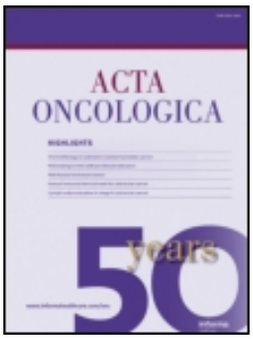

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**ORIGINAL ARTICLE**

# Interleukin-18 gene promoter polymorphisms and the risk of esophageal squamous cell carcinoma

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**Abstract**

*Background.* Esophageal squamous cell carcinoma (ESCC) is multifactorial, and the genetic background may be a crucial etiologic factor. Interleukin-18 (IL-18) is a multifunctional cytokine that induces interferon (IFN)-gamma secretion and plays an important role in antitumor immunity. Variations in the DNA sequence in the IL-18 gene promoter may lead to altered IL-18 production and/or activity, and so this can modulate an individual's susceptibility to ESCC. To test this hypothesis, we investigated the relationship of IL-18 gene promoter −137 G/C and −607 C/A polymorphisms and their haplotypes with the risk of ESCC in a Chinese population. *Methods.* Two hundred and thirty five patients with ESCC and 250 age- and sex-matched controls, using sequence specific primers-polymerase chain reaction (PCR-SSP). *Results.* Two polymorphisms, −137 G/C and −607 C/A were in strong linkage disequilibrium (LD). There were significantly differences in the genotype and allele distribution of −137 G/C polymorphism of the IL-18 gene among cases and controls. The −137 GC and CC genotypes were associated with a significantly increased risk of ESCC as compared with the −137 GG genotypes (OR=1.91, 95% CI, 1.29–2.82, p=0.001 and OR=2.95, 95% CI, 1.23–7.04, p=0.012, respectively). Consistent with the results of the genotyping analyses, the −137 C/−607 A haplotype was associated with a significantly increased risk of ESCC as compared with the −137G/−607 C haplotype (OR=1.61; 95% CI, 1.16–2.23; p=0.004). *Conclusion.* This study shows for the first time an association between IL-18 gene promoter −137 G/C polymorphism may contribute represent a genetic risk factor for ESCC in a Chinese population.

Esophageal squamous cell carcinoma (ESCC) is one of the most prevalent cancers in China. Etiological factors include tobacco smoking, alcohol consumption, nutritional deficiencies and other environmental factors [1–3]. In addition to genetic factors such as chromosomal aberrations, single nucleotide polymorphisms (SNP) of matrix metalloproteinase-7, ECRG1, and cyclin D1 also play a role in the development of ESCC [4–6]. Only a few people develop the disease in areas where ESCC is endemic even though everyone is exposed to the same environment, suggesting that genetic differences such as single nucleotide polymorphisms may contribute to ESCC carcinogenesis. However, the molecular basis of ESCC pathogenesis is not yet well defined.

Interleukin-18 (IL-18) is a cytokine of 18.3 kDa that is mainly produced by activated macrophages and, like interleukin-12 (IL-12), is able to induce interferon-gamma (IFN-gamma) and tumor necrosis factor-alpha (TNF-alpha) induction, as well as enhancing the cytotoxicity of NK cells and FasL expression [7–9]. Clinical research showed that there was a correlation between the levels of serum IL-18 and disease severity in patients with esophageal carcinoma [10]. Meanwhile, In some animal model systems, IL-18 gene transfected into tumor cells should enhance both specific and non-specific

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antitumor immune responses, which indicate if IL-18 gene were transferred into dendritic cells, it should induce highly effective antitumor immune responses [11,12]. These findings indicate the evidence of an association between susceptibility to cancer and IL-18 gene.

The human IL-18 (hIL-18) gene is located on chromosome 11q22.2–q22.3, and is composed of six exons and five introns. Several single nucleotide polymorphisms (SNPs) have been identified in the IL-18 gene [13]. Three SNPs were identified in the promoter of the IL-18 gene at positions −137, −607 and −656, relative to the transcriptional start site. The G to C substitution at position −137 abolishes a histone 4 transcription factor-1 (H4TF-1) nuclear factor-binding site and the C to A substitution at position −607 disrupts a cyclic adenosine monophosphate (cAMP)-responsive element protein-binding site. Cloning and gene expression analysis showed that two SNPs of the promoter of IL-18 gene at position −607 and −137 were suggested to cause the differences in transcription factor binding and have an impact on IL-18 gene activity [13]. Recently, genetic polymorphisms of the IL-18 gene have been implicated in the susceptibility to a range of inflammation diseases, including atopic asthma, Behcet's disease, cardiovascular disease, and rheumatoid arthritis [14–17]. Furthermore, their impact on the progression of nasopharyngeal carcinoma [18] and ovarian cancer [19] have been described. No studies, to date, have examined the association between genetic polymorphisms in IL-18 genes and ESCC. In this study, we evaluated whether IL-18 gene promoter −137 G/C and −607 C/A polymorphisms are associated with ESCC in a Chinese population.

