

Correlation between IL-4 gene polymorphisms as well as its mRNA expression and rheumatoid arthritis

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Abstract. – OBJECTIVE: This study sought to explore the correlation between IL-4-590C/T polymorphism as well as mRNA expression and the occurrence of rheumatoid arthritis (RA).

PATIENTS AND METHODS: IL-4-590C/T polymorphisms, detected by a TaqMan probe from 150 RA patients who were treated in Yantaishan Hospital from June 2014 to June 2016, and from 150 healthy people, all from the Han population, were selected for the study. Interleukin-4 (IL-4) mRNA expression was detected by Real-time Polymerase Chain Reaction (PCR), and the differences in IL-4mRNA expressions of different genotypes in RA patients were compared.

RESULTS: The difference in CC, CT and TT genotype distributions between the RA group and the healthy group were statistically significant ($p < 0.05$). The mutation frequency of the T allele in IL-4 of the RA group was significantly higher than that of the healthy group ($p < 0.01$). The mRNA expression of IL-4 in the RA group was significantly lower than that in the healthy group ($p < 0.01$). The mRNA expression of IL-4 in RA patients was gradually decreased in the following order: CC, CT and TT, and the differences among these genotypes were significant ($p < 0.01$).

CONCLUSIONS: IL-4-590C/T polymorphism may be correlated with the incidence of RA in the Chinese Han population. Carrying the T allele can significantly increase the risk of RA and reduce the mRNA expression of IL-4.

Key Words

Rheumatoid arthritis (RA), Interleukin-4 (IL-4), Single nucleotide polymorphism (SNP), mRNA.

while the incidence rate is about 0.4% in China; the onset age typically ranges from 25 to 55 years old. Moreover, the incidence rate among women is about three times higher than men^{3,4}. Modern genetics considers that, aside from traumatic conditions, almost all human diseases and traits are caused by the combined effects of genetic and environmental factors, which indicate the variable contribution of different factors in disease causation. The onset of RA is caused by the interaction between hereditary traits and environmental factors³, in which genetic factors account for about 60% of RA susceptibility^{5,6}. Studies have confirmed that the incidence of RA is closely associated with immune dysfunction, wherein the dysfunction of helper T cells is an important pathogenic factor in its etiology⁷. In this process, the activated T cells can release a variety of cytokines, resulting in the chronic inflammatory reaction that is a hallmark of RA⁸⁻¹⁰. Interleukin-4 (IL-4) is one of the most important cytokines implicated in RA. In recent years, with the development of single nucleotide polymorphism (SNP) typing techniques, a study found that IL-4-590C/T polymorphism was correlated with the onset of RA¹¹. Therefore, we selected the IL-4-590C/T SNP site for genetic analysis, detected the mRNA expression of IL-4 in peripheral blood, and investigated the correlation between RA and the IL-4 gene polymorphism, and its mRNA expression in the Chinese Han population. It is anticipated that these findings will elucidate the pathogenesis of RA, providing a reference for improving clinical treatment.

Introduction

Rheumatoid arthritis (RA) is a serious and systemic chronic autoimmune disease mainly characterized by arthrosynovitis¹. The proliferation and infiltration of inflammatory cells in the affected joints destroy articular bone and cartilage matrix, resulting in joint deformity and loss of function². The global incidence rate of RA is about 0.5-1%,

Patients and Methods

Patients

Our research team recruited 150 RA patients, who were treated in Yantaishan Hospital from June 2014 to June 2016. The diagnosis of RA was defined by the diagnostic criteria formulated by the American College of Rheumatology in 1987¹². The diagnosis could be confirmed by meeting at least

