

# IL-6 gene rs12700386 Polymorphism is Associated with Increased Risk of Knee Osteoarthritis Development in Chinese Han Population: A Case-Control Study

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## Research article

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## Abstract

**Background:** There is an association between Interleukin-6 (IL-6) polymorphism and knee osteoarthritis (OA) risk. The case-control study aims at exploring how IL-6 rs12700386 polymorphism affects the knee OA risk in Chinese Han individuals.

**Methods:** We extracted the DNA from 763 participants, thereinto, 352 were OA patients and 411 were healthy controls. The polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) assisted in genotyping the IL-6 gene polymorphism. The relative expression exhibited by IL-6 in blood samples of knee OA patients was determined via a quantitative reverse transcription PCR (qRT-PCR).

**Results:** We found that IL-6 rs12700386 enhanced the knee OA susceptibility. Based on a subgroup analysis, the loci magnified the knee OA risk in smokers, drinkers, and subjects  $\geq 55$  years old or with BMI  $\geq 25$  kg/m<sup>2</sup>. The combination of smoking and drinking and rs12700386 genotype led to an increase in the knee OA risk, indicating an underlying interaction between gene and environment. Additionally, the rs12700386 was found to be related to increased IL-6 gene levels.

**Conclusion:** These data indicate that rs12700386 polymorphism of IL-6 gene led to an increase in the knee OA risk specific to Chinese Han individuals.

## Background

Osteoarthritis (OA) is related to the damage synovial joint structure and function clinically and pathologically [1]. Worldwide it is a representative joint disease and it is estimated to affect 10% of men and 18% of women > 60 years old [2]. Clinically, OA most frequently affects the knee joint [3]. The occurrence of OA is influenced by obesity, smoking, joint damage, heredity and inflammation [4]. It is a multifactorial disease with an important genetic component [5]. In the past decade, genome-wide association studies have discovered many new genetic risk sites OA [5].

IL-6 refers to a four-helical cytokine containing 184 amino acids. A lot of cell types can secrete IL-6 after proper stimulation in the process of infection, cancer or inflammation [6]. It can effectively regulate B and T cells responses and coordinate the activities of innate as well as adaptive immune systems [7]. IL-6 is an important regulator of bone homeostasis, it can trigger osteoclast differentiation and bone resorption [8]. Goldring MB et al. found that IL-6 production underwent an up-regulation by IL-1 $\beta$  together with matrix metalloproteinase, and the up-regulated IL-6 weakened collagen-2 production, and meanwhile caused joint damage [9]. Additionally, Sakao K et.al found the positive correlation between increased IL-6 and mRNA expression and OA radiographic progression [10]. Thus, we assumed that IL-6 may be a pivotal candidate gene for OA susceptibility.

Polymorphisms of IL-6 gene, located at 7p21-24 chromosomal position, are associated with risks for OA. Nevertheless, the impact of IL-6 rs12700386 polymorphism on OA risk has never been explored in Chinese Han population. Therefore, a case-control study was conducted in the paper which contained 352 OA patients together with 411 healthy controls, aiming at investigating if IL-6 gene rs12700386 is related to knee OA risk and IL-6 gene levels in Chinese subjects.

## Methods

### Subjects

352 OA patients together with 411 healthy controls were recruited from the Affiliated Changzhou No.2 People's Hospital of Nanjing Medical University and the Second Affiliated Hospital of Jiaxing University, who were ethnic Han Chinese with no genetic relation. The Kellgren Lawrence scoring (KL scoring) system was used to diagnose each patient radiologically. The functional index of Lequesne was used to assess each patient's functional or symptomatic status. Visual Analog Scale (VAS) was employed for evaluating the pain. There were three inclusion criteria. 1) Symptoms and/or signs related to knee OA; 2) Radio-graphic abnormality (with K-L grade  $\geq 2$ ); and 3) No any other arthritis form. Demographic characteristics [gender, age, smoking, alcohol and body mass index (BMI)] and clinical characteristics of disease severity [Lequesne function index, visual analog scale (VAS), ESR and CRP] were obtained from the medical records. We selected healthy controls from patients in orthopedics clinics and general surgery in one hospital while collecting samples. Exclusion criteria: history of knee or hip replacement, application of corticosteroids or bi-immunosuppressors, Parkinson's disease, or sequelae of stroke. Table 1 lists the characteristics of all the subjects. Study had obtained the informed consent from all the subjects, and obtained the approval of the Ethics Committee of the hospital. The performance of experiment followed the tenets of the Helsinki Declaration.

