

# Impact of the Functional VNTR Variants of the Interleukin-1 Receptor Antagonist and Interleukin-4 Genes on Oral Squamous Cell Carcinoma

## Interlökin-1 Reseptörü Antagonisti ve Interlökin-4 Gen Fonksiyonel VNTR Varyantlarının Oral Skuamöz Hücreli Karsinoma Etkisi

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### ABSTRACT

**Introduction:** It has been shown that the host immune response and chronic inflammation could play a role as important risk factors for cancer. Oral squamous cell carcinoma (OSCC) is a common cancer worldwide. In this study, we aimed to evaluate the impact of interleukin-1 receptor antagonist (IL-1RA) and IL-4 variable number tandem repeat (VNTR) polymorphisms on OSCC susceptibility in a Turkish population.

**Methods:** Study subjects comprised of 36 OSCC patients and 100 healthy controls. Genotyping of the IL-1RA VNTR (rs2234663) and IL-4 VNTR (rs79071878) polymorphisms were analyzed by polymerase chain reaction.

**Results:** The frequency of IL-1RA VNTR 1/2+2/2 genotypes increased in the patients than healthy controls while IL-1RA VNTR 1/1 genotype was higher in the control group than in the patients ( $p=0.002$ ). The subjects carrying IL-1RA VNTR 1/2+2/2 genotypes showed a 12.011-fold increased risk of susceptibility to OSCC. IL-1RA VNTR allele 1 was higher in the control group than the patient group while IL-1RA VNTR allele 2 was higher in the patient group than the control group (respectively,  $p=0.000$ ,  $p=0.000$ ). The subjects carrying IL-1RA VNTR allele 2 showed a 2.609-fold increased risk of susceptibility to OSCC. The IL-4 VNTR P1/P1 and P1/P2 genotype frequencies were higher in the patient group compared to the control group ( $p=0.039$ ). IL-4 VNTR P1 allele was higher in the patients compared to the controls ( $p=0.030$ ).

**Conclusion:** The significant association between the functional VNTR polymorphisms of IL-1RA/IL-4 genes and OSCC susceptibility in a Turkish population confirmed a role of altered inflammatory process in OSCC pathogenesis.

**Keywords:** Oral squamous cell carcinoma, IL-1RA, IL-4, VNTR, polymorphism

### ÖZ

**Amaç:** Konakçı immün cevabının ve kronik enflamasyonun kanser için önemli risk faktörleri olarak rol oynadığı gösterilmiştir. Oral skuamöz hücreli kanser (OSHK) tüm dünyada yaygın bir kanser türüdür. Bu çalışmada Türk toplumunda interlökin-1 reseptörü antagonisti (IL-1RA) ve IL-4 değişken sayılı ardışık tekrar (VNTR) polimorfizminin OSHK yatınlığına olan etkisini incelemeyi amaçladık.

**Yöntemler:** Çalışma grubu 36 OSHK hastası ve 100 sağlıklı kontrolden oluşmaktadır. IL-1RA VNTR (rs2234663) ve IL-4 VNTR (rs79071878) polimorfizmlerinin genotipleme polimeraz zincirleme tepkimesi ile analiz edildi.

**Bulgular:** IL-1RA VNTR 1/1 genotipi kontrol grubunda hastalardan daha fazla iken IL-1RA VNTR 1/2+2/2 genotipleri hastalarda sağlıklı kontrollere göre artmıştı ( $p=0,002$ ). IL-1RA VNTR 1/2+2/2 genotiplerini taşıyan kişiler OSHK'ye yatınlık yönünden 12,011 kat fazla risk gösterdiler. IL-1RA VNTR allel 2 hasta grubunda kontrol grubundan daha fazla iken IL-1RA VNTR allel 1 kontrol grubunda hasta grubundan daha fazlaydı (sırasıyla,  $p=0,000$ ,  $p=0,000$ ). IL-1RA VNTR allel 2 taşıyan kişiler OSHK'ye yatınlık yönünden 2,609 kat fazla risk gösterdiler. IL-4 VNTR P1/P1 ve P1/P2 genotip sıklığı hasta grubunda kontrol grubuna göre daha fazlaydı ( $p=0,039$ ). IL-4 VNTR P1 alleli hastalarda kontrollere göre daha yüksekti ( $p=0,030$ ).

