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Association between Interleukin-6 Gene Polymorphisms and Rheumatoid Arthritis in Chinese Han Population: A Case-Control Study and A Meta-analysis

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The aim of this study was to investigate the possible association in the interleukin-6 (IL-6) gene with Rheumatoid arthritis (RA) in Chinese Han population from Shandong Province. Target regions of IL-6 gene were amplified by polymerase chain reaction (PCR) and genotyped. A logistic regression analysis was performed to detect potential associations in our case-control sample, the odd ratio(OR) and 95% confidence intervals(CIs) were calculated. Furthermore, we systematically tracked all the published studies in the field and performed a meta-analysis for the single nucleotide polymorphisms (SNPs) under study. 256 RA patients and 331 healthy controls were recruited into the case-control study. We found allele frequencies of rs1800795, rs1800797 and rs1474347 in RA patients differ from control subjects (P = 0.016, 0.024, 0.020, respectively). Significant difference was observed in haplotype frequencies of GCCGCT between RA patients and controls (P = 0.0001, OR = 4.066, 95%CI = 1.891 ~ 8.746), while GGCGCT frequencies was found lower in RA than controls (P=0.006, OR = 0.669, 95%CI = 0.501 ~ 0.894). The results of the meta-analysis showed association polymorphism within the IL-6 promoter with RA. These findings suggest that rare IL-6 gene polymorphisms may associate with RA susceptibility in Han Chinese populations; however further studies are needed to assess the validity of the association of IL-6 with RA.

Rheumatoid arthritis (RA) is a common chronic autoimmune inflammatory joint disease, characterized by erosive symmetrical polyarthritis and systemic extra-articular manifestations, affecting approximately 0.5% to 1% of the adult population worldwide[1]. Although the etiology and pathogenesis of RA is not clearly defined, a combination of risk alleles from numerous genes contributed to RA susceptibility has been found[2]. Among all these genes, HLA-DR loci was most thoroughly studied yet HLA-DR accounts only for approximately one-third of the genetic susceptibility to RA. Other loci also implicated in the predisposition to RA, such as CTLA4,PADI4,PTPN22,TNF-α,IL-1β and STAT4 et al account for relatively small additional contribution RA genetic susceptibility[2].

IL-6, one of the most well-studied pro-inflammatory cytokines, is a major mediator of the acute phase response and was increased in the synovial fluid and tissue of RA patients. Blockade of IL-6 activity with a soluble anti-IL-6 molecule tocilizumab has been shown to reduce disease activity and radiological progression in RA[3]. Data has shown IL-6 expression might be in part genetically modulated by polymorphisms located at positions rs1800795, rs1800796 and rs1800797 (also known as IL-6 -174G/C, -572G/C, -597G/A) in the promoter region of IL-6[4,5]. IL-6 promoter polymorphisms have been associated with susceptibility to RA, but with conflicting results in different populations[6].

In this study we sought to assess potential susceptibility of variants in promoter and intron area of IL-6 with RA. We designed a case-control study to evaluate these associations in a Han Chinese population. Furthermore, we performed a systematic review based on published data on various ancestral groups and performed a meta-analysis including our results.

Table 1 | Genotype distribution and allele frequencies of IL-6 gene polymorphisms in RA patients and control individuals