## Materials and methods

### *Study population*

The case-control population contained 485 adult unrelated Chinese who were selected from the same population living in China between March 2005 and February 2006 (Table I). The case group consisted of 235 ESCC patients were recruited from Department of Cardiothoracic Surgery, the second affiliated hospital of Zhengzhou University. The only selection criterion for patients was that their ESCC diagnosis had been pathologically confirmed. The patients (184 males; 51 females) had a mean (SD) age of 58.4 (9.7) years. The control group comprised 250 healthy volunteers who visited the general health check-up division at the second affiliated hospital of Zhengzhou University. Selection criteria for controls were no evidence of any personal or family history of cancer or other serious illness. The mean age of the control group (189 males and 61 females) was 57.6 (8.9) years. There was no significant difference between patients and control subjects in terms of gender and age distribution. Written informed consent was obtained from all the subjects, and the study was performed with the approval of the ethics committee of Chinese Human Genome.

Table I. Characteristics of the study population.

| Variable | ESCC patients (n = 235) | Controls (n = 250) | p |
|---|---|---|---|
| Age (mean ± SD) | 58.4 ± 9.7 | 57.6 ± 8.9 | 0.513 |
| Sex | | | |
| Male | 184 (78.3) | 189 (75.6) | 0.481 |
| Female | 51 (21.7) | 61 (24.4) | |
| Cigarette smoking | | | |
| Non-smokers | 59 (25.1) | 76 (30.4) | 0.194 |
| Smokers | 176 (74.9) | 174 (69.6) | |
| Alcohol consumption | | | |
| Non-drinkers | 81 (34.5) | 101 (40.4) | 0.178 |
| Drinkers | 154 (65.5) | 149 (59.6) | |
| Educational levels | | | |
| ≥ College | 32 (13.6) | 63 (25.2) | 0.001 |
| < College | 203 (86.4) | 187 (74.8) | |
| Clinical stages | | | |
| Stages I & II | 85 (36.2) | | |
| Stages III & IV | 150 (63.8) | | |

### *IL-18 gene promoter polymorphisms*

The single nucleotide polymorphisms at position −607(C/A) and −137 (G/C) in the promoter region of the human IL-18 gene, located at chromosome 11q22.2–q22.3, were analyzed by sequence-specific PCR method using genomic DNA isolated from peripheral blood [20]. For the position −607 specific PCR, a common reverse primer (−607 R: 5′-TAACCTCATTCAGGACTTC C-3′) and two sequence-specific forward primers (–607 FC: 5′-GTTGCAGAAAGTGTAAAAATT ATTAC-3′ and −607 FA: 5′-GTTGCAGAAAGTGTAAAAATT-ATTAA-3′) were used to amplify a 196-bp product. A control forward primer (–607 CTRL: 5′-CTTTGCTATCATTCCAGGAA-3′) was used to amplify a 301-bp fragment covering the polymorphic site as an internal positive amplification control. The PCR was performed in a 20 μl volume containing 0.4 μM of one sequence-specific primer and −607 R, 0.13 μM of −607 CTRL, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1 mM $MgCl_2$, 200 μM dNTPs, 1.0 U Amplitaq Taq polymerase and 30 ng genomic DNA. The cycling conditions were 2 min at 94°C, followed by seven cycles of 20 s at 94°C, 40 s at 64°C, and 40 s at 72°C and 25 cycles of 20 s at 94°C, 40 s at 57°C, and 40 s at 72°C.

