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Effect of Interferon-γ Polymorphisms on Ankylosing Spondylitis: A Case-Control Study

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Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: This research aimed to explore the effects of interferon-γ (*IFN-γ*) polymorphisms and expression profile on susceptibility to ankylosing spondylitis (AS) in a Chinese population.

Material/Methods: Blood samples were collected from 89 AS patients and 106 healthy controls. *IFN-γ* polymorphisms were genotyped by polymerase chain reaction (PCR) and sequencing methods. The genotype distribution of polymorphism in the control group was detected by Hardy-Weinberg equilibrium (HWE). Odds ratios (OR) with 95% confidence intervals (95%CI) were calculated using the χ^2 test to evaluate the association between AS susceptibility and *IFN-γ* polymorphisms. Moreover, serum IFN-γ level was measured by ELISA.

Results: rs1861493 and rs2430561 polymorphisms were conformed to be in HWE in genotypes distribution of the control group ($P>0.05$ for both). However, only TT genotype and T allele of rs2430561 presented significantly higher frequencies in AS patients than in healthy controls ($P=0.04$ and 0.03, respectively), indicating that they obviously increased the risk of AS in a Chinese population (OR=2.54, 95%CI=1.01–6.40; OR=1.60, 95%CI=1.04–2.46). In AS patients, serum IFN-γ level was higher than in controls, and its expression patterns showed significant association with genotypes of rs2430561.

Conclusions: *IFN-γ* rs2430561 polymorphism may contribute to the risk of AS through influencing IFN-γ expression.

MeSH Keywords: Interferon-alpha • Polymorphism, Genetic • Spondylitis, Ankylosing

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Background

Ankylosing spondylitis (AS) is a common chronic inflammatory disease involving the axial skeleton, sacroiliac joint, and periphery joints [1,2]. The clinical manifestations of AS mainly include pain, stiffness, spinal mobility limitation, and chest expansion, causing serious impacts on work and quality of life [3]. The prevalence of AS is 1.67% in Asia and 0.2–0.4% in China [4,5]. Previous reports have revealed that genetic factors play the leading role in the onset of AS [6,7], HLA-B27 is a known biomarker, and 90%-95% of AS patients are HLA-B27-positive. [8]. In addition, some other genes may also contribute to the etiology of AS, such as pentraxin 3 Gene (PTX3) and TNF-α [9]. Moreover, anti-TNF-α agents are widely used to reduce disease activity of patients with AS. However, therapeutic failure was also reported in previous studies [10]. The unclear etiology may be responsible for the failures. The etiology of AS is complex, with the involvement of multiple genetic and environmental factors. To improve the management of AS and improve patient quality of life, more investigations are required to explain the molecular mechanism of AS.

Interferon-γ (IFN-γ) is a Th1 cytokine which is the only type II interferon [11]. It is mainly secreted by natural killer cells and CD8+ T cells [12]. IFN-γ possesses not only broad-spectrum resistance for virus infection, but also has immunoregulatory functions [13]. Recently, abnormal expression of IFN-γ was reported to be associated with a variety of auto-inflammatory and immune diseases [14-16]. IFN-γ can activate inactive CD4+ cells to differentiate into Th1 cells and inhibit the proliferation of Th2. It is generally accepted that AS may be caused by vastly activated Th1 cells and weakly or hardly expressed Th2 cells [17]. The study carried out by Wang et al. reported that elevated expression of IFN-γ might contribute to the progression of AS [18]. However, few studies have explored the exact function of IFN-γ in AS.

Use of single-nucleotide polymorphisms (SNP) is becoming an important means to explore the association between genes diseases. In previous studies, a number of functional *loci* was identified in the *IFN*-γ gene [19]. Therefore, in the present study, we selected the common SNPs of *IFN*-γ to investigate their influence on AS development. In addition, serum *IFN*-γ level was also measured to reveal the mechanism of AS via *IFN*-γ polymorphisms.

Material and Methods

The cases and controls

In the prospective study, a case-control design was adopted with 89 patients with AS and 106 healthy controls. All subjects were all from Yongchuan Hospital from March 2014 to October 2015 and they had no blood relation with other study subjects. AS patients were diagnosed by clinical manifestation and CT or MRI examination based on New York criteria modified in 1984 [20]. Patients with other inflammatory or immune diseases were excluded. Finally, a total of 57 males and 32 females were included in the cases group, with an average age of 27.34±9.06 years. The controls were all from the Physical Examination Center of the same hospital during the same time and they were healthy without any diseases influencing study results, including 69 males and 37 females. The mean age was 25.66±9.38 years, with age range of 13–45 years. All subjects were of Chinese Han ethnicity, living in Chongqing. The control and case groups were matched for sex and age.

