

RESEARCH ARTICLE

Associations of IL-10 Gene Polymorphisms with Acute Myeloid Leukemia in Hunan, China

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Abstract

We investigated the possible association of interleukin-10 (IL-10) single nucleotide polymorphisms (SNPs) and susceptibility to acute myeloid leukemia (AML) in 115 patients and 137 healthy controls. Genetic analysis of IL-10 SNPs at -819 and -592 was carried out with the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) approach. The IL-10 mRNA expression of AML patients and controls with different genotype was detected by real-time quantitative polymerase chain reaction (RT-PCR). Genetic analysis of IL-10 revealed that the -819AA genotype frequencies and the -819A allele frequencies in the AML group were higher than in the controls (59.1% vs 40.9%; 75.6% vs 63.9%, respectively); there were remarkable differences in -819T/C and -592A/C gene distribution ($P < 0.05$) and the TA haploid frequencies were higher in the AML group (75.6% vs 63.9%, $P < 0.05$). IL-10 mRNA expression in incipient AML patients was obvious higher than the non-tumor group and the remission group (7.78×10^{-3} vs 2.43×10^{-3} , 3.64×10^{-3} , $P < 0.05$). The study suggested that the haploid TA and genotype TA/TA may be associated with AML in Han people in Hunan province. The IL-10 SNPs at -819 and -592 sites were associated with AML and may affect IL-10 mRNA expression in AML patients.

Keywords: Gene polymorphisms - AML - IL-10 - SNP - Hunan, China

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Introduction

Interleukin-10 (IL-10) has pleiotropic effects in immunoregulation and inflammation and influences many aspects of the immune response (Gibson et al., 2001). The IL-10 gene is located on chromosome 1 and the promoter is polymorphic (Eskdale et al., 1997). Over the past decade, the research in IL-10 gene polymorphism, specially in the promoter region, found that IL-10 single nucleotide polymorphisms (SNPs) was associated with cancer susceptibility and affected the severity, progress of the disease and the IL-10 expression level (Zhou et al., 2008; Chen et al., 2010; Zhuang et al., 2010). SNPs at -819 (C/T) and -592 (C/A) upstream of the transcription start site have been identified and become the research focus in genetic susceptibility and in the process of cancer in the recent 10 years (Cunningham et al., 2003). They can help us to understand the occurrence and development of human diseases, and the response to drug therapy. Currently, it is known that at least 93% of human genes could present SNPs (Li et al., 2012).

IL-10 has immune-stimulating (promote cancer potentially) and immuno-suppressive (inhibit cancer potentially) dual biological functions and may regulate tumor susceptibility and development (Villalta et al., 2011). Recent studies showed that IL-10 SNP was associated with the non-Hodgkin's lymphoma (Cunningham et al., 2003;

Lan et al., 2006). There was no relative research on the relation between IL-10 SNP and myeloid systemic tumor, acute myeloid leukemia specially. This study investigated the association of the IL-10 SNPs at -819, -592 and its expression, and the Susceptibility to acute myeloid leukemia.

Materials and Methods

Clinical samples

All specimens were obtained from adults diagnosed and/or treated in the Xiangya Hospital, the Second and the Third Xiangya Hospital of Central South University during March 1, 2011 to January 1, 2012 and all the participants were the Han people in Hunan province. The specimens were collected from the AML patient group, non-tumor patient group and the control group, respectively. A total of 115 AML were enrolled in the research, including 54 newly diagnosed AML patients, 35 complete remission patients treated with chemotherapy, and 26 relapsing AML patients (without complete remission). These patients did not have any hereditary blood diseases, such as hereditary hemorrhagic telangiectasia or Fanconi anemia, and they did not contact the potential drugs, poisons which would cause leukemia. All the cases met the AML diagnose standards (Zhang et al., 2008). Non-tumor patients group included 30 non-

Table 1. Primers, Restriction Enzymes and Digested Fragments of the SNPs at -819 T/C and -592 A/C of Human IL-10

	IL-10 -819 T/C	IL-10 -592 A/C
Upstream primer	5'-TCATTCTATGTGCTGGAGATGG-3'	5'-GGTGAGCACTACCTGACTAGC-3'
Downstream primer	5'-TGGGGGAAGTGGGTAAGAGT-3'	5'-CCTAGGTCACAGTGACGTGG-3'
Annealing temperature	59 °C	58 °C
Length of digested fragment	209 bp	412 bp
Restriction enzyme	MslII [5'...CAYNN ^NNRTG...3']	RsaI [5'...GT^AC...3']
Digested fragment	C: 93bp+116bp; T: 209bp	A: 176bp+236bp; C: 412bp

tumor patients (19 iron-deficiency anemia patients and 11 Leukopenia patients). There were 137 gender- and age-matched healthy physical examinees in the control group and they did not have any hereditary diseases (such as diabetes, connective tissue diseases, tumor, et al.).

