

The Association Between *Epidermal Growth Factor* rs3756261 A/G Gene Polymorphism and the Risk of Ankylosing Spondylitis in a Chinese Han Population

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Background: Epidermal growth factor (EGF) is a potent pro-angiogenic molecule promoting the angiogenic phenotype of ankylosing spondylitis (AS). Studies demonstrated that *EGF* rs3756261 polymorphism was associated with the risk of inflammatory diseases, but not including AS.

Methods: To investigate the association between *EGF* rs3756261 polymorphism and the risk of AS, we genotyped the *EGF* rs3756261 polymorphism in 208 patients with AS and 412 controls in a Chinese Han population using a custom-by-design 48-Plex SNP scan™ Kit. The serum EGF levels were measured using an enzyme-linked immunosorbent assay in 208 AS patients and 412 controls.

Results: Our data indicated that *EGF* rs3756261 polymorphism was associated with an increased risk of AS in the Chinese Han population. Stratified analyses indicated that the *EGF* rs3756261 polymorphism elevated the risk of AS among the males, smokers, drinkers and those aged <30 years. In addition, the *EGF* rs3756261 polymorphism was related to increased CRP and HLA-B27 levels in AS patients. Next, we found that the average serum levels of EGF were significantly higher in AS patients compared with controls. Meanwhile, EGF serum levels were significantly higher in AG genotype carriers when compared with AA genotype carriers in AS patients.

Conclusion: In conclusion, this study indicated that *EGF* rs3756261 polymorphism was associated with the risk of AS and EGF serum levels in a Chinese Han population.

Keywords: EGF, rs3756261 polymorphism, serum levels, ankylosing spondylitis, case-control study

Introduction

Ankylosing spondylitis (AS) is an inflammatory disease characterized by the clinical features of back pain and stiffness. It is caused by chronic inflammatory disorders, manifested as pathological bone formation, joint and stiffness of spine, spine fibrosis, leading to spine deformity, even disability.¹⁻³ The morbidity of AS is high in young adults aged 18–22 years, especially the men.⁴ However, the pathogenesis of AS is not yet clear. Genetic factors play major roles in AS pathogenesis.⁵⁻⁸ Among all relevant genes, HLA-B27 is the most important genetic factor.⁹ Other susceptibility genes (such as endoplasmic reticulum aminopeptidase-1 and mevalonate kinase) have also been reported.^{10,11}

Epidermal growth factor (EGF) is a powerful mitogen that activates DNA synthesis and cellular differentiation via mitogen-activated protein kinases (MAPKs) and other pathways.^{12,13} EGF is involved in angiogenesis in epidermal tissue.^{14,15} Studies have shown that a rapid decrease in salivary EGF levels can cause progression of the intraoral manifestations of Sjögren's syndrome.^{16,17} In rheumatoid arthritis (RA) patients, high EGF levels were found in the synovial fluid, suggesting the involvement of EGF in the pathogenesis of arthritic diseases.^{18,19} EGF and epidermal growth factor receptor (EGFR) can be downregulated by dioscin, which exerts anti-inflammatory activity by regulating the c-Jun N-terminal kinase (JNK) signaling pathway.²⁰ In the existing literature, a variety of *EGF* polymorphisms have been reported to be associated with etiology of inflammatory diseases. For instance, Wang et al revealed that *EGF*

rs11568835 G/A polymorphism was associated with susceptibility to RA.²¹ A study of *EGF* gene rs11568943 and rs2237051 polymorphisms found no evidence of a haplotype association with psoriatic arthritis (PsA).²²

Although *EGF* plays an important role in the immune response, no studies have examined the influence of the *EGF* rs3756261 A/G polymorphism on the susceptibility to AS. Functional variations in *EGF* gene may contribute to the development of AS.²³ Here, we designed this case–control study to assess the association of the *EGF* rs3756261 polymorphism and AS risk in a Chinese Han population.