For the position −137 specific PCR, a common reverse primer (−137 R: 5′-AGGAGGGCAAA ATGCACTGG-3′) and two sequence-specific forward primers (−137 FG: 5′-CCCCAACTTTTAC GGAAGAAAAG-3′ and −137 FC: 5′-CCCCAAC-TTTTACGGAAGAAAAC-3′) were used to amplify a 261-bp product. A control forward primer (−137 CTRL: 5′-CCAATAGGACTGATTAT TCCGCA-3′) was used to amplify a 446-bp fragment covering the polymorphic site as an internal positive amplification control. The PCR was performed in a 20 μl volume containing 0.5 μM of one sequence-specific primer and −137 R, 0.3 μM of −137 CTRL, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1 mM $MgCl_2$, 200 μM dNTPs, 1.0 U Amplitaq Taq polymerase and 30 ng genomic DNA. The cycling conditions were 2 min at 94°C, followed by five cycles of 20 s at 94°C, 60 s at 67°C and 25 cycles of 20 s at 94°C, 20 s at 61°C, and 40 s at 72°C. All PCR products were separated in 2% agarose gels strained with ethidium bromide.

*Statistical analysis*

Genotype and allele frequencies of IL-18 were compared between ESCC cases and controls using the $\chi^2$ test and Fisher's exact test when appropriate, and odds rations (OR) and 95% confidence intervals (CIs) were calculated to assess the relative risk conferred by a particular allele and genotype. Demographic and clinical data between groups were compared by $\chi^2$ test and by Student's t-test. Hardy-Weinberg equilibrium was tested for with a goodness of fit $\chi^2$ test with one degree of freedom to compare the observed genotype frequencies among the subjects with the expected genotype frequencies. The linkage disequilibrium (LD) between the polymorphisms was quantified using the Shi's standardized coefficient D′ (|D′|) [21]. The haplotypes and their frequencies were estimated based on a Bayesian algorithm using the Phase program [22]. Statistical significance was assumed at the $p<0.05$ level. The SPSS statistical software package version 11.5 was used for all of the statistical analysis.

## Results

*Characteristics of the study population*

The demographics of the cases and controls enrolled in this study are shown in Table I. There were no significant differences between the cases and controls for the mean age, gender distribution, smoking and drinking. However, the educational levels of in ESCC patients were significantly lower than that in healthy controls ($p<0.05$).

*The genotype and allele frequencies of IL-18 promoter*

The genotype and allele frequencies of the IL-18 promoter −137 G/C and −607 C/A polymorphisms among the controls and the cases are shown in Table II. The genotype distributions of both polymorphisms among the controls and the cases were in Hardy-Weinberg equilibrium. The frequencies of the GG, GC, and CC genotypes of −137 G/C were 70.4%, 26.4%, and 3.2% in controls, and were 54.0%, 38.7%, and 7.2% in cases, respectively. The frequencies of the CC, CA, and AA genotypes of −607 C/A were 23.6%, 49.6%, and 26.8% in controls, and were 20.4%, 52.3%, and 27.2% in cases, respectively. There were significant differences in the genotype and allele frequencies of the IL-18

Table II. The genotype and allele frequencies of IL-18 promoters of ESCC patients and controls and corresponding OR for ESCC.

| Polymorphisms | ESCC patients n = 235 (%) | Controls n = 250 (%) | OR (95% CI) | p |
|---|---|---|---|---|
| IL-18 -137 G/C | | | | |
| Genotypes | | | | |
| GG | 127 (54.0) | 176 (70.4) | 1.00 (Ref) | |
| GC | 91 (38.7) | 66 (26.4) | 1.91 (1.29–2.82) | 0.001 |
| CC | 17 (7.2) | 8 (3.2) | 2.95 (1.23–7.04) | 0.012 |
| alleles | | | | |
| G | 345 (73.4) | 418 (83.6) | 1.00 (Ref) | |
| C | 125 (26.6) | 82 (16.4) | 1.85 (1.35–2.53) | <0.001 |
| IL-18 -607 C/A | | | | |
| genotypes | | | | |
| CC | 48 (20.4) | 59 (23.6) | 1.00 (Ref) | |
| CA | 123 (52.3) | 124 (49.6) | 1.22 (0.77–1.92) | 0.393 |
| AA | 64 (27.2) | 67 (26.8) | 1.17 (0.70–1.96) | 0.539 |
| alleles | | | | |
| C | 219 (46.6) | 242 (48.4) | 1.00 (Ref) | |
| A | 251 (53.4) | 258 (51.6) | 1.08 (0.84–1.38) | 0.574 |

promoter −137 G/C polymorphism between ESCC and control groups. The −137 GC and CC genotypes were associated with a significantly increased risk of ESCC as compared with the −137 GG genotypes (OR=1.91, 95% CI, 1.29–2.82, p=0.001 and OR=2.95, 95% CI, 1.23–7.04, p=0.012, respectively). The −137 C allele was associated with a significantly increased risk of ESCC as compared with the −137G allele (OR=1.85, 95% CI, 1.35–2.53, p<0.001). However, genotype and allele frequencies of the IL-18 promoter −607 C/A polymorphism in ESCC patients were not significant difference than that in healthy controls (p>0.05).