Collection and preservation of the sample

2-5 ml bone marrow was collected after the agreement of the participants and treated with erythrocyte lysis solution. Leukocytes were then collected with centrifugation (2000 rpm, 2 min) at 4 °C and stored in Trizol (10⁷ leukocytes/ml) at -80 °C for RNA extraction.

Genetic polymorphism analysis

Genomic DNA was extracted from bone marrow using the Qiagen DNA Isolation kit (Qiagen GmbH, Hilden, Germany). IL-10 promoter polymorphisms were identified by polymerase chain reaction amplification and restriction fragment length polymorphism analysis (PCR-RFLP). The SNPs sequence of -819 T/C (rs 1800871) and -592 A/C (rs 1800872) of human IL-10 was searched through the GenBank from the National Center for Biotechnology Information (NCBI). The primer sequence of each SNP was designed by the software Primer 5.0 (Lin et al., 2011) as shown in Table 1. The genomic DNA of AML patients and healthy controls was used as template and each PCR was performed in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, USA). After amplification, all products were digested with restriction enzymes, and separated by 2.5% agarose gel electrophoresis stained with GoldView for visualization. 10% PCR-amplified DNA samples were examined by DNA sequencing by Shanghai biological engineering company to confirm the genotyping results.

Real-time PCR of IL-10 mRNA

IL-10 mRNA of the isolated bone marrow nucleated cells from AML patient or healthy controls was determined by the Real-time PCR. The procedure conditions were: pre-denaturation at 95°C for 1 min, 45 cycles of denaturation at 95°C for 15 s, annealing at 65°C for 30 s, extension at 72°C for 30 s, and a final cycle of 5min at 72 °C. Each time, the GAPDH was amplified to detect the cytokine quantity. $2^{-\Delta Ct}$ was used for the quantitate of IL-10 mRNA and $\Delta Ct = Ct_{IL-10} - Ct_{GAPDH}$.

Statistical analysis

Genotype, allele and haploid frequencies were compared by the χ^2 test to determine whether the genotype distribution of the two SNPs matched the Hardy-Weinberg equilibrium and P value > 0.05 was considered as the SNPs matched the Hardy-Weinberg equilibrium. The quantity of

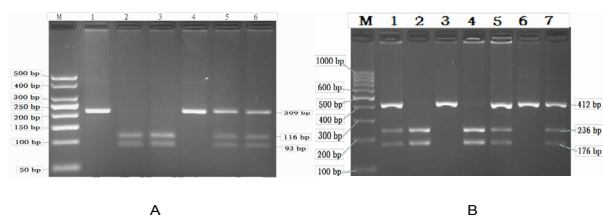


Figure 1. The PCR Electrophoregram of the SNPs of Human IL-10 DNA with Different Restriction Enzyme Digestion. A. Electrophoregram of the SNPs at -819 digested with MslII restriction enzyme (M: 50bp DNA ladder Mark; 1, 4: TT genotype; 2, 3: CC genotype; 5, 6: CT genotype); B. Electrophoregram of the SNPs at -592 digested with RsaI restriction enzyme (M: 100bp DNA ladder Mark; 1, 4: TT genotype; 2, 3: CC genotype; 5, 6: CT genotype)

IL-10 mRNA was shown as median (interquartile range, M (QR)), and the Kruskal-Wallis test was used to compare the values. The linkage disequilibrium and haplotype analysis of two SNPs used SNPStats software. Odds ratio (OR) and 95% confidence intervals (CIs) were calculated. All statistical tests were two-sided test and significant level $\alpha = 0.05$. The data were analyzed using SPSS 16.0 software.

Results

Analysis of the SNPs at IL-10 -819, -592 sites

According to the PCR electrophoregram, allele analysis revealed that the SNPs at -819 includes TT, CC and TC genotypes while the SNPs at -592 includes AA, CC and AC genotypes. When the PCR product at -819 site was digested with MslII restriction enzyme, the fragments were 93bp+116bp, 209 bp and 209bp+116bp+93bp, respectively, as shown in Figure 1A; When the PCR product at -592 site was digested with RsaI restriction enzyme, the fragments were 176bp+236bp, 412bp and 412bp+236bp+176bp, respectively, as shown in Figure 1B.

10% of the PCR samples were directly sequenced to ensure the reliability of the digestion. As in Figure 2, the sequencing results of the SNPs at -819 showed that there was only a single C peak in CC genotype, a single T peak in TT genotype and the overlapping C and T peaks in CT genotypes; similar to SNPs at -592 site, a single A peak in AA genotype, a single C peak in CC genotype and the overlapping A and C peaks in AC genotypes were found.