SNPs	Genotypes/ Alleles	RA (n = 256)	Controls (n = 331)	P value	Odds ratio	95%confidence interval
rs1800795	GG	247(96.5%)	329(99.4%)	0.049	3.235	1.005–10.412
	GC	7(2.7%)	1(0.3%)			
	CC	2(0.8%)	1(0.3%)			
	G	501(97.9%)	659(99.5%)	0.016	4.823	1.338–17.379
	C	11(2.1%)	3(0.5%)			
rs1800796	CC	108(42.2%)	138(41.7%)	0.476	1.095	0.854–1.404
	CG	125(48.8%)	152(45.9%)			
	GG	23(9.0%)	41(12.4%)			
	C	341(66.6%)	428(64.7%)	0.486	1.090	0.855–1.390
	G	171(33.4%)	234(35.3%)			
rs1800797	GG	249(97.3%)	330(99.7%)	0.076	3.419	0.879–13.305
	GA	5(1.9%)	0(0%)			
	AA	2(0.8%)	1(0.3%)			
	G	503(98.2%)	660(99.7%)	0.024	5.905	1.270–27.448
	A	9(1.8%)	2(0.3%)			
rs1524107	TT	99(38.7%)	143(43.2%)	0.376	1.116	0.875–1.422
	TC	125(48.8%)	148(44.7%)			
	CC	32(12.5%)	40(12.1%)			
	T	323(63.1%)	434(65.6%)	0.380	1.114	0.876–1.417
	C	189(36.9%)	228(34.4%)			
rs2069840	CC	199(77.7%)	278(84.0%)	0.057	1.435	0.990–2.081
	CG	52(20.3%)	49(14.8%)			
	GG	5(2.0%)	4(1.2%)			
	C	450(87.9%)	605(91.4%)	0.050	1.462	1.000–2.138
	G	62(12.1%)	57(8.6%)			
rs1474347	TT	243(94.9%)	326(98.5%)	0.019	3.488	1.227–9.915
	TG	13(5.1%)	5(1.5%)			
	GG	0(0%)	0(0%)			
	T	499(97.5%)	657(99.2%)	0.020	3.423	1.212–9.665
	G	13(2.5%)	5(0.8%)			

Results

The six SNPs of IL-6. Briefly, two hundred and fifty-six Han Chinese patients with RA [F/M: 196/60, mean age 50.26 ± 12.86 years, median disease duration 3(0.8 ~ 8.0) years] and 331 healthy controls (F/M: 239/92, mean age 48.08 ± 13.92 years) from the same geographic and ethnic background were included in this study(Supplementary Table 1). No difference in age and sex distributions were detectable between the two groups ($P > 0.05$).

Genotype and allelic frequencies of the SNPs rs1800795, rs1800796, rs1800797, rs1524107, rs2069840 and rs1474347 of IL-6 are shown in Table 1. There was a statistically significant association observed for rs1800795, rs1800797 and rs1474347. Specifically the ORs(95% CIs) were OR = 4.823 (95%CI = 1.338–17.379, P = 0.016), OR = 5.905 (95% CI = 1.270–27.448, P = 0.024) and OR = 3.423 (95% CI = 1.212–9.665, P = 0.020) for rs1800795, rs1800797 and rs1474347, respectively. Interestingly the minor allele frequencies (MAF) of these variants in the control population of our study were different from the reported European groups. In addition, no relationship was observed when we analyzed IL-6 genotypes distributions according to age, gender, mean disease duration, ESR, CRP, DAS28 and serum level of RF, aCCP, or aGPI (Supplementary Table 2, Supplementary Table 3).

Meta-analysis of IL-6 SNPs. There are four major haplotype in IL-6 polymorphism: GCTGCT, GGCGCT, GGCGGT, GCCGCT. Significant difference was observed in haplotype frequencies of GCCGCT between RA patients and controls (P = 0.0001, OR = 4.066, 95%CI = 1.891 ~ 8.746). Haplotype GGCGCT frequencies was found lower in RA than controls (P=0.006, OR = 0.669, 95%CI = 0.501 ~ 0.894). (Table 3).

Our systematic search identified greater than 4 eligible articles studying the potential association between a variant and rheumatoid arthritis for rs1800795 and rs1800796 only. Fourteen studies (13 for rs1800795 and 6 for 1800796) including our case-control study accumulating a total of 2791 RA patients and 3100 control subjects were available in the literature. Information on the primary data is shown in Table 2. Specifically the MAFs for rs1800795, rs1800797 and rs1474347 in our population were 0.5%, 0.3%, 0.8% compared to 46.5%, 47.3% and 46.0% in Caucasians. Given the difference in the MAF between Han Chinese and Europeans a sensitivity analysis by ethnic group was performed.

In a sample-size of 584/703 case/controls, Asians meta-analysis revealed an association between RA and the rs1800795 C allele ($OR = 9.75$, 95% $CI = 4.99–19.06$, $P < 0.00001$) with no observed heterogeneity ($I^2 = 0$) (Figure 1). The p-value was 0.8028 from egger's test with asymmetry in funnel plot as alternative hypothesis.

