Polymorphism in the IL-8 Gene Promoter and the Risk of Acne Vulgaris in a Pakistani Population

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ABSTRACT

Interleukin-8 (IL-8) is a well-known inflammatory chemokine and suggested to be involved in the development of acne vulgaris. This study investigates IL-8 plasma levels in acne patients and healthy controls and the molecular basis for the regulation of the IL-8 gene in a Pakistani population.

Patients with acne vulgaris (n = 264) and healthy individuals (n = 264) were enrolled in this investigation. Plasma IL-8 levels were determined by enzyme-linked immunosorbent assay (ELISA). The genotyping for IL-8 gene was performed by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP).

Our data showed a statistically significant increase in IL-8 levels from acne patients compared with healthy subjects (154.2 ± 52.1 pg/mL in patients vs. 101.6 ± 33.5 pg/mL in controls, p<0.0001). The IL-8-251T>A (rs4073) polymorphism was significantly higher in patients with acne compared with the control group (p=0.013). There was a significant difference between the T and A alleles from acne cases and controls (odds ratio OR = 1.6, 95%CI = 1.16-2.19, p = 0.003). Logistic-regression analysis showed that the increased IL-8 levels, and the IL-8-251T>A polymorphism were significantly associated with acne.

Our data suggest that the elevated IL-8 levels and the IL-8-251T>A polymorphism may be associated with acne vulgaris in the study population.

Keywords: Association; Acne vulgaris; Interleukin-8; Pakistan; Polymorphism

INTRODUCTION

Acne vulgaris is a distressing condition of pilosebaceous unit characterized by the formation of
open and closed comedones, papules, nodules, pustules and cysts. Genome-wide association studies have shown that genetic factors play an important role in the etiology and pathogenesis of acne vulgaris. Most of the genes studied are either key player of innate immune system during lesion formation or have a function in steroid hormone metabolism. More recently, additional single nucleotide polymorphisms (SNPs) in IL-8 that can be linked to cellular metabolic processes have been reported.

Little is known about the role of IL-8 in the pathophysiology of acne vulgaris. Studies have shown that IL-8 is an inflammatory chemokine, and is involved in various clinical conditions including acne, which is mediated by the migration of cells to the site of inflammation. The increased expression of IL-8 in skin biopsies showed a significant association with epidermal hyperplasia and follicular hyperkeratosis in inflammatory acne vulgaris. A significant role of IL-8 in inflammatory skin disorder is also supported by the association of the chemokine with high risk in patients with psoriasis.

Gene encoding IL-8 is located on chromosome 4q12-q21. Several functional SNP in IL-8 gene have been identified. One of them represents T to A substitution at position -251, and has a putative effect on IL-8 expression. The association of -251T>A polymorphism with susceptibility of inflammatory diseases have been widely reported. However, the association between IL-8 -251T>A polymorphism and acne vulgaris is not very clear and recommended to be studied comprehensively; therefore, it could be remarkable to study the functional relationship of IL-8 gene polymorphism and IL-8 levels with acne vulgaris. Thus, this study was designed to evaluate the association of circulating IL-8 levels and IL-8-251T>A polymorphism with acne vulgaris in a Pakistani population.

**MATERIALS AND METHODS**

**Study Population**

The work presented here includes immunological and genetic analyses of the study samples comprising of patients with acne vulgaris and their respective controls. The present study protocol is adherent to the Helsinki Declaration of 1975 as revised in 1997 and was approved from the Institutional Review Board (IRB), COMSATS Institute of Information Technology. All participants signed informed consents for the genetic and immunological analyses, including demographic observations. In this study, 264 patients (mean age 22.7 ± 6.09) with documented acne vulgaris and 264 healthy control subjects (mean age 22.5 ± 5.30) were enrolled for the case-control association of IL-8 gene polymorphism. We ascertained those patients who were not in blood relation with controls. The inclusion criterion was applied for both, the patients and the controls in this study. The diagnosis of acne vulgaris was based on physical examination, and Global Acne Grading System. Patients were divided into three sub-categories, as: acne comedonica (mild acne), acne papulo-pustulosa (moderate acne), and nodulo-cystic acne (severe acne). Healthy controls with no symptoms of acne vulgaris or other skin disease in the present as well as in the past were enrolled in this study. Control subjects were from the same ethnic region and their clinical histories were reviewed by dermatologists being unaware of the objectives of study.