### Blood Sampling and Genotyping

A TIANamp blood DNA kit (Tiangen Biotech, Beijing, China) assisted in extracting blood from all patients and extracting genomic DNA from surrounding blood leukocytes as recommended by the manufacturer. The extracted samples were stored at -20°C until further use. The IL-6 rs12700386 polymorphism was genotyped with a polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) with sequence-specific primers: 5'-GCGACAGGCTCTCCAG TCT-3' (forward) and 5'-GCAGTCACACCGGCTAGGTC' (reverse). We selected about 5% of samples in a random manner to receive repeated assays, finding that the accordance rate reached 100%.

### Quantitative real-time polymerase chain reaction (qRT-PCR)

A Trizol reagent (Invitrogen, Carlsbad, USA) was employed for isolating the total RNA from the peripheral venous blood. The oligo primer and SuperscriptII (Invitrogen) were employed to reverse-transcribe the total RNA (aliquot) from each sample into a DNA with complementary single strand. The relative gene expression of IL-6 was quantified using the Taqman method, with beta-actin as an internal reference. The forward and reverse primers for PCR include: 5'-GAG CTTTCAGGCAGGCAGTATC-3' (forward) and 5'-GTATAG ATT CTT TCCTTTGAG GC-3' (reverse); (IL-6); 5'-ACCACCATG GAGAAGGCT GG-3' (forward) and 5'-CTC AGTGTAGCCCAGGAT GC-3'(reverse)' ( $\beta$ -actin). The relative expressions were analyzed using the  $2^{-\Delta\Delta CT}$  method.

### Statistical analyses

SPSS 22.0 (SPSS Inc., USA) was applied to all the statistical analyses. The statistical graphs were applied by virtue of GraphPad Prism 5 (GraphPad Software, La Jolla, CA). A student's t-test or  $\chi^2$  test assisted in assessing the difference between patients and controls in the mean and frequency distribution regarding the clinical and demographic characteristics. The observed number of genotypes was compared with a Chi-square ( $\chi^2$ ) analysis, with that expected in accordance with Hardy–Weinberg equilibrium (HWE). An independent sample t-test or a one-way ANOVA assisted in testing the continuous variables existing in the normal distribution, with results denoted by mean  $\pm$  standard deviation. We analyzed the allele and genotype distributions between patients and the controls. Stratified analyses according to alcohol, smoking, sex, and age were conducted. The odds ratio (OR) and 95% confidence interval (CI) were calculated in a logistic regression to assess the correlation of IL-6 gene polymorphism with OA risk. A cross-over analysis was used to assess the effects of the interactions between environmental factors (smoking or drinking) and genetic factors for the OA risk. A  $P < 0.05$  implied a significant level.

## Results

### Characteristics of the study population

**Table 1** lists the information of all individuals in detail. The patients and controls were averagely aged between 61.39 to 61.03 years old, respectively. The mean BMI was 24.61 and 24.58 kg/m<sup>2</sup> for the patients and controls respectively. No significant between-group differences were identified for age, BMI, or sex. In addition, the distributions of smokers and drinkers in the cases were basically the same as the control group. **Table 1** lists the relevant indexes, including affected leg, CRP, ESR, VAS, Lequensene's index, as well as K-L grade.

### Relation of IL-6 rs12700386 polymorphism to OA risk

The allele and genotype distribution of IL-6 rs12700386 polymorphism is shown in Table 2. The HWE test found no obvious deviation in genotypic frequency among the controls, indicating that the subjects are representative of the local population. The IL-6 rs12700386 led to an obvious increase in the OA risk as seen in dominant, homozygous and allelic models. A logistic regression analyses showed that CC genotype of rs12700386 significantly intensified the OA risk (CC vs. GG: OR and 95%CI, 2.01(1.04,3.89),  $P=0.045$ ). (**Table 2**). An allele genetic analysis revealed that C allele was linked to a higher risk of OA (C vs. G: 1.38(1.08,1.75),  $P=0.010$ ) (**Table 2**). This significant association was also observed in the dominant models. Stratified analyses of age, smoking, drinking status, and BMI revealed a significantly higher OA risk in drinkers, smokers, as well as those  $\geq 55$  years old or with BMI  $\geq 25$  kg/m<sup>2</sup>. (**Table 3**). Nevertheless, the rs12700386 polymorphism did not affect the OA risk with regard to abovementioned indexes (**Table 5**).