However no difference was observed in Europeans ($OR = 1.00$, 95% $CI = 0.84–1.19$, $P = 1.00$) with high heterogeneity ($I^2 = 68\%$) (Supplementary Figure 1). Furthermore, no association with RA was found for rs1800796 C allele ($OR = 1.08$, 95% $CI = 0.54–2.16$, $P = 0.82$) in Asian populations with notable high heterogeneity ($I^2 = 92\%$) (Supplementary Figure 2) and no studies were available in European populations.

Discussion

Our study and the conducted meta-analysis supports evidence that a rare polymorphism of IL-6 may play a major role in the susceptibility to RA, at least in the Chinese population. The association was nominally statistically significant in our study with a strong association found when all studies were synthesized and with minimal observed heterogeneity.

The summary effect for this variant is null in European populations but we have shown that a different LD pattern may be observed between these two ethnic groups. A previous meta-analysis of IL-6

Table 2 | Characteristics of the individual studies included in the systematic review and meta-analysis

Year	Author	Country	Population	RA	Controls	Method	SNPs	Results
2000	Pascual M[13]	Spain	European	163	157	PCR-RFLP	rs1800795G/C	NS
2002	Dahlqvist SR[14]	Sweden	European	257	183	PCR-RFLP	rs1800795G/C	NS
2003	Martinez A[15]	Spain	European	157	163	PCR-RFLP	rs1800795G/C	NS
2005	Pawlik A[5]	Poland	European	98	105	PCR-RFLP	rs1800795G/C	NS
2007	Huang XZ[6]	China	Asian	120	168	PCR-SSP	rs1800795G/C	SA
							rs1800796G/C	SA
2008	Lo SF[16]	China	Asian	199	130	PCR-RFLP	rs1800796G/C	NS
2009	Panoulas VF[17]	UK	European	383	422	real-time PCR	rs1800795G/C	NS
2009	Palomino-Morales R[18]	Spain	European	311	226	TaqMan	rs1800795G/C	NS
2009	Trajkov D[19]	Macedonia	European	85	301	PCR-SSP	rs1800795G/C	NS
2009	Li YH[12]	China	Asian	60	84	PCR-SSP	rs1800795G/C	SA
							rs1800796G/C	SA
2009	Lu XL[11]	China	Asian	148	120	PCR-SSP	rs1800795G/C	SA
							rs1800796G/C	SA
2011	Emonts M[20]	Netherlands	European	376	463	SBE	rs1800795G/C	NS
2012	Arman A[4]	Turkey	Turkish	178	247	PCR-RFLP	rs1800795G/C	NS
							rs1800796G/C	NS
							rs1800797G/A	NS
2012	This study	China	Asian	256	331	PCR-HRM	rs1800795G/C	SA
							rs1800796G/C	NS
							rs1800797G/A	SA

NS: no significant association; SA: significant association, SBE: single base extension; RFLP: restriction fragment length polymorphism; SSP: sequence specific primers.

that attempted to summarize the effect sizes of all ethnicities[7] should be interpreted with caution given this diverse LD pattern. Indeed, it has been shown that differential ancestral effects can be anticipated and genomic risk markers may need separate further evaluation in different ancestry groups.

Polymorphism rs1800795 in IL-6 consists of a G to C substitution, which has been reported to be associated with systemic onset of juvenile idiopathic arthritis (SoJIA) and to affect levels of IL-6[8]. Serum levels of IL-6 were found to be significantly higher with genotypes containing the G allele in healthy subjects, suggesting a genetically determined difference in the degree of the IL-6[9]. In multivariate tests, the IL-6 rs1800795 G/G genotype was positively correlated with pain of RA and this may be due to higher IL-6 responses[10]. These data have shown that IL-6 rs1800795 may play functional and role in pathogenesis of RA. Although we did not find the associations of IL-6 rs1800795 with clinical parameters, further study enroll large samples combined with serum determination of cytokine network will elucidate the genetic mystery of IL-6 in RA.

We identified four major haplotype in IL-6 polymorphism: GCTGCT、GGCGCT、GGCGGT、GCCGCT. Significant difference was observed in haplotype frequencies of GCCGCT between RA patients and controls (P = 0.0001, OR = 4.066, 95%CI = 1.891 ~ 8.746). Haplotype GGCGCT frequencies was found lower in RA than controls (P=0.006, OR = 0.669, 95%CI = 0.501 ~ 0.894). The result of haplotype suggests rs1800796 has potential role in the pathogenesis of RA when in LD with other SNPs.

