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Main Article

Head and neck squamous cell carcinoma is not associated with interleukin-18 promoter gene polymorphisms: a case–control study

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Abstract

Objective: To investigate the association of two functional single nucleotide polymorphisms in the promoter region of the interleukin-18 gene, at positions −607 and −137, with head and neck squamous cell carcinoma.

Design: Genomic deoxyribonucleic acid was extracted, by the salting-out method, from peripheral blood leukocytes. Single nucleotide polymorphisms of the interleukin-18 gene at positions −607 (cytosine/adenine) and −137 (guanine/cytosine) were analysed by sequence-specific polymerase chain reaction.

Subjects: One hundred and eleven patients (86 men and 25 women; mean age 56.7 ± 13.7 years) and 212 regional controls (165 men and 47 women; mean age 53.3 ± 12.2 years) were studied. Control subjects comprised healthy volunteers or cancer-free individuals presenting with otolaryngological disease. The diagnosis of squamous cell carcinoma was confirmed histopathologically. Various clinical parameters were collected at diagnosis, including tumour site, tumour size, lymph node involvement, distant metastasis and stage.

Results: There was no significant association between the allele, genotype or haplotype frequencies of the two single nucleotide polymorphisms of the interleukin-18 promoter and the head and neck squamous cell carcinoma susceptibility or clinical parameters at diagnosis.

Conclusion: Interleukin-18 polymorphisms at positions −607 and −137 did not confer susceptibility to head and neck squamous cell carcinoma in southern Iranian patients.

Key words: Squamous Cell Carcinoma; Interleukin-18; Polymorphism; Head and Neck Neoplasms

Introduction

Head and neck carcinomas comprise a group of malignant tumours originating from the upper aerodigestive tract, including the oral cavity, pharynx and larynx.[1] More than 90 per cent of head and neck carcinomas arise from the epithelial tissue of these regions, and are termed head and neck squamous cell carcinomas (SCCs).[2]

Half a million new cases of head and neck SCC are diagnosed annually worldwide. However, the incidence varies greatly by region, being higher in developing countries.[2] Although tobacco and alcohol consumption are recognised as the most common aetiological factors for head and neck SCC, the disease occurs only in a small number of smokers. Non-users of tobacco and alcohol and young adults also develop head and neck SCC.[1–3] Head and neck SCC patients have variable prognoses, even those at the same clinical stage and receiving similar treatments.[4] These differences in head and neck SCC susceptibility and prognosis may be due to heterogeneity of study populations, specifically regarding genetic polymorphisms.

In recent decades, an increasing number of studies have assessed genetic polymorphism within head and neck SCC, aiming to identify new prognostic markers and therapeutic targets. Several studies have investigated polymorphisms in genes coding for enzymes involved in tumour suppression, growth factor pathways, tobacco-related carcinogen metabolism and the cell cycle, and have found associations between these polymorphisms and head and neck SCC susceptibility and survival.[2,4] Other studies have found an association between head and neck SCC and polymorphisms of pro-inflammatory cytokine genes.[5]

Interleukin (IL)-18 is a novel, pro-inflammatory cytokine which appears to play a key role in innate and acquired immunity. This cytokine stimulates interferon-γ production synergistically with IL-12, promotes differentiation of T cells to a Th1

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phenotype, and enhances the cytotoxic activities of natural killer cells and T cells. Administration of IL-18 results in significant suppression of tumour growth in animal models, suggesting a role for this cytokine in host defence against cancer.[6] However, interleukin-18 has also been found: to stimulate IL-4 production in the absence of IL-12; to inhibit the recognition of cancer cells by immune cells; to increase adherence of cancer cells to microvascular walls; to induce the production of angiogenic and growth factors; and to promote a prometastatic microenvironment.[7]

Interleukin-18 gene expression seems to be regulated by two single nucleotide polymorphisms at positions −607 and −137 in the promoter region of the gene. A change from cytosine (C) to adenine (A) at position −607 disrupts a potential cyclic adenosine monophosphate (cAMP)-responsive element-binding protein binding site. A change at position −137 from guanine (G) to C changes the H4TF-1 nuclear factor binding site to a binding site for an unknown factor found in the granulocyte macrophage colony-stimulating factor promoter.[8] These two single nucleotide polymorphisms have been associated with a variety of inflammatory conditions, such as autoimmune disease,[9] hepatitis C,[10] and several types of cancers, e.g. ovarian,[11] breast (Khalili *et al.* unpublished data) and prostate.[12]

The present study aimed to investigate whether polymorphisms of the IL-18 promoter in the regulatory regions of −607 (C/A) and −137 (G/C) confer a genetic risk for head and neck SCC, and to evaluate the possible correlation of these single nucleotide polymorphisms with clinical characteristics.