Based on the RT-PCR analysis, the CC genotype exhibited an obviously higher IL-6 expression relative to the GG genotype ( $P < 0.001$ ) (Figure 1).

### Cross-over analysis

The OR and 95% CI specific to two combining exposure models (IL-6 gene variants and smoking or drinking) were calculated (**Table 4**). For rs12700386 polymorphism, a GG genotype more obviously affected the OA risk compared with the CC genotype. Besides, smoking had no impact on OA risk. Notwithstanding, smokers carrying the CC genotype were more easily to suffer from OA compared with non-smokers carrying the GG genotype (OR, 2.86, 95%CI, 1.25–6.56;  $P = 0.015$ ). This indicated that CC genotype could strongly interact with smoking.

Similarly, IL-6 rs12700386 polymorphism or drinking could not affect the OA risk. Obviously, CC genotype of rs12700386 polymorphism and drinking exerted a combined effect on OA, demonstrating that CC genotype could strongly interact with drinking.

## Discussion

In this study, we found that IL-6 rs12700386 polymorphism led to an increased OA risk, especially among drinkers, smokers, and those  $\geq 55$  years old or with BMI  $\geq 25$  kg/m<sup>2</sup>. The interactions between IL-6 rs12700386 polymorphism and smoking and drinking contributed to an increased risk for knee OA. In addition, genotypes of rs12700386 polymorphism were associated with increased IL-6 gene levels. Nevertheless, the impact of rs12700386 on OA patients regarding the clinical parameters remains unknown.

OA is characterized by destruction of cartilage, remodeling of subchondral bone and synovial membrane inflammation, which actively affects the disease progression [11]. As demonstrated by studies, tibiofemoral cartilage injury progression can be clearly affected by reactive or inflammatory synovium [12]. Proinflammatory cytokines like IL-6, TNF, can dramatically mediate metabolic disorders as well as enhance the catabolism regarding OA joint tissue [11]. The synovial fluid and serum of OA patients also presented significantly increased IL-6 and sIL-6R levels [13]. On the basis of a clinical trial on OA patients, if IL-6 and CRP held a high baseline level, the cartilage loss risk would increase [14]. Increase in circulating IL-6 level and high BMI could lead to radiographic knee OA risk [15]. Furthermore, IL-6 deficiency in mice has been found to lead to reduced knee arthritis cell number as well as collagen-induced arthritis response [16]. However, how IL-6 exactly affect the OA is of considerable debate.

IL-6 gene polymorphisms may change the function and expression of gene and regulate the susceptibility to osteoporosis. Thus, we performed the case-control study for exploring the impact exerted by IL-6 gene polymorphism on the knee OA risk. Recently, as found by Monica Singh et al, despite the irrelevance between IL-6 rs12700386 and OA risk, IL-6 plasma level was significant related to the genotypes of rs12700386 [17]. However, our finding showed that rs12700386 increased the susceptibility of OA. This inconsistency may be attributed to 4 reasons. First, people living in different environments have different eating habits and living styles. Second, our data indicated the interplay between IL-6 gene rs12700386 polymorphism and some exposure factors, which are evidently diverse. Third, different genotyping approaches as well as inclusion criteria possibly come into different results. Fourth, clinical heterogeneity exhibited by OA is possibly resulted from the conflicting results due to the variation of OA malignancy degree between studies.

Furthermore, based on stratified analyses, knee OA risk increased in smokers, drinkers, and subjects  $\geq 55$  years old or with BMI  $\geq 25$  kg/m<sup>2</sup>. Thus, the cross-over analysis was used to estimate the combined effects of IL-6 gene polymorphism and smoking and drinking on knee OA risk. As expected, the combined effects of rs12700386 polymorphism and smoking and drinking conferred susceptibility to OA. In addition, we evaluated the associations between IL-6 gene polymorphism and clinical characteristic of OA. However, no evidence was found to prove that rs12700386 is associated with the clinical parameters of OA patients. To explore the underlying mechanisms, the qRT-PCR of IL-6 gene showed the rs12700386 contributed to increase IL-6 gene levels. Thus, our study indicated that the IL-6 gene polymorphism could affect the IL-6 gene level and the knee OA risk.

There are also some shortcomings in the study. 1) it had a moderate sample size, incapable of accurately exploring how IL-6 gene rs12700386 polymorphism affected the knee OA susceptibility. 2) there may have been a selection bias related to the ethnic groups as the participants were limited to Chinese population. 3) only 1 polymorphism of the IL-6 gene was examined; it could not completely cover the gene. 4) relevant experiments were not carried out for figuring out underlying mechanisms.