This study was approved by the Research Ethics Committee of Yongchuan Hospital (ID: CTER-YC-2014-01) and all subjects were informed the study objective and flow. Before sample collection, written consent was signed by every included subject.

Sample collecting

We collected 10 ml fasting peripheral venous blood from each subject in the early morning, using a blood collection tube. Blood samples were immediately centrifuged at 3000 rpm for 10 min, and the supernatant was kept at –80°C for further analyses. The archived serum specimens were used for DNA extraction and measurement of IFN-γ level.

DNA extraction and genotyping of *IFN*-γ polymorphisms

Genomic DNA was extracted using the TIANamp genomic DNA Kit purchased from TIANGEN BIOTECH CO., LTD (Beijing) [21], according to the manufacturer's instructions, and was stored at –20°C.

In this study, polymerase chain reaction (PCR) was conducted for genotyping of *IFN*-γ polymorphisms. PCR primers were designed via Primer Premier 5.0 software on the basis of the *IFN*-γ gene sequence published in GeneBank Database and were synthesized by Shanghai Sangon Biotech Co., Ltd. PCR primer sequences are listed in Table 1. In the next step, PCR system was mixed into a total of 25.0 μL solution, consisting of 20 ng DNA template, 5 μM of each primer, 2×PCR Mix, and ddH_2O. The PCR procedure was run using an Eppendorf PCR instrument (Germany) according the following steps: 95°C predegeneration for 5 min, 30 cycles of degeneration at 95°C for 30 s, 57°C annealing for 30 s, 72°C extension for 30 s, and final extension at 72°C for 5 min.

PCR amplification products were detected by 1.0% agarose gel electrophoresis (AGE) and qualified PCR products (Sangon Biotech, Shanghai) were used for sequencing for the determination of *IFN*-γ polymorphism genotypes and alleles.

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Table 1. The primer sequences of *IFN*-γ polymorphisms.

Polymorphism	Position		Primer sequence
rs1861493	Intron3	For.	5'-AGCAACAGCAAGGCGAAAAA-3'
		Rev.	5'-TGGTGGACCACTCGGATGA-3'
rs2430561	Intron1	For.	5'-TCAACAAAGCTGATACTCCA-3'
		Rev.	5'TTCTTACAACACAAAATCAAATCA-3'

Table 2. Genotype and allele distributions of *IFN*-γ polymorphisms between case and control groups.

Genotype/allele	Cases/% n=89	Control/% n=106	P	OR (95%CI)	PHWE
rs1861493					0.35
GG	16/17.98	13/12.27	0.12	1.96 (0.83–4.61)	
AG	41/46.07	42/39.62	0.16	1.56 (0.84–2.88)	
AA	32/35.95	51/48.11	–	1.00 (Ref.)	
G	73/41.01	68/32.08	0.07	1.47 (0.97–2.23)	
A	105/58.99	144/67.92	–	1.00 (Ref.)	
rs2430561					0.51
TT	15/16.85	9/8.49	0.04	2.54 (1.01–6.40)	
AT	36/40.45	39/36.79	0.27	1.41 (0.77–2.59)	
AA	38/42.70	58/54.72	–	1.00 (Ref.)	
T	66/37.08	57/26.89	0.03	1.60 (1.04–2.46)	
A	112/62.92	155/73.11	–	1.00 (Ref.)	

The measurement of IFN-γ level in serum

Serum IFN-γ level was measured using the IFN gamma ELISA Kit, Human (Thermo Scientific), according to the manufacturer's instructions.

Statistical analysis

The genotype distribution of *IFN*-γ polymorphism in the control group was tested for Hardy-Weinberg equilibrium (HWE). The genotype and allele difference comparison of *IFN*-γ polymorphism was conducted by χ^2 test. Relative risk of AS based on *IFN*-γ polymorphism is represented with odds ratios (OR) and 95% confidence intervals (95%CI). The above process was completed by PASW Statistics 18.0 software and the data are expressed by $\bar{\chi}\pm s$ or%. The serum IFN-γ level difference between the 2 groups or different genotypes of *IFN*-γ polymorphisms was compared by t-test and one-way ANOVA in GraphPad Prism 5 software. $P<0.05$ was considered as statistically significant.