A limitation of our study is the weak level of evidence for meta-analysis according to Venice criteria. Even though lack of heterogeneity supported by the p-value (0.8028) from egger's test suggests absence of critical biases, the accumulated sample size remains small for the Asian populations. Although our study was quite large compared to previously conducted studies, the number of minor alleles present in cases in this meta-analysis is rather small as expected from the observed MAF.

We did not find any association between rs1800796 in the promoter of IL-6 and RA, which was consistent with a study in a Turkish population[4]. However, this meta-analysis was rather heterogeneous with primary studies showing significant results in both directions[6,11,12].

In summary, the data from the present study indicate that a rare variant in IL-6 may play a significant role as a genetic susceptibility factor for RA in the Chinese population. Further studies with larger sample sizes are required to delineate the differences in genetic susceptibility to RA in various ancestral groups. Sequencing and functional characterization of IL-6 may reveal rare variants which may influence RA susceptibility in Chinese populations.

Methods

Patients and controls. Two hundred and fifty-six Han Chinese patients from Shandong province with RA diagnosed according to the 2008 Classification Criteria of the American College of Rheumatology and 331 healthy controls from the same geographic and ethnic background were included in this study. RA patients were recruited from the department of Rheumatology; Provincial Hospital affiliated to Shandong University, China. Controls had no history of infectious or chronic inflammatory autoimmune diseases and were unrelated to the patients. The study was approved by the Local Ethical Committee of the hospital and university, and informed consent was obtained from all subjects. The methods were carried out in accordance with the approved guidelines. Laboratory parameters were recorded including Rheumatoid factor (RF), anti-cyclic citrullinated peptide antibody (aCCP), anti-Glucose phosphate isomerase (aGPI), erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP). RF was measured using N Latex RF Kit (SIEMENS, Germany). The level of aCCP and aGPI were determined by enzyme linked immunosorbent assay (ELISA) (AESKULISA, Germany). ESR was determined by the

Table 3 | Haplotype of IL-6 polymorphisms

Haplotype*	RA	Controls	χ^2	*P*	*OR*	*95%CI*
GCTGCT	303(59.2)	407(61.5)	0.019	0.890	0.983	0.766 ~ 1.261
GGCGCT	91(17.7)	165(24.9)	7.423	0.006	0.669	0.501 ~ 0.894
GGCGGT	52(10.1)	49(7.3)	3.459	0.063	1.474	0.977 ~ 2.224
GCCGCT	26(5.1)	9(1.4)	14.942	0.0001	4.066	1.891 ~ 8.746

*Haplotype: rs1800795, rs1800796, rs1524107, rs1800797, rs2069840, rs1474347.

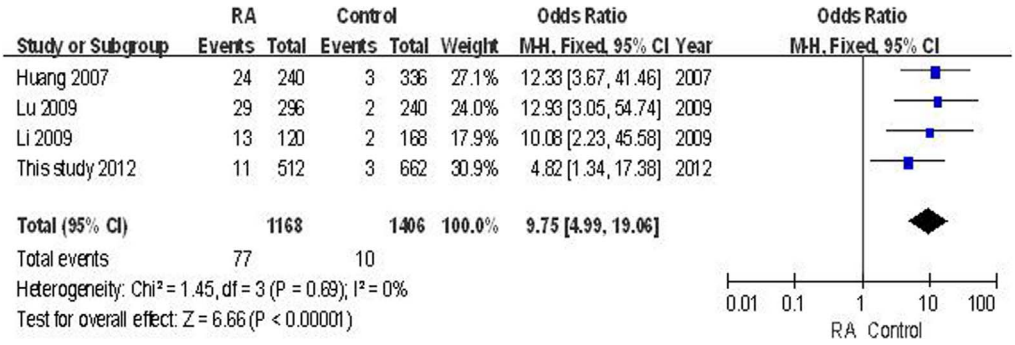

Figure 1 | Forest plot of genetic association studies of rs1800795-C and RA in Asian.

Westergren method via a Monitor-100 machine (BALZELLA, Italy) and CRP by immune turbidimetric analysis (SIEMENS, Germany).