## Conclusions

IL-6 rs12700386 polymorphism is associated with an increased IL-6 gene level and increased knee OA risk in Chinese Han population. The finding shall be verified in further studies which cover a bigger sample size.

## Abbreviations

IL-6: Interleukin-6; OA: osteoarthritis; SNP: Single nucleotide polymorphism; PCR-RFLP: polymerase chain reaction restriction fragment length polymorphism;

## Declarations

### Ethics approval and consent to participate

The study has obtained the approval of the Ethics Committee of the Second Affiliated Hospital of Jiaxing University and the Affiliated Changzhou No.2 People's Hospital of Nanjing Medical University, as well as obtained all participants' written informed consent.

## Consent for publication

The paper has obtained all participants' written informed consent for publishing their personal and clinical information.

## Availability of data and material

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

## Competing interests

The authors declare that conflicts of interests did not exist in the paper.

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The research in the paper did not receive external funding.

## Authors' contributions

DMX and GC took charge of conceiving as well as designing experiments. HY and XDZ carried out experiments. HY was responsible for data analysis. The paper was written by HY and revised by DMX. The paper had been reviewed by all authors, and gained their agreement with regard to paper content.

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## Tables

**Table 1** The general characteristics of study participants

Variable	Cases (n=352)	Controls (n=411)	<i>P</i>
Age (years)	61.39±10.42	61.03±9.89	0.629
Sex			0.884
Male	166(47.2%)	191(46.5%)	
Female	186(52.8%)	220(53.5%)	
Smoking			0.695
Yes	111(31.5%)	124(30.2%)	
No	241(68.5%)	287(69.8%)	
Alcohol			0.762
Yes	124(35.2%)	150(36.5%)	
NO	228(64.8%)	261(63.5%)	
BMI (kg/m <sup>2</sup> )	24.61±1.31	24.58±1.49	0.814
Affected leg			
Left	215(61.1%)		
Right	137(38.9%)		
ESR (mm/h)	18.47±9.64	-	
CRP (mg/L)	19.77±14.42	-	
VAS	5.66±1.59	-	
Lequesnes' index	14.50±2.06		
kellgren-Lawrence grade			
II	152(43.2%)		
III	143(40.6%)		
IV	57(16.2%)		

BMI, body mass index; ESR, erythrocyte sedimentation rate; CRP, C-Reactive protein; VAS, visual Analogue Scale;

**Table 2** The association of genotype and allele of IL-6 rs12700386 polymorphism with osteoarthritis risk

Genotype	Genotypes and alleles	Frequencies N(%)		OR (95% CI)	P
		Cases (n=351)	Controls (n=409)		
rs12700386					
Co-dominant	GG	193 (55.0%)	259 (63.3%)	1.00(reference)	
Heterozygote	GC	134 (38.2%)	134 (32.8%)	1.34(0.99,1.82)	0.063
Homozygote	CC	24 (6.8%)	16 (3.9%)	<b>2.01(1.04-3.89)</b>	<b>0.045</b>
Dominant	GG	193 (55.0%)	259 (63.3%)	1.00(reference)	
	GC+CC	158 (45.0%)	150 (36.7%)	<b>1.41(1.06-1.89)</b>	<b>0.022</b>
Recessive	GC+GG	327 (93.2%)	393 (96.1%)	1.00(reference)	
	CC	24 (6.8%)	16 (3.9%)	1.80(0.94-3.45)	0.075
Allele	G	520(74.1%)	652(79.7%)	1.00(reference)	
	C	182(25.9%)	166 (20.3%)	<b>1.38(1.08-1.75)</b>	<b>0.010</b>

The genotyping was successful in 351 cases and 409 controls for rs12700386;

Bold values are statistically significant ( $P < 0.05$ ).