4 of the following 7 items: a) early morning stiffness occurred at least 1 h every day and the course of disease lasted for at least 6 weeks; b) arthritis occurred in three or more joints and the course of disease lasted for at least 6 weeks; c) hand arthritis of proximal, metacarpophalangeal and wrist joints occurred and the course of disease lasted for at least 6 weeks; d) arthritis was symmetrical and the course of disease lasted for at least 6 weeks; e) rheumatoid nodules; f) characteristic x-ray changes of the hands (at least bone joint with erosive stenosis and where osteoporosis occurred); g) rheumatoid factor in blood was positive. Inclusion criteria: Chinese Han population who were diagnosed by the above diagnostic criteria and between 18-80 years old. Exclusion criteria: patients who had congenital genetic disease, systemic lupus erythematosus, ankylosing spondylitis or other autoimmune disease history. Exclusion criteria: patients who were pregnant and those that had complications from severe heart, liver, kidney, brain and other organ conditions and diseases. For the control group, 150 healthy volunteers were selected from the health examination center in the hospital. All healthy subjects had no history of joint, autoimmune, cardiac, respiratory, or metabolic diseases, including diabetes mellitus, hypertension and obesity. The physical and chemical indicators taken during the examination of the selected control subjects were all within the normal ranges. This study was approved by the Medical Ethics Committee of our hospital, and all subjects signed informed consent. The results of routine blood test, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and rheumatoid factor (RF) of all the subjects, were collected.

Research Methods

Extraction of DNA and RNA

3 ml venous blood from each subject was collected using an Ethylene Diamine Tetraacetic Acid (EDTA) vacuum anticoagulation tube, followed by the extraction of 1 ml venous blood and the addition of RNALock blood RNA stabilizer (Tiangen Biotech Co., Ltd., Beijing, China). DNA was extracted using a medium volume whole blood genomic DNA extraction kit (BioTeke Corporation, Beijing, China), and RNA was extracted using a blood/liquid sample total RNA rapid extraction kit (BioTeke Corporation, Beijing, China). Specific steps for extraction were conducted according to the instructions of each of the kits. The purity and concentration of samples were detected by Nanodrop-2000 Ultra Micro-volume UV Spectrophotometer (Thermo Fisher Scientific,

Waltham, MA, USA). The purity and concentration of all DNA and total RNA samples met all experimental requirements.

Analysis of IL-4 Gene Polymorphism

The TaqMan[®] SNP Genotyping Assay Kit (American Biosystems, Inc., Roanoke, VA, USA) was used for detection analysis of samples for the IL-4-590C/T SNP genotype.

mRNA Reverse Transcription

Reverse transcription was performed using the Reverse Transcription Kit (Toyobo Co., Ltd., Osaka, Japan) according to the protocol for preparing cDNA. The specific reaction system was as follows: 2 µg total RNA was collected to conduct a thermal denaturation at 65°C for 5 min and then the sample was immediately placed on ice for cooling. 4 µL 5×RT Master Mix was added followed by the addition of diethyl pyrocarbonate (DEPC) water until the total volume reached 20 µL. The reaction parameters were 37°C for 15 min, 52°C for 5 min and 98°C for 5 min.

Real-time PCR Detection

The mRNA expression of IL-4 was amplified by Real-time PCR using SYBR Green PCR Master mix reagent (TaKaRa, Otsu, Shiga, Japan). IL-4, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene primers were synthesized by Sangon Biotech (Shanghai, China) and specific sequences were shown in Table I. The reaction system was as follows: 2 µL cDNA was collected, adding 12.5 µL 2×SYBR Green PCR Master mix as well as upstream and downstream primers (each 0.5 µL, 10 µM/L). Ultrapure water was added until the total volume reached 25 µL. The sample was amplified in a Mastercycler realplex4s Real-time fluorescence quantitative PCR instrument, which was purchased from the Eppendorf Company (Hamburg, Germany). The reaction parameters were 95°C for 30 s, 95°C for 5 s and 60°C for 30 s, for a total of 40 cycles. Then, the amplification curves and Ct values of each reaction were read. With GAPDH as a reference, the relative quantitative $2^{-\Delta\Delta C_t}$ was used to compare the differences in expression among the genetic groups.

Statistical Analysis

Statistical analysis was completed by SPSS19.0 statistical software (SPSS Inc., Chicago, IL, USA). The results of measurement data were expressed as $\bar{x} \pm s$, the independent sample *t*-test was used for comparisons between two groups and com-

Table I. Primer sequences.

