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ORIGINAL ARTICLE

# Association studies of interleukin-8 gene in Graves' disease and Graves' ophthalmopathy

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**Abstract** Graves' disease (GD) is an autoimmune disorder, and the most common extrathyroidal manifestation is Graves' ophthalmopathy (GO), which is believed to be caused by a complex interaction between genetic and environmental factors. Many studies have reported that interleukin-8 (IL-8), a potent pro-inflammatory cytokine, is associated with many autoimmune diseases and could increase the degree of lymphocyte infiltration within the thyroid gland. The aim of the present study is to elucidate whether IL-8 is associated with the development of GD and GO. The serum concentration of IL-8 was tested in 39 primary GD patients, 43 treated active GO patients, and 24 healthy controls. We also performed an association study with the IL-8 gene polymorphism rs2227306 between 642 patients and 648 healthy controls in Chinese population. Our data showed that the expression level of IL-8 was associated with the development of GD, and the C-allele frequency of SNP rs2227306 was significantly higher in GD and GO patients compared with healthy controls. These results suggest that IL-8 is strongly associated with GD and GD.

**Keywords** Interleukin-8 (IL-8) · Graves' disease (GD) · Graves' ophthalmopathy (GO)

## Introduction

Graves' disease (GD) is an autoimmune disorder characterized with hyperthyroidism, resulting in increased metabolic rate. The most common extrathyroidal manifestation is Graves' ophthalmopathy (GO), which is considered to be a chronic, autoimmune inflammatory disorder that impacts all orbital tissue sections of the eye and results in various eye features. The TSH receptor has been proposed as an autoantigen in GD patients, however, the nature of autoimmune reactions in the thyroid and orbit, and the mechanisms linking GD and GO have not been fully elucidated [1, 2]. Although the pathogenesis of GD and GO remains unknown, the available evidences suggest that genetic, environmental, and immunologic factors possibly contribute to the development of GD and GO.

Interleukin-8 (IL-8) is a potent pro-inflammatory cytokine and primarily produced by monocytes and T lymphocytes; IL-1 and TNF can stimulate the secretion of IL-8 [3–5]. IL-8 is an important mediator in immune regulation and secreted during immune and host defense responses. It was originally described as a chemoattractant for neutrophils and lymphocytes, and characterized for its ability in recruitment and activation of neutrophils at inflammatory sites [6, 7]. Intradermal injection of IL-8 induces both neutrophil infiltration and T-cell migration to the site of injection, and the blocking of IL-8 significantly inhibits B-cell migration [8, 9]. Many studies have reported that IL-8 is

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associated with autoimmune diseases, such as multiple sclerosis, systemic sclerosis, and psoriasis [10–13]. In 1992, Weetman et al. first described the production of chemokine IL-8 by cultured thyroid follicular cells stimulated by IFN-$\gamma$, TNF-$\alpha$, or IL-1$\alpha$ [14]. In the initial stages of autoimmunity, local IL-1 activity enhances the production of IL-8, which in turn increases the degree of lymphocyte infiltration in the thyroid gland [15]. The IL-8, as an important cytokine, would take part in the pathogenesis of GD.

The *IL-8* gene, located at chr.4q13–q21, consists of four exons and a proximal promoter region and encodes a 99 amino acid polypeptide [16, 17]. To understand the function of IL-8 in the development of this disorder, the serum levels of IL-8 have been investigated in Chinese GD, GO patients, and control. We also performed an association study with the *IL-8* gene polymorphism rs2227306 between 642 patients and 648 healthy controls. Our results show that IL-8 is strongly associated with GD and GO.