**Table 3** Stratified analyses between rs12700386 polymorphism and the risk of osteoarthritis.

Variable	(case/control)			GC vs. GG	CC vs. GG	CC vs. GC+GG	CC+GC vs. GG
	GC	CC					
Sex							
Male	100/131	54/50	11/8	1.42(0.89-2.25); 0.156	1.80(0.70-4.64); 0.238	1.62(0.63-4.12); 0.351	1.47(0.95-2.28); 0.094
Female	93/128	80/84	13/8	1.31(0.87-1.97); 0.214	2.24(0.89-5.61); 0.107	1.99(0.81-4.91); 0.176	1.39(0.94-2.06); 0.110
Smoking							
Yes	78/103	13/12	19/9	1.29(0.57-2.94); 0.674	<b>2.51(1.09-5.78); 0.029</b>	<b>2.67(1.15-6.18); 0.025</b>	<b>2.01(1.08-3.76); 0.029</b>
No	115/156	121/122	5/7	1.35(0.95-1.91); 0.111	0.97(0.30-3.13); 0.602	0.84(0.26-2.69); 0.504	1.33(0.94-1.87); 0.116
Alcohol							
Yes	71/96	39/46	13/6	1.15(0.68-1.94); 0.687	<b>2.93(1.06-8.08); 0.049</b>	2.80(1.03-7.59); 0.054	1.35(0.83-2.21); 0.259
No	122/163	95/88	11/10	1.44(0.99-2.09); 0.058	1.47(0.61-3.57); 0.495	1.25(0.89-1.76); 0.222	1.45(1.00-2.07); 0.053
Age (years)							
<55	30/41	48/60	8/5	1.09(0.60-2.00); 0.878	2.19(0.65-7.35); 0.236	2.07(0.65-6.58); 0.254	1.18(0.65-2.13); 0.653
≥55	163/218	86/74	16/11	<b>1.55(1.07-2.25); 0.023</b>	1.95(0.88-4.30); 0.110	1.71(0.78-3.74); 0.235	<b>1.61(1.13-2.28); 0.009</b>
BMI((kg/m <sup>2</sup> ))							
<25	96/111	62/74	9/7	1.38(0.89-2.13); 0.153	1.49(0.53-4.14); 0.605	1.51(0.55-4.13); 0.453	1.01(0.67-1.54); 0.518
≥25	97/148	72/60	15/9	<b>1.83(1.19-2.81); 0.007</b>	<b>2.54(1.07-6.04); 0.049</b>	2.05(0.88-4.80); 0.138	<b>1.92(1.28-2.89); 0.002</b>

Bold values are statistically significant ( $P < 0.05$ ).

**Table 4** Genetic (G) and environmental (E) factors 2\*4 fork analysis.

G <sup>a</sup>	E <sup>b</sup>	Case	Control	OR (95%CI); P value	Reflecting information
<b>rs12700386</b>					
CC vs. GG	Smoking				
+	+	19	9	<b>2.86(1.25,6.56); 0.015</b>	G, E combined effect
+	-	5	7	0.97(0.30,3.13); 0.602	G alone effect
-	+	78	103	1.03(0.70,1.50); 0.923	E alone effect
-	-	115	156	1.00 (reference)	Common control
GC vs. GG	Smoking				
+	+	13	12	1.47(0.65,3.34); 0.402	G, E combined effect
+	-	121	122	1.35(0.95,1.91); 0.111	G alone effect
-	+	78	103	1.03(0.70,1.50); 0.923	E alone effect
-	-	115	156	1.00 (reference)	Common control
CC vs. GG	Drinking				
+	+	13	6	<b>2.90(1.07,7.83); 0.034</b>	G, E combined effect
+	-	11	10	1.47(0.61,3.57); 0.495	G alone effect
-	+	71	96	0.99(0.67,1.45); 1.000	E alone effect
-	-	122	163	1.00 (reference)	Common control
GC vs. GG	Drinking				
+	+	39	46	1.13(0.70,1.84); 0.620	G, E combined effect
+	-	95	88	1.44(0.99,2.09); 0.058	G alone effect
-	+	71	96	0.99(0.67,1.45); 1.000	E alone effect
-	-	122	163	1.00 (reference)	Common control

<sup>a</sup>G (+): IL-6 gene rs12700386 variants (Heterozygous or homozygous); G (-): wild type