**Immunological Analysis**

Standard protocol was adopted for plasma isolation from whole blood samples. Plasma IL-8 concentrations were determined by using an immunoenzymetric assay kit of DiaSource (DIA Source ImmunoAssay S.A, Rue du Bosquet, 2, B-1348 Louvain-la-Neuve, Belgium). IL-8 standards were run on micro test plates, and the antigen concentrations were measured from the standard curve using AMP Platos R 496 Micro-plate reader (AMP Diagnostics, Austria).

**Genotyping Analysis**

Genomic DNA was extracted from whole venous blood samples using standard phenol-chloroform extraction technique. Equal volumes (0.75 ml) of the blood and solution A [0.32 M Sucrose (BDH, England), 10 mM Tris of pH 7.5 (BDH, England), 5 mM MgCl2 (Sigma-Aldrich MO, USA), 1% v/v Triton X-100 (Sigma-Aldrich MO, USA)] each, were mixed in a 1.5 ml micro-centrifuge tube, mixed several times by gently inverting tube, and left for 10-15 minutes at room temperature. The tube was centrifuged at 13,000 rpm for 1 minute in a micro-centrifuge 5417 R (Eppendorf, Germany). Supernatant was discarded; pellet was re-suspended in 400 µL of solution A, and was re-centrifuged at the same speed. Supernatant was discarded and the nuclear pellet was re-suspended...
in 400 μL of solution B (10 mM Tris pH 7.5, 400 mM NaCl (BDH, England), 2 mM EDTA of pH 8.0 (BDH, England), and digested at 37°C overnight by adding 12 μL of 20% SDS solution (BDH, England) and 8 μL (20 mg/mL) of Proteinase K (Fermentas, Germany).

After complete digestion of the nuclear pellet, 0.5 ml of a freshly prepared mixture of equal volume of solution C (Sigma-Aldrich MO, USA) and solution D (24 volumes of chloroform and 1 volume of iso-amyl alcohol (BDH, England) was added in the tube; mixed and centrifuged at 13,000 rpm for 10 minutes. The aqueous phase (upper layer) was transferred in a new tube containing 500 μL of solution D, centrifuged again at 13,000 rpm for 10 minutes. Genomic DNA was precipitated out from the aqueous phase by adding 60 μL of 3M sodium acetate (pH 6) and 500 μL of isopropanol (BDH, England). The tube was then inverted for some time to settle the DNA and centrifugation was carried out again at 13,000 RPM for 10 minutes. The supernatant was discarded and the DNA pellet was washed with chilled 70% ethanol (BDH, England) and dried at 37°C. After evaporation of residual ethanol, DNA was dissolved in appropriate amount of DNA dissolving buffer (Tris-EDTA, Sigma-Aldrich MO, USA) and stored at 4°C.

The IL-8-251T>A polymorphism was investigated by the polymerase chain reaction (PCR) using forward primer 5'-CCTCCCCAATAAAATGATTGCTG-3' and reverse primer 5'-CCTTCCGTTGTTTCTTCTTG-3'. PCR amplification was carried out in 0.2 mL tubes (Axygen, CA, USA) in a total volume of 50 μL. The reaction mixture contained 3 μL of genomic DNA, 2.5 μL of each forward and reverse primer (20 μM stock), 5 μL of 10X PCR buffer (200 mM of (NH4)2SO4, 750 mM of Tris-HCl (pH 8.8), and 0.1% Tween 20), 4 μL of 25 mM MgCl2 (MBI-Fermentas, England), 1 μL of 10 mM dNTPs (MBI-Fermentas, England), and 0.5 μL (5 U/μL) of Taq DNA polymerase (MBI Fermentas, England) in 31.5 μL of PCR water. PCR reactions were performed by means of GeneAmp PCR System 9700 (Applied Biosystems Inc, Foster City, CA, USA). PCR was carried out with the following thermal cycling conditions: an initial denaturation step at 94°C for 12 min, followed by amplification for 35 cycles at 94°C for 30s, 56°C for 1 min, and 72°C for 2 min, followed by a final extension step at 72°C for 2 min. The PCR products were analyzed by 3% agarose gel electrophoresis.