Methods

Subjects

In this case–control study, 208 AS patients and 412 sex- and age-matched controls were consecutively recruited from the Changzhou Second Hospital-Affiliated Hospital of Nanjing Medical University (Changzhou, China), between September 2014 and January 2018. A diagnosis of AS was established by using the classification criteria reported by the American College of Rheumatology (Modified New York Criteria).²⁴ The controls were individuals receiving health examinations without AS, matched AS for age and sex, and recruited from the same institutions during the same time period. None of them had immune illnesses, chronic systemic disease, infection, or other serious diseases.

Each patient was interviewed by trained personnel using a pre-tested questionnaire to obtain information of demographic data and related risk factors for AS. The study was approved by the Ethics Committee of Changzhou Second Affiliated Hospital of Nanjing Medical University (Changzhou, China). All patients provided written informed consent to be included in the study. This study was complied with the Declaration of Helsinki.

Blood Sampling and Genotyping

Blood samples were collected using vacutainers and transferred to test tubes containing ethylenediamine tetra-acetic acid (EDTA). All blood samples were stored in a low-temperature refrigerator at -80°C . Genomic DNA was isolated from whole blood using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). SNP was genotyped using a custom-by-design 48-Plex SNP scanTM Kit (Genesky Biotechnologies Inc., Shanghai, China), which was based on double ligation and multiplex fluorescent polymerase chain reaction (PCR). We tested the 208 AS patients' and 412 healthy controls' EGF levels in blood serum using an enzyme-linked immunosorbent assay kit (Boster, Wuhan, China). The levels of EGF were calculated by referring to a standard curve, according to the manufacturer's instructions. Genotyping for *EGF* rs3756261 polymorphism was performed using the following primers: GCAGATGCTATGGCTGATGA (forward) and GAAGTGTGATCTGCCACCT (reverse) (Genesky Biotechnologies Inc., Shanghai, China). About 10% of selected samples were re-genotyped to verify the genotyping accuracy. The concordance of genotypes in the repeated samples was 100%.

Statistical Analyses

Differences in demographics, variables, and genotypes of the *EGF* rs3756261 A/G polymorphism were evaluated using a chi-squared test. The association between *EGF* rs3756261 polymorphism and risk of AS was estimated by calculating odds ratios (ORs) and 95% confidence intervals (CIs) using logistic regression analyses. The Hardy-Weinberg equilibrium (HWE) was tested by a goodness-of-fit chi-squared test to compare the observed genotype frequencies to the expected frequencies among controls. We analyzed the allele and genotype distributions of AS patients and controls. Stratified analyses according to drinking, smoking, sex, and age were conducted. The association of genotypes of *EGF* gene polymorphism with EGF serum levels was evaluated using the Student's *t*-test. A cross-over analysis was used to assess the effects of the interaction between environmental factors (smoking or drinking) and genetic factors on the AS risk. All statistical analyses were done with SPSS 22.0 software (SPSS Inc., Chicago, USA) and GraphPad Prism (version 8.0).

Results

Characteristics of the Study Population

The demographic and clinical characteristics of all subjects are summarized in Table 1. Subjects were adequately matched for age and sex ($P = 0.157$ and 0.406 , respectively). The genotype distributions of *EGF* rs3756261 A/G polymorphism in all subjects are illustrated in Table 2. The observed genotype frequencies for this polymorphism in controls were in HWE ($P = 0.419$).

Association Between *EGF* rs3756261 A/G Polymorphism and the Risk of AS

In this case-control study, data indicated that AG or AG+GG genotype was associated with an increased risk of AS (AG vs AA: OR, 1.64; 95% CI, 1.14–2.35; $P = 0.007$; AG+GG vs AA: OR, 1.67; 95% CI, 1.18–2.36; $P = 0.004$). In addition, G allele increased the risk of AS (G vs A: OR, 1.53; 95% CI, 1.14–2.06; $P = 0.004$). However, GG genotype was not related with the risk of AS (GG vs AA: OR, 1.98; 95% CI, 0.80–4.91; $P = 0.132$; GG vs AA+AG: OR, 1.68; 95% CI, 0.68–4.12; $P = 0.253$). Stratified analyses of sex, age, drinking, and smoking indicated that the *EGF* rs3756261 A/G polymorphism increased the risk of AS among the males, smokers, drinkers and those aged <30 years (Table 3).