Materials and methods

Patients

A total of 111 non-related patients (86 men and 25 women; mean age 56.7 ± 13.7 years) and 212 controls (165 men and 47 women; mean age 53.3 ± 12.2 years) were enrolled in this study. The patients were admitted at Khalili Hospital, Shiraz, Iran. The diagnosis of SCC was confirmed histopathologically. The mean age at the onset of the disease was 55.7 ± 13.3 years, ranging from 19 to 83 years. Information on clinicopathological parameters at diagnosis was collected, including tumour site, tumour size, lymph node involvement, distant metastasis and stage; see Table I.

Control subjects comprised 212 regional volunteers referred to Motahari Clinic, Shiraz, Iran, for routine check-ups, or cancer-free individuals presenting with otolaryngological disease.

All subjects were informed that blood samples would be used for genotyping, and their consent was obtained. The study was approved by the ethics committee of the Shiraz University of Medical Sciences.

Deoxyribonucleic acid preparation

Peripheral blood samples were obtained from patients and control subjects, in 5-ml volumes, and genomic deoxyribonucleic acid (DNA) was extracted from leukocytes by the salting-out method.[13]

TABLE I

CLINICOPATHOLOGICAL CHARACTERISTICS OF 111 PATIENTS WITH HEAD AND NECK SCC

Parameter	*n* (%)
Tumour site	
Oral cavity	46 (41.4)
Pharynx	14 (12.6)
Larynx	51 (46)
Tumour size (cm)	
≤2	16 (14.4)
2–5	87 (78.4)
>5	8 (7.2)
Lymphatic involvement	
Negative	72 (64.9)
Positive	39 (35.1)
Distant metastasis	
Negative	99 (89.2)
Positive	12 (10.8)
Stage	
I	22 (19.8)
II	21 (18.9)
III	39 (35.1)
IV	29 (26.1)

SCC = squamous cell carcinoma

Interleukin-18 gene amplification

Polymorphisms were detected by allele-specific polymerase chain reaction. For each blood sample, two separate reactions were conducted. For the −137 single nucleotide polymorphism, polymerase chain reaction was performed using a common reverse primer, 5′-AGG AGG GCA AAA TGC ACT GG-3′ (where T = thymine) and two sequence-specific forward primers, 5′-CCC CAA CTT TTA CGG AAG AAA AC-3′ and 5′-CCC CAA CTT TTA CGG AAG AAA AAG-3′. A control forward primer, 5′-CCA ATA GGA CTG ATT ATT CCG CA-3′, was used to amplify a 446-bp fragment covering the polymorphic site to serve as an internal positive amplification control. Polymerase chain reaction for the polymorphism at −607 was performed using a common reverse primer, 5′-TAA CCT CAT TCA GCA CTT CC-3′, and two sequence-specific forward primers, 5′-GTT GCA GAA AGT GTA AAA ATT ATT AC-3′ and 5′-GTT GCA GAA AGT GTA AAA ATT ATT AC-3′. A control forward primer, 5′-CTT TGC TAT CAT TCC ACG AA-3′, was used to amplify a 301-bp fragment covering the polymorphic site as an internal positive amplification control.

