGTTGTGCTTGCC-3'          | 190               |
|          |                             | 5'-GAGCTTCTCTTTCGTCC-3'          |                   |
| IL-10    | -1 082G/A                   | 5'-TCTTACCTATCCCTACTTCC-3'       | 139               |
|          |                             | 5'-CTCGTGCACCCAACTGGC-3'         |                   |
|          | -819T/C                     | 5'-AACTGAGGCACAGAGATG-3'         | 402               |
|          |                             | 5'-AACTGAGGCACAGAGATG-3'         | 402               |
| -592A/C  | 5'-GGTGAGCACTACCTGACTAGC-3' | 412                              |                   |
|          | 5'-CCTAGGTCACAGTGACGTGG-3'  |                                  |                   |
| HLA-DRB1 | 5c                          | 5'-TGC CAA GTG GAG CAC CCA A-3'  | 796 <sup>2</sup>  |
|          | 3c                          | 5'-GCA TCT TGC TCT GTG CAG AT-3' |                   |

<sup>1</sup>Variable numbers of an 86 bp tandem repeat sequence. <sup>2</sup>The inter-reference of allele-specific PCR.

## RESULTS

Table 3 summarizes the phenotypic expression deduced from the genetic polymorphism in the four selected cytokines in the patients with AIH and PBC, in healthy individuals who were blood donors. The majority of Chinese people including patients and healthy controls exhibited IL-1B 1,1 genotype, and there was no significant difference in AIH, PBC patients and controls. There were highly statistically significant differences in the distribution of the IL-1RN gene polymorphism between the patients with PBC compared with controls. The frequency of IL-1RN 1, 1 was significantly higher (90.9% vs 79.4%,  $P = 0.03$ ) and the frequency of IL-1RN 1, 2 was significantly lower in PBC patients (6.5% vs 17.5%,  $P = 0.01$ ). No statistical difference was observed between AIH patients and controls.

All of the 160 healthy controls and 62 cases of AIH patients exhibited IL-6 -174GG genotype, and there were four cases, which expressed IL-6-174GC genotype in 77 cases of PBC patients. The frequency of IL-6-174GC was markedly significantly higher in PBC patients compared with controls (5.2% vs 0%,  $P = 0.0036$ ). No statistically significant difference was found in the distribution of IL-10 promoter in AIH and PBC patients compared with controls.

**Table 3** Genotype distributions of cytokines in AIH, PBC patients vs controls

| Cytokine | Genotype              | Controls (%) | AIH (%)   | $P$       | PBC (%)   | $P$    |
|----------|-----------------------|--------------|-----------|-----------|-----------|--------|
| IL-1B    | +3 953                |              |           |           |           |        |
|          | 1,1                   | 155 (96.9)   | 61 (98.4) | 0.53      | 75 (97.4) | 0.82   |
|          | 1,2                   | 4 (2.5)      | 1 (1.6)   | 0.69      | 2 (2.6)   | 0.96   |
|          | 2,2                   | 1 (0.0)      | 0 (0)     | 0.53      | 0 (0)     | 0.46   |
| IL-1RN   | Intron 2              |              |           |           |           |        |
|          | 1,1                   | 127 (79.4)   | 51 (82.3) | 0.63      | 70 (90.9) | 0.026  |
|          | 1,2                   | 30 (17.5)    | 10 (16.1) | 0.64      | 5 (6.5)   | 0.013  |
|          | 2,2                   | 3 (1.9)      | 1 (1.6)   | 0.90      | 2 (2.6)   | 0.75   |
|          | others                | 0 (0)        | 0 (0)     | -         | 0 (0)     | -      |
| IL-6     | -174 G/C <sup>1</sup> |              |           |           |           |        |
|          | GG                    | 160 (100)    | 62 (100)  | -         | 73 (94.8) | 0.0036 |
|          | GC                    | 0 (0)        | 0 (0)     | -         | 4 (5.2)   | 0.0036 |
|          | CC                    | 0 (0)        | 0 (0)     | -         | 0 (0)     | -      |
| IL-10    | -1082G/A              |              |           |           |           |        |
|          | AA                    | 144 (90.0)   | 54 (87.1) | 0.53      | 64 (83.1) | 0.13   |
|          | GA                    | 16 (10.0)    | 8 (12.9)  | 0.53      | 13 (16.9) | 0.13   |
|          | GG                    | 0 (0)        | 0 (0)     | -         | 0 (0)     | -      |
|          | -819T/C               |              |           |           |           |        |
|          | CC                    | 13 (8.1)     | 7 (11.3)  | 0.46      | 7 (9.1)   | 0.80   |
|          | CT                    | 72 (45.0)    | 27 (43.6) | 0.84      | 41 (53.3) | 0.23   |
| TT       | 75 (46.9)             | 28 (45.2)    | 0.81      | 29 (37.7) | 0.18      |        |
| -592A/C  |                       |              |           |           |           |        |
| CC       | 16 (10.0)             | 10 (16.1)    | 0.2       | 11 (14.3) | 0.33      |        |
| CA       | 83 (51.9)             | 32 (51.6)    | 0.97      | 41 (53.3) | 0.84      |        |
| AA       | 61 (38.1)             | 20 (32.3)    | 0.41      | 25 (32.5) | 0.40      |        |

<sup>1</sup>The frequency of IL-6 -174C allele in PBC group vs control,  $P = 0.0038$ .

## DISCUSSION

The etiology of autoimmune liver diseases remains unidentified. However it is well established that a complex genetic background contributes to disease susceptibility

and severity. The previous studies showed that HLA alleles, and the polymorphisms of TNF- $\alpha$  promoter, CTLA-4, C4A were associated with an increasing prevalence of autoimmune liver diseases in Caucasians. Recently we found a significant association of CTLA-4 and vitamin D receptor gene polymorphism in Chinese AIH and PBC patients' comparison with controls<sup>[12]</sup>. In order to search more correlative gene with autoimmune liver diseases in the Chinese population, we extended study objects in this study.