Gene for detection	Primer sequence
IL-4	Upstream: 5'-TACAGCCACCATGAGAAGGAC-3' Downstream: 5'-TGATCGTCTTTAGCCTTTCCA-3'
GAPDH	Upstream: 5'-GACCTGACCTGCCGTCTA-3' Downstream: 5'-AGGAGTGGGTGTCGCTGT-3'

parisons among multiple groups were tested by one-way ANOVA followed by the S-N-K method. The likelihood ratio χ^2 test was used to analyze whether each genotype distribution conformed to Hardy-Weinberg Equilibrium (HWE). The direct counting method was adopted to calculate each genotype. The χ^2 test using R×C contingency tables was used for the comparisons of genotypes and allele frequencies among groups. $p < 0.05$ suggested that the difference was statistically significant.

Results

Comparisons of General Data

There was no significant difference in the comparison of age and gender ratios of the subjects between the two groups. The platelet (PLT), ESR, CRP, and RF of patients in the RA group were significantly higher than those of the healthy controls, and the differences were statistically significant ($p < 0.01$) (Table II).

Genetic Equilibrium Test

The likelihood ratio χ^2 test was used for determining whether the genotype distribution fre-

quency of IL-4-590C/T in people of the healthy group and the RA group was conformed to the Hardy-Weinberg Equilibrium (HWE). Also, genotype frequency distributions were met ($p > 0.05$). All of the frequency distributions were conformed to HWE, demonstrating that the sample population had good group representativeness (Table III).

Distribution of IL-4-590C/T Variation in the Healthy Group and the RA Group

The distribution frequencies of the CC, CT and TT genotypes were 72%, 26% and 2%, respectively, in the healthy group, and 59.33%, 32% and 8.67%, respectively, in the RA group. The composition of IL-4-590C/T variant genotype distribution of the two groups was different and the difference was statistically significant ($p = 0.011$). This suggests that the incidence of RA might be correlated with the IL-4-590C/T polymorphism. Further comparing the different genotypes, the odds ratio (OR) of the TT genotype to the CC genotype was 1.115, and the 95% CI was 1.029-1.208. The odds ratio of carrying the CT genotype to carrying the CC genotype was 1.131 and the 95% CI was 0.967-1.323. This suggests that the occurrence of RA was correlated with homozygous

Table II. Comparisons of general data.

Group	No.	Male/Female	Age (years)	PLT ($\times 10^9/L$)	ESR (mm/h)	CRP (mg/L)	RF (IU/ml)
Healthy group	150	51/99	53.06±7.41	234.28±24.69	14.19±4.92	23.61±4.04	10.38±4.54
RA group	150	46/104	57.25±6.68	302.72±29.33**	28.80±5.47**	76.73±8.15**	89.03±7.28**

Note: Compared with the healthy group, ** $p < 0.01$.

Table III. Detection of IL-4 genotypes by HWE.

Group	No.	CC		CT		TT		χ^2 -value	p-value
		Actual frequency	Theoretical frequency	Actual frequency	Theoretical frequency	Actual frequency	Theoretical frequency		
Healthy group	150	108	108.38	39	38.25	3	3.37	0.06	0.97
RA group	150	89	85.13	48	55.75	13	9.13	2.89	0.23

Table IV. Comparisons of genotype distributions between the two groups.

Group	No.	Genotype [case (%)]			χ^2	P
		CC	CT	TT		
Healthy group	150	108 (72.00)	39 (26.00)	3 (2.00)	9.014	0.011
RA group	150	89 (59.33)	48 (32.00)	13 (8.67)		

Table V. Comparisons of allele distributions between the two groups

Group	No.	Allele [case (%)]		χ^2	P
		C	T		
Healthy group	150	255 (85.00)	45 (15.00)	8.816	0.003
RA group	150	226 (75.33)	74 (24.67)		

mutation of IL-4-590C/T and the risk of RA was increased due to the TT homozygous mutation of IL-4-590C/T. However, the incidence of RA had no correlation with the heterozygous mutation of IL-4-590C/T (Table IV).

Comparisons of C and T Allele Distributions

The distribution frequencies of C and T alleles were 85% and 15%, respectively, in the healthy group, and 75.33% and 24.67%, respectively, in the RA group. The frequency of the allele T in the RA group was significantly higher than that in the healthy group and the difference was statistically significant ($p=0.003$). The OR for this difference was 1.128 and the 95% CI was 1.041-1.223 (Table V).

Comparison of mRNA Expression of IL-4 Between the Two Groups

The mRNA expression of IL-4 of patients in the RA group was significantly lower than that of the healthy group, showing a statistically significant difference ($p<0.01$) (Figure 1).