**Sonuç:** Türk popülasyonundaki IL-1RA/IL-4 VNTR polimorfizmleri ve OSHK yatınlığı arasındaki önemli ilişki OSHK etiopatogenezinde değişen enflamatuvar işlevlerin rolü olduğunu doğrulamıştır.

**Anahtar Kelimeler:** Oral skuamöz hücreli karsinom, IL-1RA, IL-4, VNTR, polimorfizm



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## Introduction

Oral cancer is among the most frequent cancers in the world. Its pathogenesis remains unclear. Oral squamous cell carcinoma (OSCC) originates from the squamous cells that cover the mouth mucosa and is a common type of oral cancer, responsible for more than 90% of cases (1). Risk factors for OSCC include smoking, alcohol use, and human papilloma virus infections (2), however, molecular mechanisms associated with OSCC are still being under research, and genetic predisposition is gaining interest of the scientists.

In recent years, the role of leucocytes and their relevant cytokines in inflammatory mechanisms and malignant transformation has been well investigated and documented by many studies. The interleukin-1 receptor antagonist (IL-1RA) is a significant innate cytokine that hinders IL-1 activity by binding to the IL-1 receptors without causing any signal transmission (3). The gene encoding IL-1RA (IL-1RA, also called IL-1RN) is found in the chromosome 2q14 and there is an identified variant occurring as a result of variable numbers of an 86-bp tandem repeat (VNTR) (rs2234663) in intron 2 (4). This variant causes the presence of five alleles, each linked with a distinct to a distinct number of repeats. The allele 2 of IL-1RA VNTR variant was found to be related to elevated production of the IL-1 $\beta$  *in vitro* (5).

The human IL-4 is a cytokine synthesized in CD4+Th2 cells, basophils and mast cells, which has a role in the modulation of the humoral immune response (6). IL-4 also interferes with the secretion of the pro-inflammatory cytokines tumor necrosis factor alpha (TNF- $\alpha$ ), IL-6 and IL-1. It can also stimulate the production and secretion of anti-inflammatory factors including IL-1RA (7). The gene encoding IL-4 is found in the chromosome 5q31.1 and there is a 70 bp VNTR (rs79071878) variant in its third intron that might alter the expression of *IL-4* gene; with the P1 allele increasing IL-4 expression compared to the P2 allele (7). Numerous genetic susceptibility research has been done to examine the relations between the IL-1RA and IL-4 VNTR variants and different types of cancers, but results were inconsistent. Considering the importance of IL-1RA and IL-4 cytokines in inflammatory disorders, in this study, our purpose was to evaluate the possible association between IL-1RA/IL-4 VNTR variants and an increased risk of potential OSCC in a Turkish population.

## Methods

### Study population

The study included 36 Turkish patients with histologic diagnosed OSCC, who were recruited at the Department of Medical Oncology, Gaziosmanpasa University, Faculty of Medicine, Turkey. One hundred healthy participants with no medical history of cancer were enrolled in the control group. Patients who had oral precancerous diseases including oral submucous fibrosis, leukoplakia, erythroplakia, and verrucous hyperplasia were excluded from the control group. All subjects had come from the same geographic area. Tumor degree was assessed by a pathologist using the AJCC classification. Informed consent was obtained from all participants before they were enrolled in this study. The study was carried out in compliance with the Declaration of Helsinki and was approved by the Hitit University Faculty of Medicine Regional Ethical Committee (2017/200).