MunI (Thermo Scientific, England) restriction endonuclease was used for the detection of the IL-8-251T>A polymorphism. Restriction fragment-length polymorphism was performed in 0.2 mL tubes (Axygen, CA, USA) in a total volume of 20 μL containing 12 μL of amplified products, 2 μL storage buffer (10 mM Tris-HCl (pH 7.4 at 25°C), 100 mM KCl, 1 mM DTT, 1 mM EDTA, 0.2 mg/mL BSA, and 50% (v/v) glycerol), 0.3 μL of MunI enzyme, and 5.7 μL of PCR water. Digested products were analyzed by 3% agarose gel electrophoresis and visualized under ultraviolet light.

**Statistical Analysis**

Basic and immunological variables are mentioned as mean ± SD. Comparison of these variables between patients and controls was carried out by chi-square test, one-way analysis of variance and independent samples t-test. Allele and genotype frequencies were calculated by direct counting. Hardy–Weinberg equilibrium (HWE) was calculated using the software Arlequin V3.0. Odds ratio (OR) and 95% confidence intervals were calculated using the statistical software Graph Pad Instat 3.0 for 2 way contingency table analysis (Graph Pad Software Inc., San Diego, Calif.). Multivariate analysis was carried out by using MedCalc Software (Acacialaan 22, 8400 Ostend Belgium). P values < 0.05 were considered as statistically significant.

**RESULTS**

The patient group consisted of 43.0% and 57.0%, whereas the control group of healthy subjects consisted of 44.0% and 56.0% males and females, respectively. IL-8 plasma levels were significantly higher in patients with acne vulgaris (154.2 ± 52.1 pg/mL vs. 101.6 ± 33.5 pg/mL in patients and controls, respectively, p<0.0001).

Genotype and allelic distribution of IL-8-251T>A polymorphism in both patients and controls are shown in table 1. Distribution of the genotypes in acne and the control group was consistent with the Hardy–Weinberg equilibrium (p = 0.242 for acne group and p = 0.460 for control group). The observed genotype frequencies in patient group were TT = 58.7%, TA = 34.1%, and AA = 7.2%, resulting in an A-allele frequency of 24.2%. In healthy controls, TT, TA, and AA genotypes were 70.1%, 26.5%, and 3.4%, respectively. The minor allele
A at IL-8-251 was 16.7% in the control group. There was a significant difference for IL-8-251T>A polymorphism between patients with acne vulgaris and healthy controls (chi square = 8.72, p = 0.013). The IL-8 variant genotypes (TA + AA) showed significantly high prevalence in patients (41.3%) compared with healthy controls at 29.9% (chi-square = 7.43, p=0.006, Table 1). Moreover, a significant difference was found between the T and A alleles from the patients and controls (OR = 1.6, 95%CI = 1.16-2.19, p=0.003; Table 1).

Binary logistic-regression analysis for possible association of age, gender, IL-8 plasma levels, and IL-8-251T>A polymorphism with acne is shown in Table 2. Two variables, IL-8 plasma levels (p<0.0001) and IL-8-251T>A polymorphism (p<0.0001) were independently associated with acne in the study population. However, age and gender were not associated with the disease in this study (p>0.05 for each; Table 2).

IL-8 -251T>A genotype-wise plasma concentrations are shown in figure 1. Patients with acne vulgaris showed varying concentrations of plasma IL-8 levels regarding IL-8-251T>A genotypes. Subjects with the IL-8-251 variant genotypes showed higher IL-8 levels than the cases with the wild genotype. Carriers of AA genotype showed maximal IL-8 levels (250.5 ± 7.9) followed by TA (188.8 ± 3.7) and TT (122.3 ± 2.4) genotypes, respectively. Significant difference was observed among IL-8 levels from the carriers of three genotypes (p<0.0001; Figure 1).