*Haplotype frequencies of IL-18 promoter*

Haplotype analyses were performed and found only three of the possible four haplotype frequencies are shown in Table III. IL-18 promoter −137 G/C and −607 C/A polymorphisms showed strong linkage disequilibrium (|D′|=0.97). By haplotype analyses, we found −137 C/−607 A haplotype was associated with a significantly increased risk of NPC as compared with the −137G/−607 C haplotype (OR=1.61; 95% CI, 1.16–2.23; p=0.004).

*The genotype and allele frequencies of IL-18 polymorphisms in relation to pathological indices of ESCC severity*

An association between IL-18 promoter polymorphisms and different clinical stage are shown in Table IV. Genotype and allele frequencies of the IL-18 promoter −137 G/C and −607 C/A polymorphisms in Stages I & II were not significant difference than that in Stages III & IV of ESCC patients (p>0.05).

## Discussion

To our knowledge, the present study is the first to examine the −137 G/C and −607 C/A polymorphisms of the IL-18 gene in patients with ESCC in Chinese. In this study, we have shown that the IL-18 −137 G/C polymorphism was significantly associated with the risk of ESCC. The −137 GC and CC genotypes were associated with a significantly increased risk of ESCC as compared with the −137 GG genotypes (OR=1.91, 95% CI, 1.29–2.82, p=0.001 and OR=2.95, 95% CI, 1.23–7.04, p=0.012, respectively). Moreover, and their haplotypes (−137 C/−607 A) were significantly associated with the risk of ESCC (OR=1.61; 95% CI, 1.16–2.23; p=0.004). This finding suggests that the IL-18 −137 G/C polymorphism could be used as genetic susceptibility markers of the ESCC.

In the current study, the frequencies of the −137 C and −607 A alleles among the healthy controls were 0.164 and 0.516, respectively, and these were similar to those frequencies observed in healthy Japanese and Singaporean [20,23], but the frequencies were significant difference than those of European Caucasians (0.288 and 0.424; 0.270 and 0.388, respectively) [24,25]. We also found that the −137 G/C and −607 C/A polymorphisms were in strong linkage disequilibrium (|D′|=0.97). Major haplotype frequencies of the (−137 G/−607 C) among the controls in the present study was 0.488, which was significant lower than those of study performed in the USA (0.560) [26]. However, the haplotype (−137 C/−607 C) was not detected in our Chinese population as well as in the Japanese population [20], and more prevalent in German [27], suggesting that the distribution of IL-18 gene haplotypes might vary among the different ethnic groups.

IL-18 has been initially known as a factor that is primarily involved in the inflammatory immune response and is a potent IFN-γ inducing factor [28]. However, more recently, it has been reported that IL-18 has the capacity to stimulate innate immunity and both Th1 and Th2 mediated responses [28]. IL-18 exerts antitumour action via a number of mechanisms such as enhancement of NK cell activity, induction of apoptosis via Fas/Fas ligand interaction and inhibition of angiogenesis [28]. It has been reported that serum IL-18 levels may be used as a serum marker for monitoring the clinical course of patients with some cancer types, including esophageal [10], breast [29] and gastric carcinoma [30], indicating that IL-18 could play a part in the pathogenesis of cancer. The human IL-18 (hIL-18) gene is located on chromosome 11q22.2–q22.3, and is composed of six exons and five introns. Two

Table III. Haplotype frequencies of IL-18 promoter of ESCC patients and controls and corresponding OR for ESCC.

| IL-18 gene promoter haplotypes | ESCC patients 2n=470 (%) | Controls 2n=500 (%) | OR (95% CI) | p |
|---|---|---|---|---|
| −137 G/−607 C | 212 (45.1) | 244 (48.8) | 1.00 (Ref) | |
| −137 G/−607 A | 131 (27.9) | 165 (33.0) | 0.91 (0.68–1.23) | 0.548 |
| −137 C/−607 A | 127 (27.0) | 91 (18.2) | 1.61 (1.16–2.23) | 0.004 |
| −137 C/−607 C | 0 (0.0) | 0 (0.0) | | |

Table IV. The genotype and allele frequencies of IL-18 polymorphism in relation to pathological indices of ESCC severity.