## Subjects and methods

### Subjects

In total, 642 patients with GD (147 male and 495 female, aged 10–81 years, mean age 38.9 $\pm$ 13.6 years) being treated at Ruijin Hospital were enrolled in this study. GD was diagnosed on the basis of clinical manifestations, biochemical criteria of thyrotoxicosis (TSH $<$ 0.05 mIU/l and increased free T3 and/or free T4), and the presence of TSH receptor antibodies [18]. GO was diagnosed by experienced ophthalmologists and was classified using the "NOSPECS" classification to assess GO [19–23]. For the statistical analysis, patients with classes 2–6 were considered as having GO. There were 163 GD patients with overt-ophthalmopathy (NOSPECS class 2 or more; GO) and 479 GD patients without or with mild ophthalmopathy (NOSPECS class 0 and 1).

Six-hundred and forty-eight healthy Chinese volunteers (157 male and 491 female, aged 20–85 years, mean age 51.8 $\pm$ 14.8 years) from the same region of China without family history of GD or other autoimmune diseases served as the control group. Informed consent was obtained from the GD patients and healthy controls, and the study was approved by the Ethics Committee of Ruijin Hospital, Shanghai JiaoTong University, School of Medicine.

### Measurement of IL-8 serum concentration

The concentration of IL-8 polypeptide in the different sera was determined by a commercially available ELISA kit (R&D systems, Minneapolis, MN). The following sera were tested: 39 sera of primary GD patients, 43 sera of treated active GO patients and 24 sera of healthy controls.

### *IL-8* gene SNP selection

For the selection of single nucleotide polymorphisms (SNPs), Haploview software (http://www.broad.mit.edu/mpg/haploview) was applied to conduct linkage disequilibrium and haplotype block analyses by using Hapmap phase genotype data for the chromosomal region 4:74,822,139–74,831,295 (CHB database, Hapmap release 26). The amplicon of interest was a 9.2 kb region, with *IL-8* gene and approximately 3 kb upstream and 3 kb downstream of *IL-8* gene. The selection of tag-SNP was performed by running the tagger program implemented in Haploview [24]. The criteria for $r^2$ was set at $>$0.8. Only one tag-SNP (rs2227306) located at position 781 in intron 1 was chosen.

### Genotyping

Genomic DNA from peripheral blood was extracted using a commercially available kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. The tag-SNP rs2227306 was genotyped on the GenomeLab SNPstream 12-plex Genotyping System (Beckman & Coulter Fullerton, CA) following the manufacturer's instructions. This platform uses a single-base pair extension reaction to incorporate two-color fluorescence terminal nucleotides that are detected by a specialized imager [25, 26]. To confirm the results of genotyping by the SNPstream System, 100 random samples were selected to perform a direct sequencing analysis, and the results of both methods were identical.

### Statistical analysis

The genotype distributions in both groups (control and GD) were observed using Hardy–Weinberg equilibrium. Clinical data were expressed as mean $\pm$ SD. The difference of IL-8 serum concentration were calculated using Independent-sample *T*-Test. Genotype and allele frequencies for rs2227306 polymorphisms were compared for statistically significant differences between patient and control groups using the $\chi^2$-test. Statistical analyses were carried out using the SPSS statistical package version 11.0 (SPSS, Chicago, IL).

## Results

### The different serum levels of IL-8 between disease and normal control

The serum concentration of IL-8 in the primary GD patients was significantly higher than that of healthy controls ($P < 0.05$). The mean serum concentration of IL-8 in treated active GO patients was also higher than that of

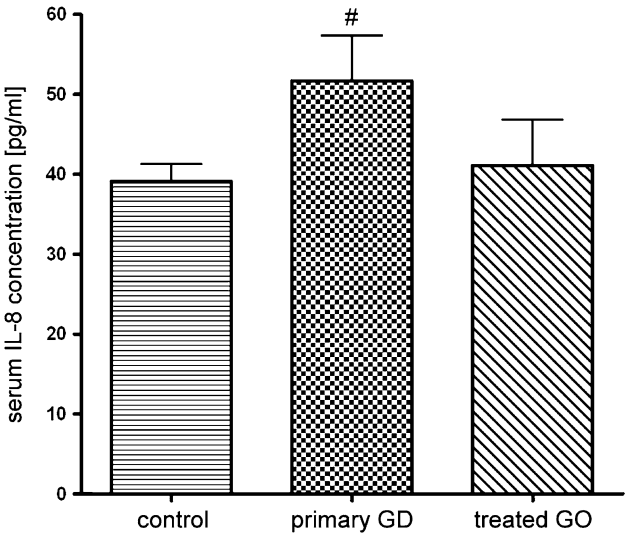


**Fig. 1** Serum levels of IL-8 in primary GD patients, treated active GO patients and normal controls. # stands for the significant difference between normal controls and the primary GD patients