Plasma IL-8 levels and their association with severity of acne is shown in figure 2. Patients were divided into three sub-categories, as: mild acne, moderate acne, and severe acne. Patients with severe acne showed maximal IL-8 plasma levels (210.9 ± 8.8 pg/mL) followed by moderate acne (169.9 ± 7.4 pg/mL) and mild acne (138.44 ± 3.1 pg/mL). One-way analysis of variance showed a significant association of plasma IL-8 concentration with severity of acne vulgaris (p<0.0001, Figure 2).

### Table 1. The genotype and allele frequencies of IL-8-251T>A polymorphism in the study population

<table>
<thead>
<tr>
<th>IL-8-251T&gt;A SNP</th>
<th>Acne Patients (n = 264)</th>
<th>Controls (n = 264)</th>
<th>Chi square (P-Value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TT</td>
<td>155 (58.7%)</td>
<td>185 (70.1%)</td>
<td>8.72 (P = 0.013)</td>
</tr>
<tr>
<td>TA</td>
<td>90 (34.1%)</td>
<td>70 (26.5%)</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>19 (7.2%)</td>
<td>9 (3.4%)</td>
<td></td>
</tr>
<tr>
<td>TA+AA</td>
<td>109 (41.3%)</td>
<td>79 (29.9%)</td>
<td>7.43 (p= 0.006, TT vs. TA+AA genotype)</td>
</tr>
<tr>
<td>A allele</td>
<td>128 (24.2%)</td>
<td>88 (16.7%)</td>
<td>p=0.003 (A vs T allele)</td>
</tr>
<tr>
<td>T allele</td>
<td>400 (75.8%)</td>
<td>440 (83.3%)</td>
<td>OR = 1.6, 95%CI = 1.16-2.19</td>
</tr>
</tbody>
</table>

Values are given in numbers and percentage
P-Values were calculated by Chi-square test
OR, Odd Ratio; 95%CI, 95% confidence interval

### Table 2. Binary Logistic regression analysis in all subjects when acne was used as a response variable and age, gender, IL-8 and IL-8-251T>A polymorphism were taken as independent variables

<table>
<thead>
<tr>
<th>Variables</th>
<th>Coefficient</th>
<th>SE</th>
<th>OR</th>
<th>95%CI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.029931</td>
<td>0.019565</td>
<td>1.030</td>
<td>0.99-1.07</td>
<td>0.1261</td>
</tr>
<tr>
<td>Gender</td>
<td>0.030815</td>
<td>0.17554</td>
<td>1.0313</td>
<td>0.73-1.45</td>
<td>0.8607</td>
</tr>
<tr>
<td>IL-8 levels</td>
<td>-0.037125</td>
<td>0.0036207</td>
<td>0.9636</td>
<td>0.95-0.97</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>-251T&gt;A</td>
<td>-4.51877</td>
<td>0.73543</td>
<td>0.0109</td>
<td>0.002-0.04</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Constant</td>
<td>14.2481</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SE, standard error; OR, odds ratio; 95%CI, 95% confidence interval; -251T>A, IL-8-251T>A polymorphism (TT vs. TA+AA)
Association of Interleukine-8 with Acne Vulgaris

Figure 1. Influence of IL-8-251T>A polymorphism on the circulating levels of IL-8 in acne vulgaris patients. Data represent mean ± SEM, *P*<0.0001

Figure 2. Relationship of the acne severity with circulating levels of IL-8 in patients. The data represent mean ± SEM. Plasma IL-8 levels in mild, moderate, and severe acne vulgaris were compared by 1-way analysis of variance (*p*<0.0001).

**DISCUSSION**

The present systematic study examined the IL-8 SNP at -251 and circulating IL-8 levels in acne vulgaris from Pakistani population. Our findings indicate that IL-8-251T>A promoter SNP may function as an important risk marker for acne vulgaris in our high risk population. In this study, we have observed significantly high levels of IL-8 from acne cases compared with healthy individuals (*p*<0.0001). Moreover, the increased levels of IL-8 were significantly associated with the severity of acne vulgaris. The association of IL-8 plasma levels with acne remain significant in multivariate analysis when confounding variables were taken in consideration. Besides, we also observed a significant increase of IL-8 levels in patients with severe acne vulgaris.