Cross-Over Analysis

Due to the findings of stratified analyses, we performed the cross-over analysis to evaluate the potential interaction between exposure factors (smoking or drinking) and genetic factors (Table 4). Drinkers carrying the AG genotype were more easily to suffer from AS compared with non-drinkers carrying the AA genotype (OR, 1.71, 95% CI, 1.04–2.81; $P = 0.035$). This indicated that AG genotype could strongly interact with drinking. The similar phenomenon was also shown in smokers carrying the AG genotype (AG vs AA: OR, 2.08; 95% CI, 1.26–3.44; $P = 0.004$). These data indicated that there was a strong interaction between the AG genotype of rs3756261 and exposure factors (smoking or drinking).

Table 1 Demographic Factors in Ankylosing Spondylitis and Control

Variable*	Cases (n = 208)	Controls (n = 412)	P
Age (years)	32.43 (± 8.47)	33.54 (± 10.47)	0.157
Sex			0.406
Male	155(74.50%)	294(71.36%)	
Female	53(25.50%)	118(28.64%)	
Smoking			0.156
Yes	130(62.50%)	233(56.55%)	
No	78(37.50%)	179(43.45%)	
Drinking			0.643
Yes	109(52.40%)	224(54.37%)	
No	99(47.60%)	188(45.63%)	
CRP positive, no. (%)		–	–
Positive	123(59.13%)		
Negative	85(40.87%)		
HLA-B27 positive, no. (%)		–	–
Positive	169(81.25%)		
Negative	39(18.75%)		
Grading of sacroiliac joint			
Grade I	0(0.00%)	–	–
Grade II	64(30.76%)	–	–
Grade III	103(49.51%)	–	–
Grade IV	41(19.71%)	–	–
EGF levels** (pg/mL)	135.9(109.0–155.7)	95.2(76.3–111.4)	0.000

Notes: HLA-B27: **The EGF levels were expressed as median (P25, P75), Bold values are statistically significant ($P < 0.05$).

Abbreviation: *CRP, C-reactive protein.

Table 2 Logistic Regression Analysis of Associations Between *EGF* rs3756261 A/G Polymorphisms and Risk of Ankylosing Spondylitis

Genotype	Cases* (n=208)		Controls* (n=412)		OR (95% CI)	P
	n	%	n	%		
AA	118	57.8	286	69.6	1.00	–
AG	77	37.7	114	27.7	1.64 (1.14–2.35)	0.007
GG	9	4.4	11	2.7	1.98 (0.80–4.91)	0.132
AG+GG	86	42.2	125	30.4	1.67 (1.18–2.36)	0.004
AA+AG	195	95.6	400	97.3	1.00	–
GG	9	4.4	11	2.7	1.68 (0.68–4.12)	0.253
A allele	313	76.7	686	83.5	1.00	–
G allele	95	23.3	136	16.5	1.53 (1.14–2.06)	0.004

Notes: *The genotyping was successful in: 204 cases and 411 controls for *EGF* rs3756261 A/G. Bold values are statistically significant ($P < 0.05$).

Table 3 Stratified Analyses Between *EGF* rs3756261 A/G and the Risk of Ankylosing Spondylitis