### Genotyping

For all participants, peripheral blood was collected into tubes with EDTA as an anticoagulant. Genomic DNA was extracted from the samples by the DNA extraction kit (Sigma-Aldrich, St. Louis, MI, USA) and was kept at -20 °C until investigation. The VNTR polymorphisms in *IL-1RA* and *IL-4* genes were examined by the polymerase chain reaction (PCR) method described previously, respectively (4,8). The identification of IL-1RA VNTR polymorphism was done by the PCR technique using the forward 5'-CTC AGC AAC ACT CCT AT-3' and reverse 5'-TTC CAC CAC ATG GAA C-3' primers following these conditions: first, an initial denaturalization at 94 °C for 4 minutes; second, 30 cycles at 94 °C, 51 °C for 30 seconds, and 72 °C for 45 seconds at each temperature; and third, final extension at 72 °C for 5 minutes. PCR products were separated by electrophoresis within a 3% agarose gel and visualized by ethidium bromide staining. Five different alleles of IL-1RA were identified as follows: allele 1, four repeats (410 bp); allele 2, two repeats (240 bp); allele 3, five repeats (500 bp); allele 4, three repeats (325 bp), and allele 5, six repeats (595 bp).

For *IL-4*, amplification was done using the forward 5'AGG CTG AAA GGG GGA AAG C-3' and reverse 5'-CTG TTC ACC TCA ACT GCT CC-3' primers, with initial denaturation at 95 °C for 5 minutes, 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 58 °C for 45 seconds, extension at 72 °C for 1 minutes and final extension at 72 °C for 10 minutes. PCR was done in a 25  $\mu$ l reaction mixture containing 50 ng DNA, 0.8  $\mu$ M of each primer, 200  $\mu$ M of each dNTP, 2.5 mM MgCl<sub>2</sub>, 1.5 units Taq polymerase, 2.5  $\mu$ l 10 $\times$ KCl buffer. The resultant PCR products were examined by gel electrophoresis. The PCR products were of 183 bp for the P1 allele and 253 bp for the P2 allele.

### Statistical Analysis

The SPSS software version 20.0 (Chicago, IL, USA) and OpenEpi ver. 3.01 was used for all statistical analyses. Continuous data were presented as mean  $\pm$  standard deviation (SD) and minmax. Chi-square test was used to determine the significance of differences in allele frequency and genotype distributions between the groups. The odds ratio (OR) and 95% confidence intervals (CIs) were calculated. A p value of less than 0.05 was considered statistically significant.

## Results

This study included 36 OSCC patients (22 males and 14 females; mean age  $\pm$  SD, 63.94 $\pm$ 13.96 years) and 100 healthy unrelated subjects as the control group (65 males and 35 females; mean age  $\pm$  SD, 57.31 $\pm$ 11.85 years). The baseline demographic and clinical features of the studied cases were summarized in Table 1.

### IL-1RA genotyping

The allelic and genotype frequencies of the IL-1RA VNTR polymorphism among the patients with OSCC and the healthy control subjects are summarized in Table 2. The significant difference between patients and controls was observed for both genotype and allele frequencies of IL-1RA VNTR polymorphism. In our population, we did not find individuals carrying the following genotypes: 1/4, 1/5, 2/3, 2/4, 2/5, 3/4, 3/5, 4/4, 4/5 and 5/5. The genotypic frequencies of the IL-1RA VNTR polymorphism