Results

HWE test

The genotype distributions of both *IFN*-γ rs1861493 and rs2430561 polymorphisms in the control group conformed to HWE ($P=0.35$, 0.51 respectively), which showed that this study group was a representative Mendelian population.

Effects of *IFN*-γ polymorphisms on the susceptibility to AS

Genotype, allele frequencies of *IFN*-γ polymorphisms, and their effects on AS risk are shown in Table 2. Neither AG nor GG genotype frequency of rs1861493 were significantly different between the case and control groups, when compared with AA genotype frequency ($P>0.05$ for both). G allele of rs1861493 had no obvious difference between AS patients and healthy controls. These results indicated that neither genotype nor allele of rs1861493 polymorphism were associated with the risk of AS. However, rs2430561 TT genotype frequency in AS patients

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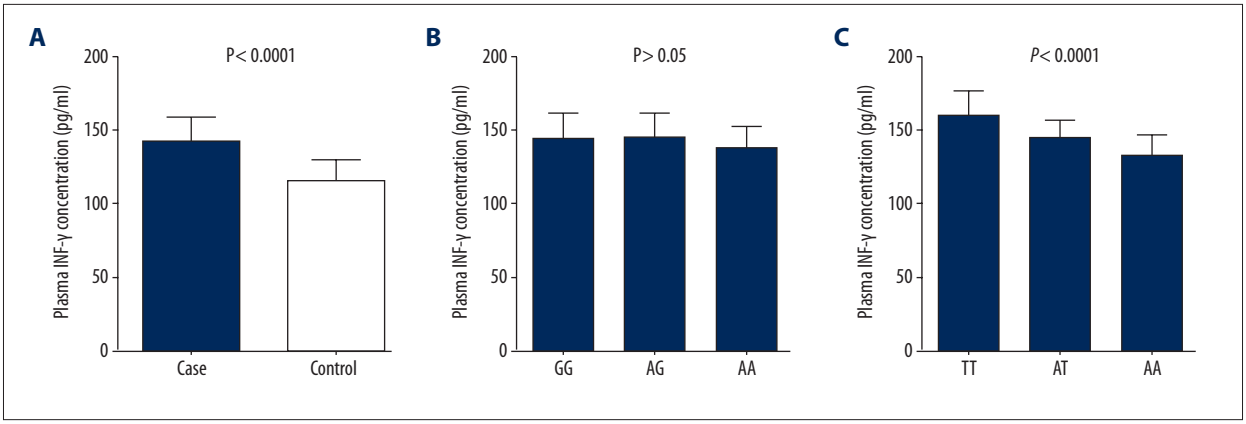

Figure 1. The expression patterns of serum IFN-γ in the case and control groups (**A**); The comparison of serum IFN-γ level between GG, AG, and AA genotypes of rs1861493 in patients with ankylosing spondylitis (**B**); The comparison of serum IFN-γ level based on genotypes of rs2430561 in patients with ankylosing spondylitis (**C**).

was significantly higher than that in the controls, in comparison with AA genotype (*P*=0.04), suggesting that TT genotype might contribute to the risk of AS (OR=2.54, 95%CI=1.01-6.40). Furthermore, T allele was also correlated with elevated susceptibility to AS (*P*=0.03, OR=1.60, 95%CI=1.04-2.46).

Effect of *IFN*-γ polymorphisms on serum IFN-γ level

The mean serum IFN-γ level of AS patients was 141.69±16.97pg/ml, and that in the control group was 115.90±16.97pg/ml. The difference between the 2 groups was statistically significant (*P*<0.0001) (Figure 1A). However, serum IFN-γ levels did not show an obvious association with rs1861493 genotypes (*P*=0.16) (Figure 1B). Additionally, IFN-γ levels were distinctly different between AS patients carrying different genotypes of rs2430561 (*P*<0.0001) (Figure 1C), and TT genotype was associated with increased IFN-γ level.