Variable	rs3756261 (Case/Control)			AG vs AA OR (95% CI); P	GG vs AA OR (95% CI); P	GG vs AG+AA OR (95% CI); P	AG+GG vs AA OR (95% CI); P
	AA	AG	GG				
Sex							
Male	90/214	60/74	3/5	1.93(1.27–2.94); 0.002	1.43(0.33–6.10); 0.699	1.15(0.27–4.89); 1.000	1.90(1.26–2.87); 0.002
Female	28/72	17/40	6/6	1.09(0.53–2.24); 0.808	2.57(0.77–8.65); 0.180	2.49(0.76–8.13); 0.188	1.29(0.66–2.50); 0.458
Age (years)							
<30	73/189	45/51	5/6	2.28(1.41–3.70); 0.001	2.16(0.64–7.29); 0.304	1.70(0.51–5.67); 0.517	2.27(1.43–3.62); 0.000
≥30	45/97	32/63	4/5	1.10(0.63–1.90); 0.748	1.72(0.44–6.73); 0.473	1.66(0.43–6.37); 0.482	1.14(0.67–1.95); 0.630
Smoking							
Yes	74/159	49/68	5/5	1.55(0.98–2.45); 0.061	2.15(0.60–7.65); 0.301	1.85(0.52–6.50); 0.336	1.59(1.02–2.49); 0.042
No	44/127	28/46	4/6	1.76(0.98–3.07); 0.056	1.92(0.52–7.14); 0.460	1.60(0.44–5.85); 0.491	1.48(0.88–2.49); 0.139
Drinking							
Yes	56/164	46/53	5/6	2.54(1.55–4.18); 0.000	2.44(0.72–8.31); 0.164	1.77(0.53–5.95); 0.344	2.53(1.56–4.10); 0.000
No	62/122	31/61	4/5	1.00(0.59–1.70); 1.000	1.57(0.41–6.07); 0.495	1.57(0.41–6.00); 0.495	1.07(0.65–1.78); 0.785

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

Correlation Between the *EGF* rs3756261 A/G Polymorphism and Clinical Characteristics of AS

We evaluated the association between the *EGF* rs3756261 A/G polymorphism and clinical characteristics of patients with AS (Table 5). We found that the *EGF* rs3756261 A/G polymorphism was associated with increased CRP and HLA-B27 levels in AS patients. However, no significant relationship was found in the analyses of grading of sacroiliac joint.

Association of *EGF* rs3756261 A/G Polymorphism with the Serum *EGF* Levels

The average serum levels of *EGF* were significantly higher in AS patients compared with controls (Table 1). We compared serum *EGF* levels on the basis of genotypes of *EGF* rs3756261 A/G polymorphism and demonstrated that, in AS patients, *EGF* serum levels were significantly higher in AG genotype carriers than AA genotype carriers ($P < 0.05$, Figure 1).

Discussion

AS is a chronic inflammatory disorder characterized mainly by new bone formation that results in ankylosis of the sacroiliac and intervertebral joints. Angiogenesis plays an important role in the pathogenesis of chronic inflammatory

Table 4 Genetic (G) and Environmental (E) Factors 2*4 Fork Analysis

G ^a	E ^b	Case	Control	OR (95% CI); P value	Reflecting Information
GG vs AA	Smoking				
+	+	5	5	2.89(0.80,10.45); 0.137	G, E combined effect
+	-	4	6	1.92(0.52,7.14);0.460	G alone effect
-	+	74	159	1.34(0.87,2.09); 0.188	E alone effect
-	-	44	127	1.00 (reference)	Common control
AG vs AA	Smoking				
+	+	49	68	2.08(1.26,3.44); 0.004	G, E combined effect
+	-	28	46	1.76(0.98,3.07);0.056	G alone effect
-	+	74	159	1.34(0.87,2.09); 0.188	E alone effect
-	-	44	127	1.00 (reference)	Common control
GG vs AA	Drinking				
+	+	5	6	1.64(0.48,5.59); 0.516	G, E combined effect
+	-	4	5	1.57(0.41,6.07);0.495	G alone effect
-	+	56	164	0.67(0.44,1.03); 0.070	E alone effect
-	-	62	122	1.00 (reference)	Common control
AG vs AA	Drinking				
+	+	46	53	1.71(1.04,2.81); 0.035	G, E combined effect
+	-	31	61	1.00(0.59,1.70);1.000	G alone effect
-	+	56	164	0.67(0.44,1.03); 0.070	E alone effect
-	-	62	122	1.00 (reference)	Common control

Notes: ^aG (+): EGF rs3756261 A/G variants (Heterozygous or homozygous); G (-): wild type. ^bE(+): Smoking/drinking; E(-): non-smoking/non-drinking. Bold values are statistically significant ($P < 0.05$).

