

# A correlation study between gene polymorphism of Th cell expressed chemokine receptor CXCR3 and its ligand levels with HCV infection prognosis

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**Abstract. – OBJECTIVE:** Chemokine receptor and its ligand participate in viral immunity and HCV infection, which are important inflammatory mediators. The current study showed the different roles of Th cell secreted chemokines CXCR3, CCR5 and CCR6 in chronic liver inflammation after HCV infection. As one important chemokine receptor, the role of polypeptide property and ligand level in HCV prognosis is still unclear. This study aims to investigate gene polymorphism of chemokine genes and ligand level, and their correlation with patient liver function, to provide evidence for HCV prognosis and chronic transition mechanism.

**PATIENTS AND METHODS:** Whole blood samples were collected. Participants were divided into chronic hepatitis, HCV cirrhosis and self-clearance groups. Chemokine level, gene polymorphism of CXCR3 gene at loci rs2280964 and liver index were measured to analyze their correlation with HCV infection or prognosis.

**RESULTS:** Gene polymorphism of CXCR3 at loci rs2280964 is one factor-affecting prognosis of HCV patients. CG genotype at these loci is one independent risk factor affecting chronic HCV infection. IP-10, Mig and I-TAC levels were significantly elevated in chronic hepatitis group or HCV cirrhosis group ( $p < 0.05$  compared to self-clearance group).

**CONCLUSIONS:** Gene polymorphism at rs2280964 locus of chemokine receptor CXCR3 is one possible reason explaining differential processes of chronic transition. CXCR3 ligands IP-10, Mig and I-TAC levels were all significantly elevated in chronic hepatitis and HCV cirrhosis patients, possibly functioning as one clinical index for HCV prognosis.

## Key Words

Hepatitis C virus, CXCR3, Gene polymorphism, Disease prognosis.

## Introduction

Hepatitis C virus (HCV) infection is difficult to be cleared by body immune system due to its unique property and host immune function. About 50%-80% HCV patients develop into chronic hepatitis, in which 20%-30% progresses into liver cirrhosis<sup>1</sup>. In those cirrhosis patients, about 1%-4% eventually develop into liver cancer. Thus, the study of HCV prognosis is one major challenge. Chemokines are one group of cytokines that exert their functions via G-protein-coupled receptor (GPCR) super-family. It mainly directs the targeted movement of cells during development of immune tissues/organs, immune response, pathogen infection and clearance, and tumor clearance<sup>2</sup>. After HCV infection, chronic inflammation follows with Th1 immune response predominance. In this process, the distribution and expression of chemokines along with their receptors determine the recruitment mode of lymphocytes. For example, CCR5 initiates lymphocyte recruitment into hepatic portal veins<sup>3</sup>, CXCR6 mediates hepatocyte recruitment<sup>4</sup>, whilst CXCR3 induces recruitment from hepatic sinusoidal to parenchyma<sup>5</sup>. CXCR3 is highly expressed in activated T lymphocytes. Belonging to Th1 cell surface chemokine receptor, it is abundantly distributed in HCV-infected liver lymphocytes<sup>6</sup>, accompanied with HCV infection-induced expressional potentiation of CXCR3 ligands including IP-10, Mig and BLC. Potentiated expression of CXCR3 ligand is closely correlated with attraction of activated lymphocytes into injury liver, and causing Th1 cellular immunity initiated tissue damage.

Other reports<sup>7</sup> indicated significant correlation between intrahepatic IP-10 and I-TAC levels and severity of HCV disease.

A recent study<sup>8</sup> showed the important role of gene polymorphism, which can alter disease susceptibility and affect disease prognosis. Single nucleotide polymorphism (SNP) refers to difference of single base pair in genomes across individuals. Such mutation can modulate gene transcription when occurred in regulatory domains, and can affect protein function in coding region, whilst those non-functional loci may work as the marker for pathogenic mutations<sup>9</sup>. Generally speaking, population-wide study for allele frequency showed the correlation between SNPs and disease. CXCR3 is one important chemokine receptor during HCV infection. The correlation between its gene polymorphism or ligand expression level and HCV infection prognosis is still unclear. This work thus investigated gene polymorphism of CXCR3 at rs2280964 loci, and the distribution of its ligands across HCV positive, HCV continuous infection group and negative group. Also, we analyzed the correlation between gene polymorphism and HCV infection prognosis, to reveal the clinical implication of CXCR3 gene polymorphism at rs2280964 loci and its ligands IP-10, Mig and I-TAC levels in HCV prognosis.