| Variable | ESCC patients | | | |
|---|---|---|---|---|
| Clinical stages | Stages I & II n=85 (%) | Stages III & IV n=150 (%) | OR (95% CI) | p |
| IL-18 −137 G/C genotypes | | | | |
| GG | 48 (56.5) | 79 (52.7) | 1.00 (Ref) | |
| GC | 32 (37.6) | 59 (39.3) | 1.12 (0.64–1.96) | 0.691 |
| CC | 5 (5.9) | 12 (8.0) | 1.46 (0.48–4.40) | 0.501 |
| alleles | | | | |
| G | 128 (75.3) | 217 (72.3) | 1.00 (Ref) | |
| C | 42 (24.7) | 83 (27.7) | 1.17 (0.76–1.79) | 0.485 |
| IL–18 −607 C/A genotypes | | | | |
| CC | 23 (27.1) | 25 (16.7) | 1.00 (Ref) | |
| CA | 41 (48.2) | 82 (54.7) | 1.84 (0.93–3.63) | 0.077 |
| AA | 21 (24.7) | 43 (28.7) | 1.88 (0.87–4.07) | 0.105 |
| alleles | | | | |
| C | 87 (51.2) | 132 (44.0) | 1.00 (Ref) | |
| A | 83 (48.8) | 168 (56.0) | 1.33 (0.92–1.95) | 0.134 |

common −137 G/C and −607 C/A polymorphisms of the IL-18 promoter have been reported to influence gene activity and expression of IL-18 [13,31]. IL-18 and IFN-γ expression analysis by RT-PCR showed that subjects homozygous for haplotype (−137 G/−607 C) had higher levels of IL-18 mRNA compared to other haplotypes [13]. Thus it is possible that a higher promoter activity of haplotype −137 G/−607 C of the IL-18 gene might increase expression of IL-18, resulting in up-regulation of the IFN-γ producing T-cells. Furthermore, the frequency of haplotype (−137 C/–607 A), which had a lower promoter activity and IFN-gamma mRNA level than the other haplotypes, was significantly higher in the proctitis-type patients than in controls ($p=0.01$) [31]. In the current study, we found haplotype(−137 C/−607 A) was significantly higher in the ESCC patients than in controls too ($p=0.004$). Our results suggest that the haplotype (−137 C/−607 A) of IL-18 gene may play a facilitative role in the development of ESCC.

A few studies have investigated the association between the IL-18 polymorphisms and the risk of different cancer types. Pratesi et al. [18] have reported that −137 G/C and −607 C/A polymorphisms of the IL-18 in nasopharyngeal carcinoma patients were not significant difference than that in healthy controls ($p>0.05$). However, in nasopharyngeal carcinoma patients with C/C or G/C combined IL-18 genotypes of −137 G/C polymorphism showed an increased risk of being with Stages III-IV (OR=2.1, 95% CI: 1.2–6.6). Results suggest that IL-18 genetic variants may represent a genetic risk factor for tumor aggressiveness. Bushley et al. [19] also reported that there was no significant difference in genotype and allele distributions of IL-18 gene −137 G/C polymorphism between ovarian cancer patients and controls; however, the IL-18 G137 C variant may be a marker for ovarian cancer progression or metastasis. In this study, our data suggest that the variant IL-18 genotypes were not associated with progression risk in ESCC. Our data are inconsistent with these two previous studies. The reason for these discrepancies remains unclear, but several possibilities should be considered. First, it may be due to the genetic trait differences, IL-18 gene polymorphisms were distinct in specific populations, various ethnic groups and geographic regions. Furthermore, cancer is a multifactorial disease and individual exposure to various environmental factors, and genetic susceptibility might have caused the different results. In addition, the inadequate study design such as non-random sampling and a limited sample size should be also considered. The possible selection bias that might have been present in the hospital-based, case-control study is a relevant issue. Finally, we cannot exclude that the observed association depends on a gene in linkage disequilibrium with the IL-18 gene or on the effect of IL-18 on another peptide.

In summary, we found that the −137 G/C polymorphism of IL-18 and their haplotype (−137 C/−607 A) were significantly associated with the risk of ESCC. These results suggest that the IL-18 gene may contribute to an inherited predisposition to ESCC although additional studies with larger sample sizes will be necessary to confirm our findings. Because genetic polymorphisms often vary between different ethnic groups, further studies are needed to clarify the association of the IL-18

polymorphism with the risk of ESCC in diverse ethnic populations.

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