Table 5 The Associations Between EGF rs3756261 A/G Polymorphism and Clinical Characteristics of Ankylosing Spondylitis

Characteristics	Genotype distributions			
	AA	AG	GG	AG+GG
CRP status				
Positive / Negative	60/58	56/21	5/4	61/25
OR (95% CI); P-value	1.0 (reference)	2.58(1.39–4.78); 0.002	1.21(0.31–4.72);0.785	2.36(1.31–4.25); 0.004
HLA-B27 status				
Positive / Negative	92/26	69/8	6/3	75/11
OR (95% CI); P-value	1.0 (reference)	2.44(1.04–5.71); 0.036	0.57(0.13–2.42);0.714	1.93(0.89–4.15); 0.091
Grading of sacroiliac joint				
III+IV / I+II	85/33	54/23	4/5-	58/28
OR (95% CI); P-value	1.0 (reference)	0.91(0.48–1.72); 0.774	0.31(0.08–1.23);0.081	0.80(0.44–1.47); 0.581

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

disorders such as RA and PsA.^{22,25} Sacroiliitis is the primary clinical feature of AS patients. Fibrovascular synovial tissue infiltrated with inflammatory cells makes incursions into the joint, implying that new vessel formation plays a pivotal role in the pathogenesis of sacroiliitis.²⁶ Many cases of sacroiliitis and peripheral arthritis in AS indicate that vascularity is increased in the synovial tissues.^{27,28} The inflammatory processes of the synovial membranes of peripheral arthritis in AS are similar to those of RA.^{27,29} Furthermore, Francois et al showed that changes in synovitis and subchondral bone marrow might result in joint destruction.³⁰ The cytokines involved in angiogenesis included VEGF and EGF, which were also upregulated in AS, RA, and psoriasis.²² Together, these findings suggested that EGF may be associated with the pathogenesis of AS.

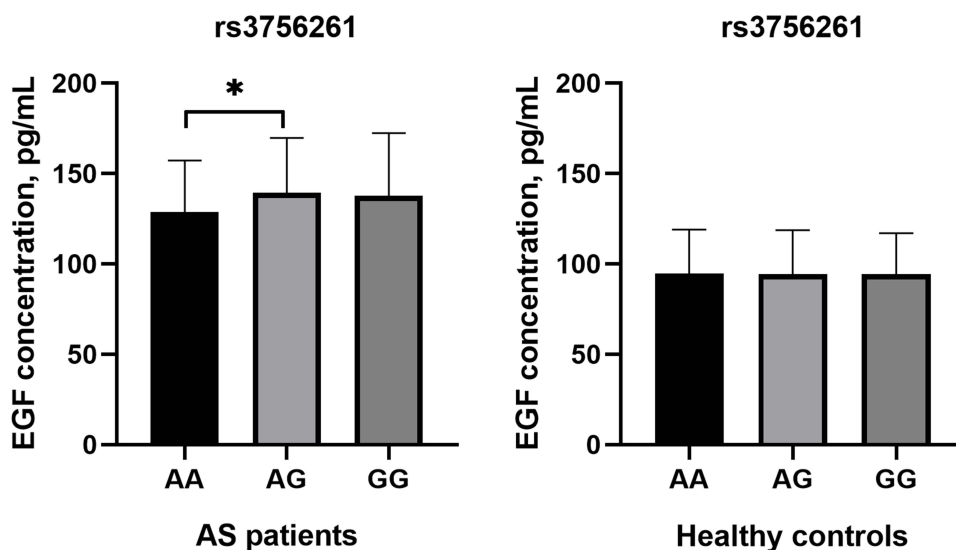


Figure 1 Serum EGF levels among AS patients and controls in each genotype of rs3756261 polymorphism (* indicated $P < 0.05$).

