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Genetic Variations of miRNAs and the Risk of Oral Squamous Cell Carcinoma: A Case-control Study

Oral Skuamoz Hücre Kanseri Riski ve miRNA'ların Genetik Varyasyonları: Olgu-kontrol Çalışması

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Abstract

Aim: In this study, we investigated the association between two miRNA variants and the risk of oral squamous cell carcinoma (OSCC), and explored the interaction between clinical factors in the Turkish population.

Methods: In this case control study, a total of 142 subjects were genotyped by polymerase chain reaction-restriction fragment length polymorphism to analyze *miR-146aG/C* (rs2910164) and *miR-149C/T* (rs2292832) variants. Associations between OSCC risk and clinicopathological characteristics were analyzed by chi-square test

Results: There was a significant difference in genotype and allele frequencies of *miR-146aG/C* variant between patients and control individuals. *miR-146aG/C CC* genotype and C allele were higher in the patient group compared to the control group (p=0.000, p=0.0001, respectively). Significant differences were also observed when the patients and the controls were compared according to CC vs GG+GC (p=0.0002) and GG vs GC+CC (p=0.002). In combined analysis, CC-CT combined genotype increased in patient group compared to controls (p=0.002), while GC-CT combined genotype increased in controls compared to patients (p=0.028),

Conclusion: Our study provides evidence that *miR-146aG/C* variant may play an important role in susceptibility to OSCC in the Turkish population.

Keywords: Oral squamous cell carcinoma, microRNA, variant

Öz

Amaç: Bu çalışmada, iki miRNA varyantı ve oral skuamoz hücreli kanser (OSHK) riski arasındaki ilişkiyi inceledik ve Türk popülasyonundaki klinik faktörlerle arasındaki etkileşimi araştırdık.

Yöntemler: Bu olgu-kontrol çalışmasında, *miR-146aG/C* (rs2910164) ve *miR-149C/T* (rs2292832) varyantlarını analiz etmek için toplam 142 kişi polimeraz zincir reaksiyonu- sınırlayıcı enzim parça uzunluk polimorfizmi ile genotiplendi. OSHK ve klinikopatolojik özelliklerin ilişkisi χ^2 testi ile analiz edildi.

Bulgular: Hastalar ve kontrol bireyleri arasında *miR-146aG/C* varyant genotip ve alel sıklıklarında önemli fark vardı. *miR-146aG/C CC* genotipi ve C aleli hasta grubunda kontrol grubuna göre daha yüksekti (p=0,000, p=0,0001, sırasıyla). Hastalar ve kontroller kıyaslandığında CC'ye göre GG+GC (p=0,0002) ve GG'ye göre GC+CC (p=0,002) önemli farklar saptandı. Kombine analizde, GC/CT kombine genotipi kontrollerde hastalara göre artmışken (p=0,0028), CC-CT kombine genotipi hasta grubunda kontrollere göre artmıştı (p=0,002).

Sonuç: Çalışmamız *miR-146aG/C* varyantının Türk popülasyonunda OSHK'ye yatkınlıkta önemli rol oynayabileceği kanıtlarını göstermektedir.

Anahtar Sözcükler: Oral skuamoz hücreli kanser, mikroRNA, varyant

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Introduction

Oral squamous cell carcinoma (OSCC) is the most prevalent oral malignancy, accounting for up to 80-90% of all malignant neoplasms of the oral cavity (1). Tobacco use (chewing with or without smoking), alcohol consumption, and human papilloma virus (HPV) infection are among the major risk factors for OSCC, yet, molecular mechanisms involved in OSCC are still unclear, whilst genetic tendency has been studied extensively (2). Recognition of biomarkers for screening high-risk status for increased tendency to cancer is crucial for prevention of this malignancy.

MicroRNAs (miRNAs) are small (ranging often between 18 to 25 nucleotide in size), single stranded, and noncoding RNAs. Particularly, miRNAs can modulate gene expression at the post-transcriptional level by repressing translation of protein coding genes, or cleaving target mRNAs, based on the level of complementarity between the miRNA and its target mRNA (3). These small miRNAs are involved in a numerous physiologic and pathological events including cell cycle, differentiation, growth, and metabolism along with myriad diseases like cancer (3). Studies have reported that microRNAs may be critical elements in oncogenesis, acting as tumor suppressors and/or oncogenes, and influence the etiology of several cancer types (4). Single nucleotide polymorphisms (SNPs) found in miRNA encoding gene (miR-SNPs) have attracted attention due to their potent involvement in cancer. It is already known that miR-SNPs can affect the transcription of the target gene, modify the processing of pri-miRNA or pre-miRNA and have impact on interaction between miRNA and mRNA (5). Studies have shown that miR-146aG/C (rs2910164), and *miR-149C/T* (rs2292832) SNPs play a major role in carcinogenesis owing to their targeting on various vital genes and are involved in diverse types of cancers (6).