**Table 1. Baseline clinical and demographics features of the patients with OSCC**

Characteristics	Controls (n=100)	Patients (n=36)
Gender, male/female, n (%)	65/35 (65.0/35.0)	22/14 (61.1/38.9)
Age, mean ± SD, years	57.31±11.85	63.94±13.96
<b>Smoking</b>	-	-
Yes, n (%)	-	5 (13.9)
No, n (%)	-	26 (72.2)
Ex-smoking, n (%)	-	5 (13.9)
<b>Smoking onset age, Yes/No, n (%)</b>	-	-
<b>Smoking duration</b>	-	-
10-20 years, n (%)	-	2 (5.6)
20-30 years, n (%)	-	5 (13.9)
>30 years, n (%)	-	3 (8.3)
<b>Daily cigarette consumption</b>	-	-
One package, n (%)	-	3 (8.3)
> One package, n (%)	-	6 (16.7)
<b>Alcohol consumption, Yes/No, n (%)</b>	-	6/30 (16.7/83.3)
<b>Frequency of alcohol consumption</b>	-	-
Daily, n (%)	-	4 (11.1)
Social drinker, n (%)	-	2 (5.6)
Family history, Yes/No, n (%)	-	29/7 (80.6/19.4)
Response to treatment, Yes/No, n (%)	-	27/9 (75.0/25.0)
<b>Patients status</b>	-	-
Alive, n (%)	-	29 (80.6)
Exitus, n (%)	-	7 (19.4)
<b>Disease State</b>	-	-
Complete response, n (%)	-	26 (72.2)
Stable disease, n (%)	-	1 (2.8)
Metastatic disease, n (%)	-	9 (25.0)
<b>Disease area</b>	-	-
Intra-oral, n (%)	-	3 (8.3)
Floor of the mouth, n (%)	-	2 (5.5)
Buccal, n (%)	-	1 (2.8)
Roof of the mouth, n (%)	-	2 (5.5)
Tongue, n (%)	-	9 (25)
<b>Lip, n (%)</b>	-	<b>16 (44.4)</b>
Oral mucosa, n (%)	-	1 (2.8)
<b>Tonsil, n (%)</b>	-	<b>1 (2.8)</b>
Cheek mucosa, n (%)	-	1 (2.8)

SD: Standard Deviation, OSCC: oral squamous cell carcinoma

in patients were as follows: 8 individuals (22.22%) had 1/1 genotype, 26 (72.22%) had 1/2+2/2 genotype, and 2 (5.56%) had 1/3+3/3 genotype. In the control group, the genotypic frequencies of the VNTR IL-1RA polymorphism were as follows: 54 individuals (54%) had 1/1 genotype, 40 (40%) had 1/2+2/2 genotype, and 6 (6%) had 1/3+1/3 genotype. The frequencies of IL-1RA 1/2+2/2 genotypes were more common in the patients than healthy controls while IL-1RA VNTR 1/1 genotype was higher in the control group than in the patients ( $p=0.002$ ,  $X^2=12.011$ ).

The allele frequencies of IL-1RA VNTR showed a significant difference between the patient and the control groups. IL-1RA VNTR allele 1 was higher in the control group than the patient group while IL-1RA VNTR allele 2 was higher in the patient group than the control group ( $p=0.000$ , OR: 0.369, 95% CI: 0.204-0.656;  $p=0.000$ , OR: 2.609, 95% CI: 1.503-4.580, respectively). The subjects carrying allele 2 showed a 2.609-fold increased risk of susceptibility to OSCC.

**Table 2. Genotype and allele frequencies of IL-1RA VNTR variant in OSCC patients and controls**

Genotypes				$\chi^2$	p
IL-1RA	1/1 n (%)	1/2+2/2 n (%)	1/3+3/3 n (%)	12.011	0.002
Patients (n=36)	8 (22.22)	26 (72.22)	2 (5.56)		
Controls (n=100)	54 (54.00)	40 (40.00)	6 (6.00)		
Alleles					
	IL-1RA 1 (+/-)	IL-1RA 2 (+/-)	IL-1RA 3 (+/-)	-	
Patients	24/48 (33.33/66.67)	44/28 (61.11/38.89)	2/70 (2.78/97.22)		
Controls	113/87 (56.50/43.50)	75/125 (37.50/62.50)	12/188 (6.00/94.00)		
OR (CI 95%)	<b>0.369 (0.204-0.656)</b>	<b>2.609 (1.503-4.580)</b>	0.448 (0.066-1.833)		
p	<b>0.000</b>	<b>0.000</b>	>0.05		

The results that are statistically significant are typed in bold  
 IL-1RA: OSCC: oral squamous cell carcinoma

**Table 3. Genotype and allele frequencies of IL-4 VNTR variant in OSCC patients and controls**

Gene	OSCC patients, n=36 (%)	Controls, n=100 (%)	$\chi^2$	p	OR (CI 95%)
IL-4 VNTR					
Genotypes					
P1/P1	1 (2.78)	0 (0.00)	6.514	0.039	2.646 (1.13-6.10)
P1/P2	10 (27.78)	14 (14.00)			
P2/P2	25 (69.44)	86 (86.00)			
Alleles					
P1	12 (16.67)	14 (7.00)		0.030	
P2	60 (83.33)	186 (93.00)			