EGF gene is located on chromosome 4q25-27 and contains 24 exon and 23 introns. Rs3756261 A/G polymorphism is located on the promoter region of *EGF* gene. Butt et al investigated the relationship between *EGF* gene polymorphisms and PsA risk but observed no association of *EGF* gene loci with PsA susceptibility.²² Wang et al addressed the association between *EGF* rs11568835 G/A and rs3756261 A/G polymorphisms and RA risk, and found that *EGF* rs3756261 A/G polymorphism was not associated with susceptibility to RA.²¹ As these inflammatory diseases may share common susceptibility loci, investigating the association of *EGF* rs3756261 A/G polymorphism with AS risk is warranted. Thus, we performed this case–control study and found that the *EGF* rs3756261 A/G polymorphism was associated with an increased risk of AS in a Chinese Han population. This finding was not observed in the study by Wang et al, and we assumed that *EGF* rs3756261 A/G polymorphism may be a specific locus for some disorders such as AS. We found that individuals carrying AG genotype or G allele were more likely to develop AS. To our knowledge, this is the first study to reveal an association between the *EGF* rs3756261 A/G polymorphism and the risk of AS.

Next, we performed subgroup analyses of sex, age, drinking, and smoking, and found *EGF* rs3756261 A/G polymorphism increased the risk of AS among the males, smokers, drinkers, and subjects aged <30 years. It is likely that interactive contributions of genetic and environmental factors may contribute to the development of AS. To further evaluate the impact of the interactions between environmental and genetic factors on AS susceptibility, we performed cross-over analyses, and data suggested that drinkers or smokers carrying the AG genotype were more prone to from the occurrence of AS. It indicated that the interaction between drinking and smoking and AG genotype increased the risk of AS. A previous study by Ding et al also observed the interaction between *FCRL4* gene and suspected environmental factors in patients with AS.³¹ Those studies provide evidence that AS is a chronic inflammatory disease, which is induced by genetic and environmental factors. In addition, we explored the association of *EGF* rs3756261 A/G polymorphism and clinical characteristics of AS, and found that this SNP was related to increased levels of CRP and HLA-B27 in AS patients.

Last, we measured the serum EGF levels among AS patients and controls, and found that AS patients showed higher EGF levels in comparison with healthy controls. However, Przepiera-Będzak et al revealed that serum EGF levels were similar in AS and controls.²³ Potential factors may account for inconsistent findings of these studies. First, ethnic group of these patients was different. Przepiera-Będzak et al investigated this issue in a Polish population,²³ while this study explored it in a Chinese Han population. Second, the sample sizes were significantly distinct. Limited sample sizes in the study by Przepiera-Będzak et al may produce false-negative findings. In addition, we also found that EGF serum levels were significantly higher in AG genotype when compared with AA genotype carriers in AS patients, suggesting that *EGF*

rs3756261 A/G polymorphism was related to the levels of EGF. Thus, we assumed that *EGF* rs3756261 A/G polymorphism may increase the risk of AS via affecting the EGF serum levels, which needs further studies to verify it.

Overall, the results obtained in our study might be helpful for early screening of individuals at high-risk of AS in the Chinese Han population. However, several limitations of the present study need to be addressed. First, this was a hospital-based case–control study; thus, selection bias was unavoidable. Second, the polymorphism investigated, based on functional consideration, may not offer a comprehensive view of the genetic variabilities of *EGF* gene. Third, environmental factors differed between Chinese and other populations; The *EGF* gene may be associated with different degrees of genetic risk in different ethnic groups with diverse environmental exposures. Fourth, the potential mechanisms of *EGF* rs3756261 A/G polymorphism affecting the incidence of AS should be investigated. Last, the most important factor was MRI bone marrow edema, which was not investigated in this study.

In conclusion, this study indicated that *EGF* rs3756261 A/G polymorphism was associated with an increased risk of AS and in a Chinese Han population. Drinking and smoking may be risk exposure factors to take a combined action with *EGF* rs3756261 A/G polymorphism in patients with AS. In addition, the EGF level of AS patients was found to be higher compared to the healthy controls.

Data Sharing Statement

The data of this research has been presented in the article.

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Disclosure

The authors declare that they have no conflict of interest.

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