Comparisons of IL-4 mRNA Expression in Different Genotypes of the RA Group

The patients in the RA group were divided into three subgroups according to different genotypes and the mRNA expression of IL-4 across the different genotypes was compared. The results showed that the mRNA expression of IL-4 was gradually decreased across the genotypes in the following order: CC, CT and TT. Therefore, compared with the CC genotype, the expression of IL-4 mRNA in the CT and TT genotypes were significantly decreased and the differences were

statistically significant ($p<0.01$). The expression of IL-4 mRNA in the TT genotype was significantly lower than that in the CT genotype, showing a statistically significant difference ($p<0.01$) (Figure 2).

Discussion

RA is a chronic inflammatory disease with unclear mechanisms of causation. The body's abnormal immune response and inflammatory reaction are associated with RA, and these changes lead to the onset of RA and the delay of disease^{7,8}. Genetic researchers have confirmed that RA has a certain familial aggregation, and the prevalence of RA increases in first-degree relatives of RA patients, ranging from 2.8% to 12.1%. Various susceptible genes are involved in the early pathogenesis of RA, which leads to a series of complex pathophys-

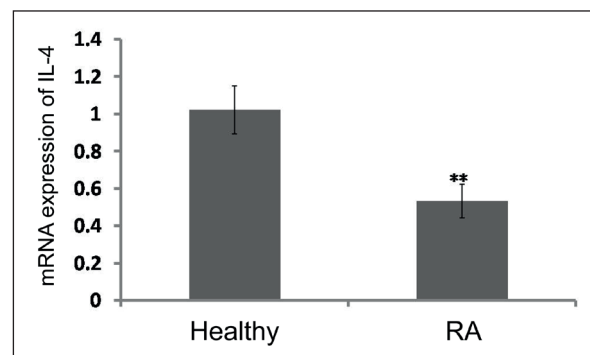


Figure 1. Comparison of mRNA expression of IL-4 between the two groups. IL-4 expression in the healthy group and the RA group as detected by Real-time PCR, showing that the expression was lower in the RA group than in the healthy group. Note: Compared with the healthy group, ** $p<0.01$.

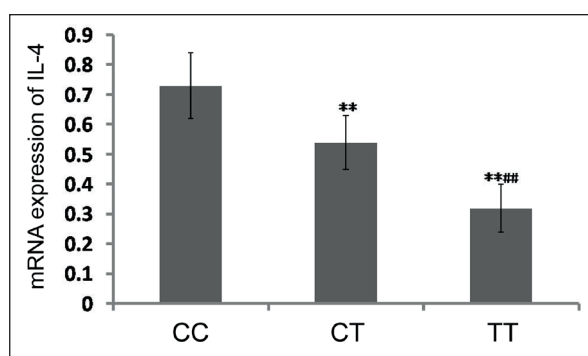


Figure 2. Comparisons of IL-4 mRNA expression levels in different genotypes of the RA group. The patients in the RA group were divided into three subgroups according to the different genotypes, showing that IL-4 mRNA expression in CT and TT genotypes were significantly lower than in the CC genotype, and the expression in the TT genotype was significantly lower than in the CC genotype. Note: Compared with the CC genotype, ** $p < 0.01$; Compared with the CT genotype, ### $p < 0.01$.

iological changes^{5,6}. Currently, it is still uncertain which gene plays a decisive role in the pathogenesis and susceptibility of RA. Therefore, it is of great significance to explore the effects of RA-related genes in the incidence and development of the disease by advancing RA-directed genetic research. This research aimed to understand the biological mechanisms and genetic factors implicated in RA. The genome project found that the two SNP sites located at 6q32 and chromosome 9 were independently associated with the risk of RA¹³⁻¹⁵. In recent years, with the development of genome-wide association analysis and SNP analysis techniques, more and more RA-related polymorphic sites have been discovered. The studies indicate that 49A/G and CT60G/A polymorphisms in cytotoxic T lymphocyte-associated antigen 4 have correlations with the occurrence of RA^{16,17}. The rs1343151 and rs10489629 sites of the IL-23 receptor are polymorphic sites of RA that show a significant correlation with RA susceptibility^{9,18}. The TNF- α promoter gene, 380A/G polymorphism, also affects the onset of RA^{19,20}. Research on an Asian population sample revealed that the rs3093024 polymorphic site in CCR6 was associated with susceptibility of RA²¹. Thus, RA is caused by the combined action of multiple genes. Moreover, multiple SNP sites can affect the susceptibility of RA. The IL-4 gene is located at 5q23-31 and has a length of about 10kb. IL-4, which is synthesized and secreted by the activated CD4⁺ cells, basophils and clasmatoblast, is the major cytokine of Th2 cells and has a strong anti-inflammatory effect. Due to its immunosup-