All polymerase chain reactions were performed in a mixture containing 0.3 μg of genomic DNA, 0.8 pM of common reverse primer, 0.8 pM of sequence-specific forward primer, 0.3 pM of control forward primer (primers from Tib Molbio, Berlin, Germany), 0.3 mM of deoxyribonucleotide triphosphate (dNTP) (Boehringer, Ingelheim, Germany), 0.3 mM of $MgCl_2$, 2 units of Taq DNA polymerase (CinnaGen, Tehran, Iran), 2.5 μl polymerase chain reaction buffer (CinnaGen, Tehran, Iran) and double distilled H_2O added to make up a final

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volume of 25 μl. The cycling conditions for the −137 single nucleotide polymorphism were 2 minutes at 94° C followed by five cycles of 20 seconds at 94° C, 40 seconds at 64° C, 70 seconds at 72° C, and then 25 cycles of 20 seconds at 94° C, 40 seconds at 57° C and 40 seconds at 72° C. The cycling conditions for the −607 single nucleotide polymorphism were 2 minutes at 94° C followed by seven cycles of 20 seconds at 94° C, 30 seconds at 64° C, 80 seconds at 72° C, and then 25 cycles of 20 seconds at 94° C, 40 seconds at 57° C and 50 seconds at 72° C. All polymerase chain reaction products were separated in 2 per cent agarose gels and stained with ethidium bromide. Amplification products of 196 and 261 bp were detected for the −607 and −137 single nucleotide polymorphisms, respectively.

Statistical analysis

All genotype frequencies were tested for the Hardy–Weinberg equilibrium. The fit to the equilibrium was tested by calculating the chi-square test. Haplotype frequencies were calculated by Arlequin population genetic software (http://anthropologie.unige.ch/arlequin). Data were analysed using the Statistical Package for the Social Sciences software (version 11.5.0; SPSS, Chicago, Illinois, USA). Pearson's chi-square test and Fisher's exact probability test were used, when appropriate, to estimate the differences in the distribution of alleles, genotypes and haplotypes in the groups studied. Findings were considered statistically significant at a p value less than 0.05.

Results

Neither patient nor control genotype frequencies significantly differed from those expected according to the Hardy–Weinberg equilibrium.

As shown in Table II, the frequencies of the interleukin (IL)-18 single nucleotide polymorphisms at positions −607 and −137 did not differ significantly, comparing patients and controls. At position −607, the respective frequencies of CC, CA and AA genotypes were 43 (38.7 per cent), 53 (47.7 per cent) and 15 (13.5 per cent) in patients, versus 82 (38.7 per cent), 101 (47.6 per cent) and 29 (36 per cent) in controls. At position −137, the respective frequencies of GG, GC and CC genotypes were 65 (58.6 per cent), 37 (33.3 per cent) and nine (8.1 per cent) in patients, versus 116 (54.7 per cent), 79 (37.3 per cent) and 17 (8 per cent) in controls. The allele distribution of the single nucleotide polymorphisms at positions −607 and −137 showed no significant difference, comparing patients and controls (Table II). The haplotype frequencies also did not differ, comparing patients and controls (Table III).

The frequency of the IL-18 alleles and genotypes was also compared with subjects' clinical parameters at diagnosis, including tumour size, tumour stage, lymph node involvement and metastasis. No statistically significant correlation was observed (data not shown).

TABLE II

GENOTYPE AND ALLELE FREQUENCIES OF IL-18 GENE PROMOTER IN 111 HEAD AND NECK SCC PATIENTS AND 212 CONTROLS

Parameter	Patients (n (%))	Controls (n (%))
Alleles		
Position −607		
– C	139/222 (62.6)	265/424 (62.5)
– A	83/222 (37.4)	159/424 (37.5)
Position −137		
– G	167/222 (75.2)	311/424 (73.3)
– C	55/222 (24.8)	113/424 (26.7)
Genotypes		
Position −607		
– CC	43 (38.7)	82 (38.7)
– AC	53 (47.7)	101 (47.6)
– AA	15 (13.5)	29 (13.7)
Position −137		
– GG	65 (58.6)	116 (54.7)
– GC	37 (33.3)	79 (37.3)
– CC	9 (8.1)	17 (8)

No statistically significant difference was found between patient and control groups for any comparison (chi-square test on 2 × 3 or 2 × 2 tables). IL = interleukin; SCC = squamous cell carcinoma

Discussion

The development of head and neck SCC is a multifactorial process affected by genetic factors and also environmental agents, including tobacco and alcohol consumption, viral infection and chronic inflammation.[1,2] The association of head and neck SCC with inflammatory cytokine genes has been the subject of several studies.[5,14,15] Single nucleotide polymorphisms at positions −607 and −137 of the interleukin (IL)-18 promoter have been reported to cause differences in transcription factor binding and to have an impact on the genetic expression of this pro-inflammatory cytokine. Upon stimulation, higher promoter activity has been observed for C and G alleles in the −607 (C/A) and −137 (G/C) positions, respectively.[8] These two single nucleotide polymorphisms of the IL-18 promoter have been found to affect the susceptibility to and prognosis of several types of malignant tumours, including ovarian[11] and breast (Khalili *et al.* unpublished data).