Hardy-Weinberg equilibrium test

The allele frequencies of the SNPs at -819 and -592 sites in AML cases and controls were test in Hardy-Weinberg equilibrium. There were no statistically significant difference in the distribution of alleles between

Table 2. Allele and Genotype Distribution of the SNPs at -819 in AML Cases and Controls

Group	Case	Genotype			Allele number	Allele	
		CC	TT	TC		T	C
AML	115	9(7.8%)	68(59.1%)	38(33.1%)	230	169(75.6%)	61(24.4%)
control	137	18(13.1%)	56(40.9%)	63(46.0%)	274	175(63.9%)	99(36.1%)
χ^2			8.494			8.153	
<i>P</i>			0.014			0.004	

Data are reported as number with percent in parentheses

Table 3. Allele and Genotype Distribution of the SNPs at -592 in AML Cases and Controls

Group	Case	Genotype			Allele number	Allele	
		CC	TT	TC		T	C
AML	115	9(7.8%)	68(59.1%)	38(33.1%)	230	169(75.6%)	61(24.4%)
control	137	18(13.1%)	56(40.9%)	63(46.0%)	274	175(63.9%)	99(36.1%)
χ^2			8.494			8.153	
<i>P</i>			0.014			0.004	

Data are reported as number with percent in parentheses

Table 4. The IL-10 mRNA Expression in Bone Marrow of AML Patients (M(QR))

Group	Case (n)	$2^{-\Delta Ct}$	d.f.	<i>P</i>
Newly diagnosed AML	54	7.78×10^{-3} (3.31×10^{-3})		
Remission AML	35	3.64×10^{-3} (0.98×10^{-3})	2	<0.05
Non-tumor controls	30	2.43×10^{-3} (1.22×10^{-3})		

d.f. = degrees of freedom

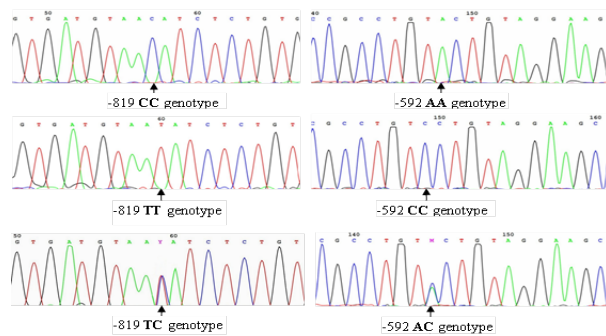


Figure 2. Sequencing Results of the IL-10 SNPs at -819, -592 Sites

patients and controls ($P > 0.05$). The results suggested that the genotype distribution was in the Hardy-Weinberg equilibrium.

Allele and genotype distribution of the SNPs at -819 and -592 in AML cases and controls

Three kinds of genotype were found respectively in the IL-10 SNPs and Table 2 and Table 3 showed the Allele and genotype distribution of the two SNPs. For SNPs at -819, allele analysis revealed that the T allele frequency was 75.6% in AML patients and 63.9% in controls ($P = 0.004$). A significant difference was observed in the genotype distribution between cases and controls. TT prevalence was markedly higher in cases than in controls (59.1 vs 40.9%, $P = 0.014$). The prevalence risk of the -819 TT was 2.492 times higher than the -819 CC genotype (OR=2.492; 95% CI: 1.013-5.825). Similar to SNPs at -819, significant differences were observed in the -592AA genotype percentage and -592A allele frequencies between case and control groups ($P < 0.05$). The number of individuals with the AA genotype was significantly higher in cases than in controls (59.1 vs 40.9%, $P = 0.014$). The A allele

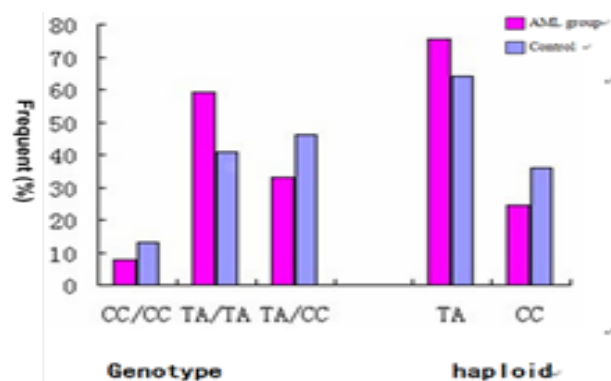


Figure 3. Genotype and Haploid Frequencies of IL-10 in AML Cases and Controls

frequency was markedly higher in cases than in controls (75.6 vs 63.9%, $P = 0.004$). The prevalence risk of the -592AA genotype was 2.492 times higher than the -592 CC genotype (OR=2.492; 95% CI: 1.013-5.825).