healthy controls, 41.1 vs. 39.1 pg/ml, however, there was not statistically significant difference ($P > 0.05$). The results were shown in Fig. 1.

rs2227306 in *IL-8* gene association between Graves' disease and control

The observed genotype distributions in both groups (control and GD) were in accordance with Hardy–Weinberg equilibrium. The CC genotype frequencies of polymorphism rs2227306 were significantly higher in GD patients than in controls (45 vs. 38%; CC genotype vs. CT and TT genotypes, odds ratio, 1.347 [95% CI, 1.079–1.683] $\chi^2 = 6.92$, $P = 0.009$; Table 1). The C-allele frequencies

**Table 1** *IL-8* gene polymorphisms in patients with GD and healthy control subjects

| rs2227306 Polymorphism | Graves' disease ($n = 642$) | Control subjects ($n = 648$) | $\chi^2$-test *P*-value |
|---|---|---|---|
| Genotype frequencies | | | |
| CC | 287(45) | 243(38) | $\chi^2 = 6.92$ |
| CT | 285(44) | 325(50) | $P = 0.03$ |
| TT | 70(11) | 80(12) | |
| Dominant mode | | | |
| CC | 287(45) | 243(38) | $\chi^2 = 6.92$ |
| CT + TT | 255(55) | 405(62) | $P = 0.009$ |
| Allele frequencies | | | |
| C | 859(67) | 811(63) | $\chi^2 = 5.28$ |
| T | 425(33) | 485(37) | $P = 0.02$ |

Values in parentheses are percentages of the group

*P*-values were calculated with $\chi^2$-test, comparing patients with GD and healthy control subjects

**Table 2** *IL-8* gene polymorphisms in GO and healthy control

| rs2227306 Polymorphism | Graves' ophthalmopathy ($n = 164$) | Control subjects ($n = 648$) | $\chi^2$-test *P*-value |
|---|---|---|---|
| C781T Genotype frequencies | | | |
| CC | 76(46) | 243(38) | $\chi^2 = 10.59$ |
| CT | 81(50) | 325(50) | $P = 0.005$ |
| TT | 7(4) | 80(12) | |
| Dominant mode | | | |
| CC | 76(46) | 243(38) | $\chi^2 = 4.29$ |
| CT + TT | 88(54) | 405(62) | $P = 0.04$ |
| Allele frequencies | | | |
| C | 233(71) | 811(63) | $\chi^2 = 8.16$ |
| T | 95(29) | 485(37) | $P = 0.004$ |

Values in parentheses are percentages of the group

*P*-values were calculated with $\chi^2$-test, comparing patients with GO and healthy control subjects

at position 781 in the intron 1 of the *IL-8* gene in GD patients were significantly higher than controls (67 vs. 63%; odds ratio, 1.209, [95% CI, 1.028–1.421]; $\chi^2 = 5.28$, $P = 0.02$; Table 1).

rs2227306 in *IL-8* gene association between Graves' ophthalmopathy and control

There was a strong significant difference in genotype and allele frequencies of *IL-8* gene polymorphism between GO and controls (Table 2). The genotype frequencies at position 781 of *IL-8* gene between GO and controls reached a significant difference ($\chi^2 = 10.59$, $P = 0.005$; Table 2). The C-allele frequencies in GO were significantly higher than in healthy controls (71 vs. 63%; odds ratio, 1.467 [95% CI, 1.127–1.909]; $\chi^2 = 8.16$, $P = 0.004$; Table 2).