SNPs selection. The three promoter region polymorphisms rs1800795, rs1800796 and rs1800797 in IL-6 had been previously reported as associated with RA in Asian population with contradictory results across studies. We expanded the investigation in this genetic area by also searching for additional SNPs identified in IL-6 in the database of dbSNP (http://www.ncbi.nlm.nih. gov/SNP/). We selected 3 additional SNPs with minor allele frequency (MAF) > 1% in Chinese Han population. Along with the previously 3 reported SNPs, in total, we included six SNPs in our study; rs1800795 (G/C), rs1800796 (G/C), rs1800797 (G/A), rs1524107 (T/C), rs2069840 (C/G) and rs1474347 (T/G).

Genotyping. Genomic DNA was isolated by RelaxGene Blood DNA System(TIANGEN,China) and the target polymorphisms of IL-6 were amplified by polymerase chain reaction(PCR) using TaKaRa Taq Hot Start Version (TaKaRa, Japan) and analyzed by HRM methods using LightCycler480(ROCHE, Germany). We confirmed our genotyping results by direct sequencing using an ABI3730 sequencer (ABI, America).

Statistical analysis. The distribution of genotypes and allele frequencies of RA patients for the 6 IL-6 SNPs was compared with control groups using the Pearson's chi-square test or Fisher's exact test as appropriate. All p values were two sided and using Bonferroni correction for the 6 comparisons significance can be deemed at the $p = 0.008$ level. Hardy-Weinberg equilibrium was examined by chi-square goodness-of-fit test using gene frequencies of the healthy individuals. We assessed the LD of the SNPs in our sample by using SHEsis (http://analysis.bio-x.cn). We assessed the association between the IL-6 variants and RA using a per-allele model and running a logistic regression. The distribution of age, disease duration, ESR, CRP, DAS28 and autoantibodies with genotypes of each polymorphism with RA was tested using the one-way ANOVA test or Kruskal-Wallis test. The association of gender and autoantibody status was measured by the Pearson's chi-square test. P values < 0.05 were deemed significant. LD was investigated using Haploview.

Meta-analysis. To compute the association between the IL-6 SNPs studied with RA, we scrutinized PubMed, Embase, HuGNet and China National Knowledge Infrastructure (CNKI) using the keywords 'rheumatoid arthritis', 'RA', and 'IL-6'. RA was defined according to the 1987 or 2010 ACR (American College of Rheumatology) criteria. The data were last accessed on Dec 30, 2012 and we did not set any language restrictions. The eligibility criteria included case- control or cohort studies that quantitatively assessed the relationship of an IL-6 gene polymorphism and risk of RA. Cases with RA were eligible regardless of age, gender and ethnicity. Studies without data on allelic counts in cases and controls and studies focusing on survival or other clinical outcome in RA were excluded from the study. In the case of overlapping cases/controls in different studies, we retained only the studies with the largest sample size.

For each study, we recorded the first author, year of publication, the ethnic group of the study population, the method of genotyping, the number of genotyped cases and controls, the source of control population, the standard errors (SE) or the 95% confidence interval (CI), and the odds ratio (OR). We extracted the calculated ORs in each study so as to reflect the same allelic contrast. We performed meta-analysis when 4 or more studies were available for each SNP. All eligible articles were independently screened by two researchers and consensus reached.

The heterogeneity of the studies was assessed using the Cochran's Q test (considered significant for $P < 0.10$) and was quantified by the I^2 statistic. I^2 ranges between 0 to 100% and were considered low, moderate, large and very large for values 0%–25%, 25%–50%, 50%–75% and >75% respectively. Both fixed effects (Mantel-Haenszel) and random effects (DerSimonian and Laird) models were used to combine the data. Random effects are more conservative incorporating an estimate of the between-study variance and thus providing relatively wider 95% confidence intervals when heterogeneity exists. Egger's test was conducted with R(www.r-project.org) to evaluate the publication bias. The credibility of the associations was evaluated using the Venice criteria. Briefly this includes an assessment of the amount of evidence, replication and protection from bias that is simplified into three levels of evidence: weak, moderate and strong. The analyses were conducted with Revman5.1 package. All p values were two sided.

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Author contributions

Study concept and design: Z.P., F.L. and E.E.; Acquisition of data: Z.P. and F.L. Analysis and interpretation of the data: Z.P., F.L., J.X., J.S., J.Z., Y.Z. and H.S. Write the paper: Z.P., F.L., E.E. and K.Z.

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