<sup>b</sup>E(+): smoking/non-smoking; E(-): non-smoking/non-drinking

**Table 5** Comparison of studied according to IL-6 genotypes in all osteoarthritis cases.

		OA(n=351)							
		GG(n=193)	GC(n=134)	CC(n=24)	<i>P</i>	GC+CC(n=158)	<i>P</i>	GG+GC(n=327)	<i>P</i>
IL-6 rs12700386									
ESR, mm/h	Mean ± SD	18.14±10.61	18.82±8.40	19.29±8.33	0.065	18.89±8.37	0.468	18.42±9.76	0.558
CRP, mg/L	Mean ± SD	20.29±14.16	19.49±15.06	17.96±14.41	0.509	19.25±14.74	0.505	19.96±14.52	0.477
VAS	Mean ± SD	5.64±1.51	5.75±1.57	5.50±2.06	0.262	5.71±1.65	0.698	5.69±1.54	0.671
Lequesnes' index	Mean ± SD	14.38±2.04	14.70±2.12	14.46±1.79	0.539	14.66±2.07	0.204	14.51±2.08	0.144
Affected leg	Left/right, n	117/76	81/53	16/8	0.838	97/61	0.913	198/129	0.667
KL grading	III+ IV/II, n	107/86	82/52	11/13	0.305	93/65	0.588	190/138	0.288

SD, Standard Deviation; Bold values are statistically significant ( $P < 0.05$ ).

## Figures

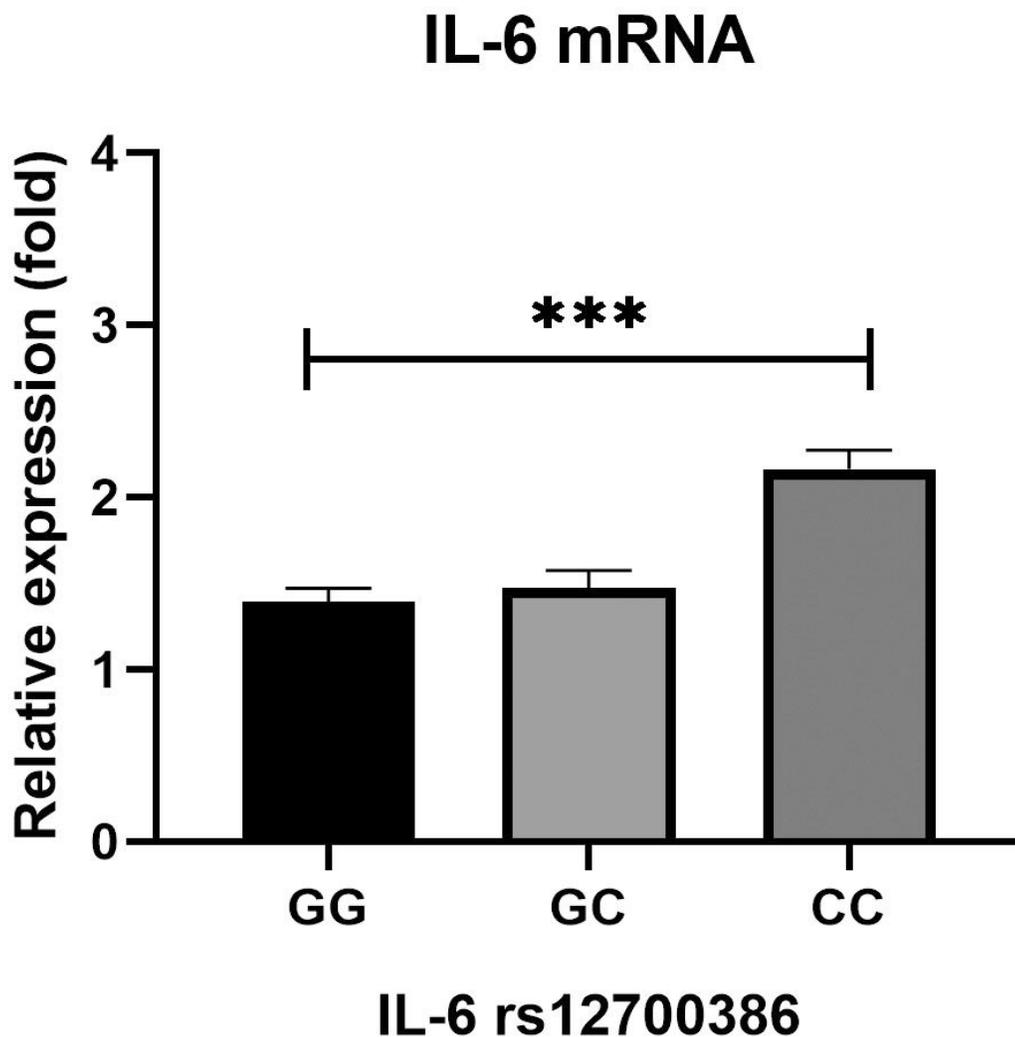


Figure 1

Expression levels of IL-6 in 3 different genotypes.