Discussion

In this research, we investigated the effects of *IFN*-γ rs1861493 and rs2430561 polymorphisms on AS susceptibility. The results showed that rs2430561 was significantly associated with the individual susceptibility to AS, but independent association was not detected based on genotypes or alleles of rs1861493 for the risk of AS. Moreover, serum IFN-γ level in AS patients was higher than that in healthy controls. The difference reached the extremely significant level. The conclusion was consistent with a previous study by Wang et al., who reported that peripheral Vδ1 T cell could inhibit the pathogenesis of AS via suppressing the expression of IFN-γ [10]. Serum IFN-γ level was also obviously different in AS patients with different genotypes of rs2430561 polymorphism. Therefore, rs2430561 might participate in the onset of AS through altering the expression of IFN-γ. This article was the first study to investigate the association of *IFN*-γ polymorphisms with AS development, especially in a Chinese population.

IFN is a class of cytokines with broad-spectrum resistance to viruses. It includes 3 classes according to homology and specific receptor [21]. IFN-γ is the only type IFN. In addition to its antiviral property, IFN-γ also play an important role in immune system and inflammatory response [22]. As an immunomodulatory factor, IFN-γ promotes the differentiation of CD4+ cells into Th1 cells and stimulates unregulated expression of some cytokines, such as interleukin-2 (IL-2), IL-12, and tumor necrosis factor-α (TNF-α), thus activating the immune system. IFN-γ also increases reactive sensibility of macrophages for lipopolysaccharide and then stimulates macrophages to kill microorganisms by accelerating pro-inflammatory cytokines production [23]. It is well known that AS is a common chronic immune inflammatory disease and is associated with multiple pro-inflammatory cytokines [24]. Based on the above research, we speculated IFN-γ might be involved in the etiology of AS.

In humans, IFN-γ is encoded by IFN-γ gene (*IFNG*) located on chromosome 12q14, including 4 exons and 3 introns. Recently, multiple SNPs in *IFN*-γ have been identified. These SNPs are reported to influence disease occurrence via altering *IFN*-γ transcription and expression level. Rs1861493 is a mutation with the substitution of G/A in intron 3 of *IFN*-γ, which was reported to regulate the expression of IFN-γ [13]. Several articles have discussed to the role of rs1861493 in disease development. Abhimanyu et al. reported that rs1861493 influenced the individual susceptibility to pulmonary tuberculosis in North Indians [25] and Kumar et al. revealed that rs1861493 was obviously associated with the risk of asthma [26]. Rs2430561 (+874T>A), a common mutation identified in intron 1 of *IFN*-γ with A/T alleles substitution and +874 T allele, can enhance the expression of *IFN*-γ [24]. This SNP has been studied widely in various diseases, including immune diseases [27,28]. A

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meta-analysis by Lee et al. revealed that rs2430561 (+874T>A) was significantly correlated with autoimmune diseases [28]. Hirankarn et al. reported that rs2430561 AA genotype combined with *IL-18* (-137) GC genotype was found to increase the risk of arthritis in systemic lupus erythematosus (SLE) patients [29]. However, the role of *IFN*-γ polymorphisms in AS risk had been rarely reported.

AS is a common chronic inflammatory disease influenced by multiple factors. A number of genes have been shown to be involved in the pathogenesis of AS, including *HLA-B27*, multiple cytokines genes (*IL-6*, *IL-23*, *IL-12* and *TNF*-α), and endoplasmic reticulum aminopeptidase 1 (*ERAP1*) [30]. But these efforts fail to completely explain the etiology of AS, and more related factors need to be discovered. Previous studies have shown that IFN-γ has a minor effect on AS. For example, Wang et al. concluded that mRNA and protein expression level of IFN-γ was significantly higher in AS patients than in health controls [18], but the detailed explanation was lacking. In addition, AS severity is affected by region differences such as the specific genetic background and geographical environment. Healey reported the independent association between disease severity and region, concluding that AS patients living in areas with low socioeconomic status need better healthcare [31]. The distribution of *IFN*-γ polymorphisms also has regional divergence. Therefore, it was necessary to design and conduct the present study. However, due to its small sample size and single group, as well as the lack of analysis of environmental factors, the results obtained in our study might be limited. More well-designed studies were required to verify the function of *IFN*-γ polymorphisms in AS risk.

Conclusions

In conclusion, *INF*-γ rs2430561 polymorphism, but not rs1861493, significantly increases the risk of AS in the Chinese Han population. Serum IFN-γ level in AS patients is higher than that in healthy people, so serum IFN-γ level may be a biomarker for AS diagnosis. The expression profile of IFN-γ exhibits obvious association with rs2430561 polymorphism, suggesting that rs2430561 minor allele may regulate the expression of IFN-γ, thus contributing to AS risk.

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