It is believed that tumourigenesis is largely influenced by the pleotropic, systemic IL-18 cytokine, either in protective or permissive ways.[7] Interleukin-18 was initially identified as a potent inducer of interferon-γ production, a cytokine

TABLE III

HAPLOTYPE FREQUENCIES OF IL-18 GENE PROMOTER IN HEAD AND NECK SCC PATIENTS AND CONTROLS

Haplotype	Patients* (n (%))	Controls† (n (%))	p‡
−607C/−137G	129 (58.1)	250 (59)	NS
−607A/−137C	46 (20.7)	99 (23.3)	NS
−607A/−137G	37 (16.7)	60 (14.2)	NS
−607C/−137C	10 (4.5)	15 (3.5)	NS

*$2n = 222$; †$2n = 424$; ‡For differences in frequency of a given haplotype, comparing patients and controls; chi-square test. IL = interleukin; SCC = squamous cell carcinoma; C = cytosine; G = guanine; A = adenine; NS = not significant

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which in turn strengthens the cellular arm of the immune response. Anticancer effects of IL-18 were demonstrated in animal models treated with this cytokine.[6] Conversely, serum IL-18 levels were reported to be higher in patients with cancer compared with healthy donors.[7] Interleukin-18 levels have also been observed to increase as the pathological stage of cancer progresses, and the serum IL-18 level has been suggested as a non-invasive marker for suspected metastasis in certain types of cancer, e.g. breast cancer.[7,16] Several mechanisms have been suggested by which IL-18 could promote a pro-metastatic environment.[7]

Therefore, we investigated two functional single nucleotide polymorphisms in the promoter region of IL-18 as possible genetic risk factors for head and neck SCC. We found no significant association between polymorphisms of the IL-18 gene at positions –607 and –137 and head and neck SCC susceptibility or clinical parameters at diagnosis.

- **Inflammation plays an extremely complex role in cancer and involves interaction of several inflammatory cytokines**
- **This study aimed to investigate the association between interleukin-18, pro-inflammatory cytokine and head and neck squamous cell carcinoma (SCC)**
- **Interleukin-18 promoter polymorphisms were not observed to confer susceptibility to head and neck SCC in southern Iranian patients**

In agreement with our results, previous investigation of IL-18 polymorphism and oral SCC found no significant association between IL-18 promoter polymorphisms and head and neck SCC in a Greek population.[14] Furthermore, serum IL-10, IL-12 and IL-18 levels have been measured in head and neck SCC patients in a UK population; systemic IL-10 and IL-12 concentrations were found to be significantly altered in patients compared with non-tumour controls, but no such differences in IL-18 levels were observed.[17] Head and neck cancers often drain to the lymph nodes of the neck, and cervical lymphadenopathy is often the first manifestation of disease at the time of diagnosis.[18] Immunoregulatory molecules secreted within the central nervous system and effluxing along with cerebral extracellular fluid into the cervical lymph nodes provide an immunoregulatory microenvironment.[19] It is suggested that these immunoregulatory molecules (such as transforming growth factor-beta) modulate antigen-presenting cells in cervical lymph nodes.[19] Macrophages are an important group of antigen-presenting cells, and are thought to be the major site of IL-18 production.[6] One could argue that, since the major source of IL-18 is modulated by the exceptional microenvironment of the cervical lymph nodes, the effect of this cytokine on head and neck SCC may be too small to detect in a small sample.

Conclusion

This study found that interleukin (IL)-18 promoter polymorphism did not contribute to head and neck SCC in an Iranian population. More data from a larger number of patients are required in order to exclude a possible minor effect of IL-18 gene polymorphism on head and neck SCC susceptibility and prognosis.

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