## Patients and Methods

### Patients

A total of 426 patients were included in Shenzhen Center for Disease Control and Prevention of Guangdong, including a chronic hepatitis group with 212 cases with HCV RNA positive for more than 6 months, 123 patients with anti-HCV antibody positive, and 91 patients having history of chronic HCV infection, presenting liver dysfunction and portal vein hypertension plus liver cirrhosis in the imaging system. All participants came from patients admitted to our hospital. The diagnosis was made according to the Guideline of HCV stipulated by Hepatology Sub-committee of Chinese Medicine Association. No patients received interferon or ribavirin, or with infections of other hepatitis virus. The study protocol was approved by the Research Ethics Committee of Shenzhen Center for Disease Control and Prevention of Guangdong, and all patients gave their informed consent before study commencement.

### Experimental materials

Human IP-10, Mig and I-TAC dual-antibody sandwiched enzyme-linked immune reagents were purchased from Boster Co., Ltd., (Wuhan, China). Human whole blood genome DNA was extracted by QuickGene whole blood DNA kit (Fuji Film, Singapore, SG). PCR primer was synthesized by Toyobo Co., Ltd., (Osaka, Japan).

#### Chemokines level assay

All participants have signed the consent forms and were collected for fasted blood samples, which were centrifuged for separating the serum for aliquot and storage at -80°C. Test procedures for chemokine levels followed the manual instruction of test kit. The standard curve was plotted using standard samples in the test kit. A microplate reader was used to measure absorbance values at 450 nm. Content of serum IP-10, Mig and I-TAC was calculated based on standard curves.

### Separation of CD4+T cells by immune magnetic beads

A total of 2 mL whole blood samples were centrifuged to collect the supernatant. Antibody cocktail mixture was added for 10 min incubation at 4-8°C, followed by 500 ×g centrifugation to remove the supernatant. 320 μL buffer and 80 μL magnetic beads were added for 15 min incubation at 4-8°C. After rinsing and centrifugation in buffer, cells were re-suspended in 500 μL buffer. MS separation column was then used for elution in 1500 μL. The elute was collected and centrifuged to separate Th cells.

### Genomic DNA extraction

Extraction of genomic DNA followed the manual instruction of test kit. Briefly, 30 μL EDB was mixed with 200 μL Th cell suspensions. 250 μL LDB was added and mixed well in pipette. The mixture was vortexed for 15 s, and was incubated at 56°C for 2 min. 250 μL absolute ethanol was added for 15 s high-speed vortex. After well mixture of samples and ethanol, the mixture was transferred to QG-810 for extracting genomic DNA, which was determined by purity and stored at -80°C for further use.

### PCR combined sequencing analysis of CXCR3 gene at locus rs2280964

PCR primer was designed by primer blast based on sequences in Genbank-dbSNP database (<http://ncbi.nih.gov>). Expected amplified product length was 183bp, with forward primer (5'-CGCCC CCTCC ATTTT GCAGA-3') and reverse primer (5'-GGCCG CATGG GTTGT G-3').

**Table I.** Demographic and clinical features of patients.

Item (unit)	Chronic hepatitis (n=212)	Cirrhosis (n=91)	Self clearance (n=123)
Age (year)	46.28±10.82	52.38±10.98**	50.28±9.78**
Sex (M/F)	159/53	61/30	92/31
ALT (U/L)	82.5 (30.3-408.2)	87.3 (8.1-921.3)	62.15 (9.2-683.2)**
AST (U/L)	50.3 (31.2-225.7)	87.5 (18.2-802.9)**	38.2 (15.7-211.8)**

PCR was performed in a 20 µL system, following the manual instruction of test kit. Reaction conditions were: 50°C 30 min and 95°C 5 min, followed by 40 cycles each consisting 95°C 30 s, 55°C 30 s and 72°C 50 s, and ending in 72°C elongation for 5 min. Real-time PCR amplification curve and solving curve were recorded. PCR products were sequenced and genotyped.