Association between *IL-8* gene polymorphisms and the age of GD onset

There was no significant difference in genotype or allele frequencies of *IL-8* gene polymorphisms between the subjects with an earlier onset of GD (<40 years) and the subjects with a later onset (≥40 years). However, the C-allele frequency of polymorphism rs2227306 in GD patients with the earlier onset age appeared greater than that in controls (68 vs. 66%; odds ratio, 1.095 [95% CI, 0.864–1.387]; $\chi^2 = 0.56$, $P = 0.45$).

## Discussion

IL-8, a member of the chemokine family, principally involves in the initiation and amplification of inflammatory

process. It plays an important role in diseases in which inflammation is a substantial pathophysiologic feature; it can induce neutrophils infiltration and lymphocytes migration. Many studies have shown that IL-8 participates in many diseases such as bronchial asthma, multiple sclerosis, systemic sclerosis, and psoriasis.

GD, with the thyroid as a major target for autoimmunity, is characterized by reactivity to self-thyroid antigens and by lymphocyte infiltration of the gland [27–30]. Although the etiology of GD remains unclear, it is believed to be caused by a complex interaction between genetic and environmental factors. Several studies have shown that elevated levels of some interleukins and inflammatory cytokines (such as IL-12, IL-6, IL-10, and TNF) are associated with GD and GO. Different cytokines participate in different roles in the pathogenesis of GD and GO, such as IL-10 can enhance B-cell proliferation, and TGF$\beta$ is another cytokine with important modulatory functions, including an inhibitory role in B-cell maturation. Other studies demonstrate that thyrocytes express IL-8 upon stimulation with the proinflammatory cytokines IFN-$\gamma$, tumor necrosis factor-$\alpha$ (TNF-$\alpha$), and IL-1$\alpha$ [14]. It has been reported that circulating serum levels of IL-8 are higher in GD patients than in nonautoimmune controls [31, 32]. The positive expression of IL-8 mRNA in most extraocular muscle samples from patients with GO is reported in another study, suggesting the role of IL-8 in mediating lymphocyte infiltration in GO [33].

In the present study, we found that the IL-8 serum level in GD patients was significantly higher than that of the healthy controls, which is in accordance with the results of the previous studies [31, 34]. This suggests that IL-8 plays a role in the pathogenesis of GD. However, there is no significant difference in the IL-8 serum level between treated active GO patients and the healthy controls that drug intervention on GO patients might account for this. We also investigated *IL-8* gene polymorphism for the risk of GD in Chinese GD patients. The case–control association study results show that the C-allele frequency of SNP rs2227306 is significantly higher in GD and GO patients comparing with healthy controls. It suggests that C781T polymorphism in *IL-8* gene is a risk factor for the development of GD and GO.

SNPs located in the 5′-flanking region and within the transcription factor binding sites of the gene have been identified to be involved for gene expression. We performed the sequence analysis of rs2227306 variant with the online TFSEARCH program: Searching Transcription Factor Binding Sites (http://www.cbrc.jp/research/db/TFSEARCH.html) [34]. Uploading the two different allele sequences of rs2227306, the results from TFSEARCH gave a recognized site, ACMGGAWRTT, c-Ets-1 protein binding site, with a high score of 86.2 (T allele of rs2227306 located at the last nucleotide site). The Ets-1 protein is a member of ETS transcription factor families that regulate numerous genes and are involved in stem cell development, cell senescence and death, and tumorigenesis. However, there is no report available about Ets-1 protein regulation on *IL-8* gene, which might help to determine any association of this transcription factor with *IL-8*, and future studies are required to clarify it.

In conclusion, the present study has shown that the high serum level of IL-8 is associated with the development of GD, and *IL-8* gene polymorphism rs2227306 is identified with susceptibility to GD and GO in Chinese patients. The role of cytokine IL-8 would affect the pathogenesis of GD and related ophthalmopathy through immune regulation and genetics regulation. However, further studies are required to examine the precise role of IL-8 in the pathogenesis of GD and to confirm *IL-8* gene polymorphism association in different ethnics.

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