Our study was designed to analyze the potential association between cytokine gene polymorphism and the patients with AIH, PBC. We found a statistically significant difference in IL-1RN phenotype between the patients with PBC and the healthy controls. The frequency of IL-1RN 1, 1 was significantly higher and the frequency of IL-1RN 1, 2 was significantly lower in PBC patients. This result was partly coincident with Donaldson's, but we did not find any significant difference in the distribution of IL-1B genotype<sup>[11]</sup>. IL-1 is a proinflammatory cytokine, which interacts with both tumor necrosis factor and IL-6, and may be important in both inflammation and fibrosis. The IL-1 gene family on chromosome 2q13, encodes three proteins, including IL-1 $\alpha$ , IL-1 $\beta$ , and the IL-1 receptor antagonist (IL-1RA). IL-1RA competes with IL-1 $\beta$  (and IL-1 $\alpha$ ) for binding to the IL-1 receptors and is a potent inhibitor of IL-1 activity<sup>[15]</sup>. IL-1RN is the second association with PBC to be described by our group, which maps to chromosome 2. The other associated gene is CTLA-4 (2q33) which appears to be associated with AIH and PBC. These two studies based on the same series of patients identify chromosome 2q as an important location for further genetic studies in PBC. The IL-1B\*2 allele is associated with high IL-1 $\beta$  production, the IL-1RN\*2 allele is associated with high IL-1RN production. There is a genetic link between IL-1RN\*2 and IL-1B\*1, they both contribute to lower IL-1 $\beta$  production<sup>[16-18]</sup>.

IL-6 is a pleiotropic cytokine capable of regulating proliferation, differentiation and activity of a variety of cell types, and plays a pivotal role in acute phase response and in the balancing of the pro-inflammatory/anti-inflammatory pathways<sup>[14]</sup>. IL-6 gene maps to chromosome 7p21, there is G>C single nucleotide polymorphism at the promoter -174, and this base transition is associated with different IL-6 plasma levels in healthy subjects, and affects the rate of IL-6 gene transcription<sup>[19,20]</sup>. Previous studies showed that IL-6 -174G/C polymorphism was associated with multiple diseases, including juvenile chronic arthritis, atherosclerosis and diabetes. The IL-6 -174C allele contributes to lower production *in vitro* and *in vivo*, the IL-6 -174CC homozygosity expressed in the renal transplant donor and the IL-6 -174GG homozygosity expressed in receptor had been shown to associate significantly with the incidence and severity of graft-versus-host disease (GVHD), both acute GVHD and chronic GVHD<sup>[21,22]</sup>. In this investigation, we also found that frequency of IL-6 -174C was strong and significantly increased in PBC patients compared with controls among the Chinese. This result supported the IL-6 -174G/C polymorphism contributing to the change of susceptibility to PBC for some people.

IL-10 is an important suppressor factor for both

immunoproliferative active and inflammatory responses<sup>[23,24]</sup>. It is produced by numerous cell types including activated CD4<sup>+</sup> T cells, cytotoxic CD8<sup>+</sup> T cells, monocytes, Kupffer cells, hepatocytes, and hepatic stellate cells. IL-10 inhibits antigen specific activation, proliferation, and cytokine production by reducing the antigen presenting capacity of monocytes, associated with downregulation of class II HLA molecules and B7 expression on their surface. In contrast, in B lymphocytes, IL-10 stimulates proliferation, immunoglobulin secretion, and isotype class switching from IgM to IgA. IL-10 also has potent anti-inflammatory effect. It downregulates the synthesis of proinflammatory cytokines and chemokines by monocytes and Kupffer cells stimulated by endotoxin, including IL-1, TNF- $\alpha$ , IL-6, IL-8, and IL-12, and upregulates the synthesis of the IL-1R antagonist. Finally, IL-10 may also exert antifibrotic effects in the liver through inhibition of collagen gene transcription and increased collagenase expression by hepatic stellate cells<sup>[25,26]</sup>.

The IL-10 gene is located on the long arm of chromosome 1 in the 1q32 band and has been shown to contain three distinct SNPs within its promoter region, at nucleotide position -1082 (G→A), -819 (C→T), and -592 (C→A), with the SNP at position -1082 giving rise to differential IL-10 production. These three polymorphisms have been shown to be in strong linkage disequilibrium, and three main haplotypes (GCC, ACC, and ATA) are known to segregate in most populations<sup>[27]</sup>. In this study we have shown the haplotypes distribution of three SNPs in IL-10 promoter region in controls were all quite different from that of the European Caucasians, and similar with that of the Singapore Chinese and Japanese population<sup>[28,29]</sup>. In Italian PBC patients, the frequency of homozygosity for G/G at position -1082 was significantly higher than that of local controls, and the frequencies of haplotype GCC possibly linked to higher IL-10 production, were also significantly higher in PBC patients than local controls. However, in Japanese population, there were no significant difference in the three SNPs and haplotypes between PBC patients and controls. Our research has also shown that there were no significant differences in the three SNPs and haplotypes among AIH, PBC patients and controls in the Chinese population. Excessive production of IL-10 may play an important role in some populations in modulating the onset of PBC. Further, immunogenetic studies of AIH, PBC should take into account ethnic and geographic variations, this knowledge can then be utilized to clarify their etiologies, and develop new treatment options including manipulation of the cytokine network, an approach which is currently being applied to several autoimmune diseases with some success<sup>[30]</sup>.

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