**Taqman probe for genotyping**

Amplification primer for genotyping and Taqman probe was obtained by blast. PCR primer, amplification primer and Taqman FAM/HEX fluorescent probe sequences were: FAM-CGCCCCCCCCATTTTGC-TAMRA, HEX-CGCCCCCTCCATTTTGCAGA-TAMRA. Reaction conditions were described as above. Products were analyzed by Taqman SNP Genotyping Assays system to read fluorescent value and genotype. At least three blank samples were included in each test.

**Serum AST and ALT level assay**

Fasted blood samples were collected and centrifuged to obtain the serum, which was tested in an automatic biochemical analyzer.

**Hardy-Weinberg equilibrium test**

All research subjects were firstly tested for Hardy-Weinberg, to determine if gene frequency at SNP locus of research population fitted inheritance equilibrium. A good fitting, or inheritance equilibrium was identified when  $p > 0.05$ , which makes the sample as representative of the population.

**Gene frequency calculation**

Gene frequency at SNP locus was directly calculated.

**Statistical Analysis**

SPSS software (SPSS Inc. Chicago, IL, USA) was used for statistical analysis. Those measurement data fitted normal distribution were presented as mean±standard deviation (SD), while other data that did not fit normal distribution were presented

as median and range. A between-group comparison was performed by student *t*-test or rank-sum test.  $\chi^2$  analysis was used to compare allele frequency and genotype difference between groups. Multi-variate unconditioned Logistic regression was used to calculate ratios plus 95% confidence interval (CI) and to evaluate the relationship between SNP locus genotype and HCV prognosis. Statistical significance was defined when  $p < 0.05$ .

**Results**

**Demographic and clinical features of research subjects**

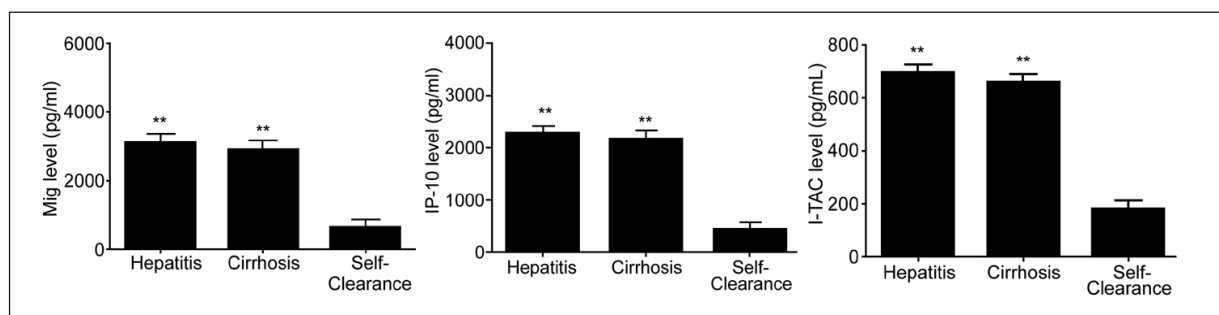
We compared age, sex and serum AST, ALT levels among all groups (Table I). Compared to chronic hepatitis group, liver cirrhosis group had significantly older age. Self-clearance group also had older age than hepatitis group. No significant difference existed between HCV-cirrhosis group and self-clearance group. The sex ratio among all groups was comparable. ALT level was indifferent between chronic hepatitis and cirrhosis group, both of which had higher ALT and AST than self-clearance group ( $p < 0.05$ ).

**Hardy-Weinberg equilibrium test**

Shesis software (Shanghai, China) was used to test the fitness of SNP genotype frequency in samples from self-clearance group against Hardy-Weinberg inheritance equilibrium. Results showed that in self-clearance group, the gene frequency distribution of CXCR3 gene SNPs at rs2280964 loci fitted Hardy-Weinberg equilibrium ( $p > 0.05$ , Table II).