The results that are statistically significant are typed in bold  
 IL-4: OSCC: oral squamous cell carcinoma

#### IL-4 genotyping

The allelic and genotype frequencies of the IL-4 VNTR polymorphism among the patients with OSCC and the healthy controls are shown in Table 3. The frequencies of P1/P1, P1/P2, and P2/P2 genotypes of IL-4 VNTR polymorphism in the control group were 0%, 14%, and 86%, respectively; and 2.78%, 27.78%, and 69.44% in OSCC patients, respectively. There was a significant difference in the distribution of both genotypic and allelic frequencies between OSCC patients and the control group. The IL-4 VNTR P1/P1 and P1/P2 genotype frequencies were higher in the patient group compared to the control group while the P2/P2 genotype frequency was higher in the controls compared to the patients ( $p=0.039$ ,  $\chi^2=6.514$ ) (Table 3). IL-4 VNTR P1 allele was higher in the patients compared to the controls while IL-4 VNTR P2 allele was higher in the control group than the patients ( $p=0.030$ ; OR: 2.646, 95% CI: 1.13-6.10).

#### Discussion

In the present study, we scrutinized whether functional VNTR polymorphisms of the IL-1RA and IL-4 were associated with OSCC in a Turkish population. OSCC is an aggressive epithelial cancer. Despite early diagnosis and several treatments, the overall survival rate of patients with OSCC is still low (9). The immune response to inflammation is commonly involved in the cancer pathogenesis. Genetic changes also take place in the inflammatory response that could be linked with the risk of several

cancers, including the OSCC. Functional DNA polymorphisms affecting gene expression of inflammatory molecules were reported to predispose the individual to disease and worsen the prognosis. Studies suggest that serum levels of inflammatory cytokines (including TNF- $\alpha$  and TNF- $\beta$  (TNF- $\alpha$  and TNF- $\beta$ ), IL-6, IL-8) and anti-inflammatory cytokines (such as IL-10) are likely to be associated with carcinogenesis via multiple and usually controversial pathways, thus they may have a prognostic importance in OSCC and other cancer types (10-12).

IL-1RA is a crucial immunologic modulator and its expression inhibits pro-inflammatory signals from cellular damages during wide immunologic responses (3). In intron 2 of the *IL-1RA* gene, a variant due to the presence of variable numbers of an 86-bp VNTR was described. Although this IL-1RA VNTR-polymorphism is found in intron 2, it is probably responsible for a change in IL-1RA synthesis (13). The number of repeats, present in intron 2 of the *IL-1RA* gene, may manifest a functional role as it is thought that each repeat has considerable number of binding sites for transcription factors. Thus, a higher amount number of repeats would result in a higher transcriptional activity. IL-1RA alleles 1-5 contain 4 repeats, 2 repeats, 5 repeats, 3 repeats, and 6 repeats, respectively. The role of IL-1RA VNTR polymorphism has been investigated for years in the development of inflammatory diseases. Actually, some studies reported different relations of IL-1RA VNTR alleles with the occurrence of some cancers, including gastric (14),