The linkage disequilibrium and haploid analysis of the IL-10 SNPs at -819 and -592

The SNPStats software (<http://bioinfo.iconcolgia.net/snpstats/start.htm>) was used to analyze the linkage disequilibrium of the IL-10 SNPs at -819 and -592. The results were $D' = 1$ and $r^2 = 1$ ($D' > 0.8$ suggested that the two SNPs had strong linkage and $r^2 > 1/3$ meant that the two SNPs were linkage disequilibrium) and suggested that -819C and -592C were complete linkage and -819T and -592A were co-inherited. There were only two kinds of haploids consisted of the two sites, which were TA and CC. With the analysis of the IL-10 SNPs at -819 and -592 in AML patients and healthy controls, the genotype and haploid distribution, as in Figure 3, suggested that there were significant differences in the IL-10 genotype and haploid frequencies between AML patients and healthy controls. The percentage of TT/AA genotype and TA haploid frequencies were higher in AML patients than healthy controls. We also found that the risk of the TA/TA genotype suffered from AML was 2.492 times higher than the CC/CC genotype (OR=2.492).

The IL-10 mRNA expression of AML patients

The IL-10 expression were detected in the bone

marrow of 54 newly diagnosed AML patients, 35 remission patients and 30 non-tumor controls. Ct value was calculated through the fluorescence intensity curves with RT-PCR and $2^{-\Delta Ct}$ represented the expression of IL-10 mRNA in every sample ($\Delta Ct = Ct_{IL-10} - Ct_{GAPDH}$). If the results did not accord with normal distribution, M (QR) was chosen to be the results. Table 4 revealed that the IL-10 expression in the newly diagnosed AML patients was higher than the non-tumor controls; the remission patients was lower than the newly diagnosed ones, but still higher than the non-tumor controls ($P < 0.05$).

Discussion

Helper T lymphocyte subsets are consisted of Th1 and Th2 subsets and IL-10 are mainly secreted by Th2 subsets. The recent study pointed out that IL-10 down-regulated the perforin and granzyme B gene expression, so as to inhibit the function of cytotoxic T lymphocytes. The study focused on the relation between IL-10 SNP at -819, -592 and the susceptibility to AML. The observed gene polymorphism were in Hardy-Weinberg genetic equilibrium with good representative of population.

Mok CC, et al. found that the allele frequencies of IL-10 SNPs at -819T/C and -592A/C were significantly different in different peoples (Mok et al., 1998). We analyzed that between the AML patients and healthy controls of Han people in Hunan province. Results suggested that the allele frequencies of healthy controls in Hunan at -819T/C (T 63.9%, C 36.1%) and -592A/C (T 63.9%, C 36.1%) were similar to Guangxi province (-819T 65.9%, C 34.1%; -592A 65.9%, C 34.1%) (Lan et al., 2007).

According to the genetic analysis, the remarkable higher value of -819AA and -819A allele frequencies in AML patients and the significant differences in -819T/C and -592A/C gene distribution ($P < 0.05$) indicated that -819T and -592A alleles probably were the inherent predisposing genes of AML. The linkage disequilibrium analysis of -819T/C and -592A/C genotype showed that the two SNP sites were with complete linkage disequilibrium ($D' = 1$). The IL-10 gene -819 and -592 has only TA, CC two haploid. The TA haploid frequencies and the TA/TA genotype distribution in AML group were higher than that in healthy control group ($P < 0.05$), which suggested that the TA haploid and TA/TA genotype of IL-10 were associated with the susceptibility of AML in Han people of Hunan province.

In this study, we also use RT-PCR to analyze the IL-10 mRNA expression. Our study showed that the IL-10 mRNA expression of AML group was remarkable higher than the non-tumor group (7.78×10^{-3} vs 2.43×10^{-3} , $P < 0.05$). This indicated the leukemic cells in bone marrow could secrete IL-10 in an autocrine way as the local secretion of IL-10 in the tumor growth process, may cause the loss of the sensitivity to cytotoxic T lymphocytes and make the growth of tumor cells easier. This result was similar to the published paper (Yang et al., 2002), IL-10 mRNA of remission group (by chemotherapy) was significantly decreased when compared with the newly diagnosed AML group (3.64×10^{-3} vs 7.78×10^{-3} , $P < 0.05$).

Maybe the chemotherapy inhibits the growth and activity of the leukemic cells and affects the function of the patients' immune system (Hsieh et al., 2000).

In addition, we found that IL-10 mRNA of the TA/TA genotype was the lowest in the AML patients ($P < 0.05$) and non-tumor controls, while the CC/CC genotype was the highest ($P < 0.05$). So the genotype of IL-10 at -819 and -592 sites may affect the expression of IL-10 mRNA. But the mechanism of the IL-10 promoter gene polymorphisms affect IL-10 expression is not clear and needs further study.

In conclusion, the study indicated that the IL-10 SNPs at -819 and -592 was associated with the AML. The -819T and -592A may be the susceptibility gene. IL-10 gene polymorphism may affect the expression of IL-10 mRNA expression in AML patients.

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