**Table II.** Hardy-Weinberg equilibrium test of self-clearance group.

Group	CXCR3 rs2280964	N (%)	p-value
Self-clearance (n=123)	AA	47.15%	0.228
	AG	37.48%	
	CG	15.37%	



**Figure 1.** Serum IP-10, Mig and I-TAC levels. \*\*,  $p < 0.01$  compared to self-clearance group.

### Chemokines level assay in all patients

CXCR3 ligands IP-10, Mig and I-TAC levels were measured in all groups. As shown in Figure 1, compared to self-clearance group, IP-10, Mig and I-TAC levels were all significantly elevated. No significant difference existed between chronic hepatitis and liver cirrhosis group.

### SNP genotype distribution at rs2280964 loci of CXCR3 gene

By DNA sequencing and PCR analysis, we analyzed genotype frequency of CXCR3 gene at locus rs2280964 (Table III). Dot-plotted distribution of CXCR3 SNPs at locus rs220964 was shown in Figure 2. A between-group comparison of genotype distribution showed significant difference. These results showed chronic prognosis of HCV infection was correlated with SNP distribution of CXCR3 gene at locus rs2280964.

### Multi-variate Logistic regression analysis between SNP at locus rs2280964 of CXCR3 gene and HCV infection prognosis

Using self-clearance as the control group, with the removal of age/sex factors, a multi-variate Logistic regression analysis was performed to find host factors affecting HCV chronic transition. Results showed that CG genotype at rs2280964 locus of CXCR3 gene is one independent risk factor for chronic transition of HCV infection (OR=2.369, 95% CI=1.633-4.006,  $p < 0.001$ , Table IV).

### Discussion

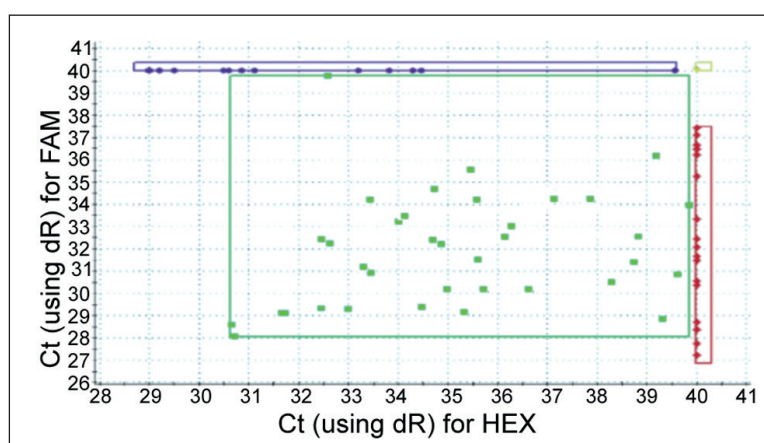
HCV infection is one important reason causing type C hepatitis. Common routes for HCV infection include blood transfusion, venous drug abuse, maternal-fetal transmission and sexual transmission<sup>10</sup>. In recent years, the infection rate of HCV is rapidly increasing<sup>11</sup>. Two outcomes, self-clearance and continuously chronic infection follow HCV infection. Without effective treatment, patients with chronic infection may develop into liver cirrhosis or liver cancer, thus severely affecting patient's life quality and lifespan. Currently it is still unclear the mechanism of self-clearance after continuous infection. Therefore, the study of factors related with clinical prognosis after HCV infection is of critical importance for predicting disease progression of HCV infection and for providing new treatment strategy for patients with chronic infection. Increasing evidence showed that chemokine receptor and its ligands worked as important mediators of inflammatory response in the process in viral immunity or HCV infection. The current study showed different roles of Th cell expressing chemokine receptors CXCR3, CCR5, CCR6 and their ligands in chronic hepatitis after HCV infection. In a study on HBV patients, serum and tissue levels of CXCL10 were elevated<sup>12</sup>, and were positively correlated with serum HBV-DNA content and ALT level. These results indicated the close correlation between CXCL10 expres-