esophageal (15), bladder (16), breast (17), colorectal (18), lung (19), brain (20), and nasopharyngeal (21). In a meta-analysis evaluating the relationship between IL-1RA VNTR variant and cancer, Zhang et al. (22) suggested that the IL-1RA VNTR variant might play a role in the genetic susceptibility to gastric cancer. Shiiba et al. (23) reported that expression of IL-1RA mRNA was significantly downregulated in OSCCs compared to normal tissues. Also, they demonstrated that IL-1RA expression level was lower in the oral premalignant lesion cases with severe dysplasia compared to those with mild/moderate dysplasia. Gupta et al. (24) reported that the percentage of IL-1RA 1/2 individuals was higher in patients with OSCC than controls. Additionally, in carriage rate analysis, they reported that 2 allele of IL-1RA was to be significantly associated with OSCC. In the present study, statistical analysis of the IL-1RA allele and genotype frequencies in the OSCC patient group affirms the significance of the IL-1RA VNTR variant in OSCC in comparison to the control population. Despite the small sample size in our study, we found a significant association with OSCC and IL-1RA VNTR variant. IL-1RA VNTR 1/2+2/2 genotypes were more common in patients than healthy controls. The subjects carrying IL-1RA VNTR 1/2+2/2 genotypes showed a 12.011-fold increased risk of susceptibility to OSCC. (Table 2). IL-1RA VNTR allele 1, associated with high IL-1RA levels, was lower in patients. Conversely, IL-1RA allele 2, leading to an inadequate synthesis of IL-1RA protein or to an overproduction of IL-1 $\beta$  in response, was higher in the patient group ( $p=0.00$ ). The subjects carrying allele 2 showed a 2.609-fold increased risk of susceptibility to OSCC. This condition could explain why carriage of IL-1RA allele 2 could influence an individual's susceptibility to OSCC. Our results were consistent with those reported by Gupta et al. (24).

IL-4 is a fundamental differentiation cytokine that stimulates development of Th2 subset of lymphocytes. This group of lymphocytes activates granulocytes and eosinophils and inhibits angiogenesis, making it crucial in surveillance and clearance of tumor cells (25). IL-4 is a strong modulator of anti-tumor immune responses with both tumor-promoting and tumor-inhibiting features because it acts as both immunosuppressive and anti-angiogenic factor (26). More and more epidemiologic studies have been performed to examine the impact of some IL-4 variants on human cancer risk. The IL-4 VNTR variant could change messenger ribonucleic acid splicing, which leads to different splice variants (27). There is strong evidence that the IL-4 intron 3 VNTR polymorphism might affect the synthesis of IL-4, with the P1 (two 70-bp repeats) allele enhancing IL-4 expression compared to P2 (three 70-bp repeats) allele (8). In a study, Duan et al. (25) evaluated eight eligible case-control studies including 1583 cases and 1638 controls with respect to the relationship of IL-4 VNTR variant and cancer. They indicated that the IL-4 VNTR RP2 allele was related to a reduced cancer risk compared to the RP1 allele. In subgroup analyses, they stratified by ethnicity and they found that there was evidence in the Asian population for an association between this variant and cancer risk. In a meta-analysis performed on 1896 patients and 2526 controls for IL-4 VNTR variant, Jia et al. (28) reported that IL-4 VNTR variant was associated with a higher risk of bladder cancer risk. Also, they showed that IL-4-33CT (rs2070874) variant was correlated with leukemia and oral carcinoma. Yang et al. (29) demonstrated that IL-4 VNTR P1/P1 genotype was associated with an increased risk of OSCC, particularly early-stage OSCC.

In the present study, IL-4 VNTR P1/P1 genotype and P1 allele, a higher IL-4 production allele and genotype, were more common in the patient group ( $p=0.039$ ,  $p=0.030$ , respectively). The subjects carrying IL-4 VNTR P1/P1 genotype showed a 6.514-fold increased risk of susceptibility to OSCC (Table 3). Our findings are compatible with the potentially growth-promoting effect of IL-4 on OSCC.

## Conclusion

The results of this study revealed an association of both IL-1RA and IL-4 VNTR polymorphisms with the occurrence of OSCC. To the best of our knowledge, this is the first research study to evaluate an association between IL-1RA and IL-4 VNTR polymorphisms and the risk of OSCC in a Turkish population. In conclusion, within the limitations of ethnicity, sample size and sample selection, the IL-1RA and IL-4 VNTR polymorphisms seem to be associated with developing OSCC.

**Ethics Committee Approval:** The study was carried out in compliance with the Declaration of Helsinki and was approved by the Hitit University Faculty of Medicine Regional Ethical Committee (decision no: 2017/200)

**Informed Consent:** Informed consent was obtained from all participants before they were enrolled in this study.

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