Acne is a common skin disease being explained as chronic inflammation of pilosebaceous part of skin.\(^1\) In the development of acne, the keratinocytes are basic cells of skin which differentiate slowly to form different skin layers. This differentiation phenomenon is often affected by various stimuli which carries the signals of activation for various skin cells. Consequently affected cells may lead to abnormal proliferation of cells. These cellular and morphological changes are the results of abnormal function of cell surface receptors, keratins, adhesion molecules and inflammatory cytokines.\(^2\) Studies have shown that IL-8 is released by a variety of cell types including macrophages, monocytes, T-lymphocytes, fibroblasts endothelial cells and keratinocytes in response to inflammatory stimulus.\(^3\)–\(^14\) Moreover, IL-8 attracts and degranulates neutrophils in the site of inflammation.\(^15\) This degranulation releases potential key regulators of cell signaling during inflammation such as serine proteases, cathepsin G, leucocyte elastase and proteinase, triggering IL-8 and subsequently activate different IL-8 receptors.\(^16\) Subsequently, the increased expression of IL-8 in skin biopsies showed a significant association with epidermal hyperplasia and follicular hyperkeratosis in inflammatory acne vulgaris.\(^8\) A significant role of IL-8 in inflammatory skin disorders is also supported by the association of this chemokine with higher risk in patients with psoriasis.\(^6\)–\(^7\)

It is proven that there is a genetic basis for most inflammatory diseases, including acne vulgaris. Evidence from classic twin studies suggested that genetic determinants were significant modifiers of the acne vulgaris phenotype.\(^17\) The clarification of the genetic basis of acne vulgaris should permit a better understanding of disease etiology, allowing the improved classification, diagnosis, and treatment. Thus, enormous efforts have been expended to identify gene polymorphisms associated with the risk of acne vulgaris. Several SNPs in pro-inflammatory genes including TNF-alpha and interleukine-1 have been associated with the pathogenesis of acne.\(^2\) In this study, we have observed a significant link between IL-8-
251T>A polymorphism and acne vulgaris in our high risk population. The real picture of genetic association of IL-8-251T>A polymorphism with acne vulgaris has not been clear. In contrast to findings of this study, Sobjanek and associates have reported non-significant association of IL-8 gene polymorphism with acne in a polish population.3 This inconsistency between the findings has been always attributed to the difference in ethnic variations and gene-environment interaction. Nonetheless, our finding of the IL-8-251T>A polymorphism association with acne vulgaris in a Pakistani population is consistent with link between minor allele A at IL-8-251 and inflammatory conditions like periodontitis 8 and oral cancer 18 from other ethnic regions. Although our data indicate that IL-8 high levels and IL-8-251T>A polymorphism are associated with acne vulgaris in the study population, at this stage, it is not certain whether IL-8 is a causative factor for acne or not. Therefore, these findings should be replicated from larger cohorts with additional SNPs to confirm the pioneer findings of this study.

In our patient group, the IL-8-251 genotype-wise variations were observed in plasma levels of IL-8. The carrier of AA genotype at -251 showed maximal mean concentration of TNF-alpha followed by TA genotype. Experimental study has demonstrated that the IL-8 AA genotype at -251 is associated with enhanced transcriptional activity compared with the wild TT genotype.19 Moreover, the -251A allele has been linked with higher induced levels of IL-8.4 It is considered that high IL-8 concentrations may increase the susceptibility of acne.7 The direct functional effect of IL-8 variant genotype on circulating IL-8 levels and their association with acne in patients has not been investigated prior to this study. However, more studies would be quite desirable to establish a correlation between the circulating IL-8 levels and acne vulgaris.

In conclusion, for the first time, we demonstrate a trend for association of increased IL-8 levels and IL-8-251T>A polymorphism with acne from Pakistan. The AA genotype at -251 contributes to increased levels of IL-8 in patients. Subsequently, a significant link of plasma IL-8 levels was also observed with severity of acne. However, the interpretation of the findings in the study population is limited by small sample size and one promoter SNP. This study should be considered as an exploratory investigation demonstrating an association of the said SNP with disease phenotype, and not the causative factor of the disease.

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