**Table III.** Genotype distribution of SNP at locus rs2280964 of CXCR3 gene.

	n	Genotype		
		AA	AG	CG
Chronic hepatitis	212	65 (30.66%)	77 (36.32%)	70 (33.02%)
Liver cirrhosis	91	24 (26.41%)	38 (41.76%)	29 (31.87%)
Self-clearance	123	58 (47.15%)	46 (37.48%)	19 (15.37%)



**Figure 2.** Scattered plot of SNPs genotypes at rs2289064 of CXCR3 gene. Each dot represented one sample. Blue dots in left-upper quadrant represented AA genotype with HEX fluorescent signal only. Green dots in left-lower quadrant represented AG type with both HEX and FAM signals. Right dots in right-lower quadrant represented GG type with FAM signals. Yellow area on right-upper quadrant was a negative control.



sion level and hepatic injury-induced higher inflammation<sup>13-15</sup>. As the major receptor of IP-10, CXCR3 is mainly expressed in Th1 type cell surface, and has been shown to be up-regulated in liver lymphocytes of HCV infectious patients<sup>6</sup>. A previous study<sup>16</sup> showed that the interaction between virus and host immune system, largely determines constitutive viral infection and chronic inflammation. Viral infection and related liver injury are believed to be caused by the suppression of host immune system<sup>17,18</sup>. There are various studies regarding host gene mutation and HCV prognosis. Some reports<sup>19,20</sup> showed that those patients with TG or GG genotype at rs8099917 locus of IL-28b gene had higher risk of developing into chronic hepatitis type C comparing to those with TT genotype. All these researches suggested that gene polymorphism of host genes may cause difference in host immune status, leading to differential prognosis of HCV infection. CXCR3 plays a critical role in inflammation and immune response. Located in chromosome Xq13, this gene consists of two exons and one intron<sup>21</sup>. A total of 7 mutation sites of CXCR3 were found, including rs59881122, rs3761523, rs3091306, rs2280964, rs2230438, EXON1 and EXON2. Except for rs2280964 locus in this study, other loci all show pseudo-mutation<sup>22,23</sup>. In a study about CXCR3 gene, mutation at rs2280964 locus is one risk factor for asthma<sup>23</sup>. This study, therefore, selected rs2280964 locus of CXCR3 gene as the research object. This work recruited

HCV patients admitted to our hospital and collected blood samples from healthy volunteers, whose liver ALT/AST levels, gene polymorphism at rs2280964 locus of CXCR3 gene, and major ligands of CXCR3 including IP-10, Mig and I-TAC were measured. Statistical analysis concluded that gene polymorphism at rs22890964 locus of CXCR3 gene is one of factors causing differential clinical prognosis of HCV patients. CG genotype at rs22890964 locus of CXCR3 gene is one independent risk factor for chronic transition of HCV infectious patients. In either of chronic hepatitis or HCV-related liver cirrhosis group, IP-10, Mig and I-TAC levels were all significantly elevated ( $p < 0.05$  compared to self-clearance group). In summary, this study performed a retrospective study and attributed CG genotype at rs22890964 locus of CXCR3 gene as one independent risk factor of chronic transition of HCV infection. Also, this work indicated that host gene polymorphism might affect hos immune status, leading to differential clinical outcomes of HCV infection.

### Conclusions

Chemokines can also cause hepatocyte damage with the participation of HCV clearance. Its differential level may affect HCV infection and chronic transition. Gene polymorphism of chemokine receptor CXCR3 is thus one possible reason responsible for differential processes of chronic transition.

**Table IV.** Multi-variate Logistic regression analysis of host factors for chronic HCV infection patients using self-clearance group as the control group.

SNP	$\beta$	$\chi^2$ value	p-value	OR	95%CI
rs2280964 CG/AA+AG	0.994	12.936	0.000	2.369	1.633-4.006

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## Conflict of Interest

The Authors declare that they have no conflict of interests.

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