pressive action, IL-4 can mediate humoral immune reaction, which can inhibit inflammation, graft rejection and other immune responses caused by Th1. It has a long-term inhibitory effect on Th1 cells in the microenvironment^{22,23}. Meanwhile, IL-4 can inhibit the mRNA transcription of interferon- γ and suppress interferon- γ inducing B cells to generate IgG antibody, thereby inhibiting interferon- γ from exerting its biological effects. Also, IL-4 has an inhibitory effect on the secretion of pro-inflammatory factor. IL-4 can inhibit the secretion of pro-inflammatory factor IL-1 by enhancing the IL-1 receptor antagonist. IL-4 has a protective effect on cartilage degradation. By inhibiting the production of matrix metalloproteinase, IL-4 can reduce joint injury resulting from matrix metalloproteinase in synovium. In recent years, genetic studies have found that there are multiple SNP sites, including IL-4-590C/T polymorphism^{24,25}, IL-4-589C/T polymorphism²⁶, and IL-4-33C/T gene polymorphism²⁷ in the promoter of IL-4 gene. Studies have confirmed that these polymorphic sites affect the susceptibility of many diseases. A study on European populations has found that the IL-4-590C/T gene polymorphism is associated with severe joint injury²⁸. Meanwhile, Hussein et al¹¹ observed that carrying the TT genotype can significantly increase the risk of RA. The current study on the Chinese Han population showed that the IL-4-590C/T gene polymorphism was significantly correlated with the risk of RA, which was increased due to the TT homozygous mutation of IL-4-590C/T. Thus, carrying the T-allele can significantly increase the risk of RA in the Chinese Han population. IL-4 participates in multiple stages in the pathogenesis of RA. Firstly, IL-4 can promote macrophages to move to vascular endothelial cells, followed by overflowing blood vessels and adhering to the position of inflammation. Secondly, IL-4 can inhibit the release of TNF- α and the production of matrix metalloproteinases and pro-inflammatory cytokines, including IL-1, IL-6 and IL-8. This further promotes the generation of soluble TNF receptor and IL-1 receptor. Thirdly, IL-4 can produce anti-platelet antibodies and decrease the number of platelets, thereby reducing the blood hypercoagulable state. Finally, by promoting the proliferation of Th2 cells, IL-4 can increase the release of other anti-inflammatory cytokines and inhibit the proliferation of Th1 cells. This reduces the imbalance of the proportion of Th1/Th2 cells. This work indicated that the mRNA expression of IL-4 in RA patients was significantly lower than that of comparable healthy subjects. By further grouping RA patients according to different genotypes of IL-

4-590C/T, we showed that the mRNA expression of IL-4 was gradually decreased in the following order: CC, CT and TT. Thus, it is speculated that the mutation of the TT gene in IL-4-590C/T can lead to a significant reduction of mRNA expression of IL-4, which, through a series of pathways, results in the incidence of RA.

In this study the IL-4-590C/T polymorphisms in RA patients and healthy subjects were explored as well as the relationships between mRNA expression of IL-4 and different genotypes of IL-4-590C/T in RA patients. The results confirmed that the IL-4-590C/T polymorphism is correlated with the onset of RA and that carrying the T-allele can significantly increase the risk of RA in the Chinese Han population. The mRNA expression of IL-4 is significantly decreased in RA patients. Moreover, the mRNA expression of IL-4 was gradually decreased across genotypes, from CC to CT to TT.

Conclusions

The incidence of RA results from the combined action of multiple genes, including IL-4 expression and environmental factors. This work should be further expanded upon using larger samples and multicenter studies as well as a broader focus on the interaction among multiple genes.

Conflict of Interest

The authors declare that they have no conflict of interest.

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