Therefore, we performed a study to investigate the association between two common miRNA polymorphisms and the risk of OSCC in a Turkish population.

Methods

Study Population

A cohort of 142 individuals, including 42 patients with OSCC and 100 healthy controls was enrolled in this case control study. Patients were treated at the Department of Medical Oncology, Training and Research Hospital, Gaziosmanpaşa University, Tokat, Turkey. The diagnosis of OSCC was based on pathological features of tissue specimens. Clinical information, including age, gender, smoking, alcohol consumption and tumor-node-metastasis stages, were obtained from patient's medical charts. 100 age- and sex-matched healthy individuals, who did not have any evidence of OSCC or any other overt oral diseases, constituted the control group. Informed written consent was obtained from all subjects. This study protocol was approved by the Local Ethics Committee (Gaziosmanpasa University, Medical Faculty, decision no: 17-KAEK-061), in accordance with the ethical standards for human experimentation established by the Declaration of Helsinki.

Genotyping

2 mL of venous blood was obtained from each participant (OSCC patients and healthy controls) and DNA was extracted from all the samples using a commercial kit (Sigma-Aldrich, Taufkirchen, Germany) according to the manufacturer's instructions. Using genomic DNA, miRNAs variants were identified by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) methods as previously described (7,8).

Statistical Analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (IBM SPSS Statistics, version 20) and OpenEpi software package version 3.01 (www.openepi.com). The sample size was assessed using the Power and Sample size Calculation software. The relationships between variants and the clinical characteristics of patients were analyzed by using the chi-square test or analysis of variance (ANOVA) statistics. Chi-square test and Fisher's exact test were used to compare categorical variables appropriately. Odds ratio (OR) and 95% confidence interval (CI) were used for the assessment of risk factors. All p values were 2-tailed and p values less than 0.05 were considered statistically significant.

Results

A total of 42 OSCC patients and 100 age and gender matched healthy controls were genotyped for *miR 146aG/C* and *miR-149C/T* variants. Demographic characteristics of the study participants are shown in Table 1.The distribution of *miR 146aG/C* and *miR-149C/T* variants genotypes and allele frequencies in the study and control groups are shown in Table 2.

miR 146aG/C

The frequency of GG, GC, and CC genotype was 31.0% vs 45.2%, 23.8% vs 59%, and 40% vs 1% in OSCC patients and control individuals, respectively. There was a statistically significant difference in genotype and allele frequencies of *miR 146aG/C* variant between the patients and the control subjects. *miR-146G/C CC* genotype and C allele were found to be increased in the patient group compared to the control group (p=0.000 and p=0.0001, respectively). Statistically significant differences were also observed when the patients and the controls were compared according to CC vs GG+GC (p=0.0002, OR:

30.9, CI: 95%: 3.81-251.1) and GG vs GC+CC (p=0.002, OR: 3.21, CI: 95%: 1.49-6.90).

miR 149C/T

The frequency of the CC, CT, and TT genotypes of the *miR-149C/T* variant in the patients was 28.6%, 59.5%, and 11.9% respectively; and in the controls, the frequency was 40%, 51%, and 9%, respectively. The genotype and allele distribution of *miR-149C/T* variant did not show any statistically significant difference between the patients and the controls (p=0.425 and p=0.253, respectively).

Furthermore, we also analyzed whether any differences existed in the clinical and demographic characteristics of patients according to distributions of *miR-146aG/C* and *miR-149C/T* genotypes (Table 3). There was no significant difference between the variants and the clinical characteristics (p>0.05). The associations between *miR-146aG/C/miR-149C/T* and the combined genotypes with OSCC susceptibility were further evaluated by stratification analysis for OSCC patients (Table 4). In combined analysis, CC-CT genotype increased in patient group compared to controls (p=0.002), while GC/CT combined genotype increased in controls compared to patients (p=0.028),

Table 1. The demographical characteristics of the study subjects

		Patient group (n=42) (%)	Control group (n=100) (%)
Age		63.05±13.250	60.37±8.666
Gender	Female	15 (35.7)	34 (34)
	Male	27 (64.3)	66 (63.3)

Discussion

OSCC is an important cause of morbidity and mortality all over the world. Although enormous advances occurred in diagnostic and therapeutic methods, the prognosis of OSCC is still poor, with a 5-year survival rate of nearly 50% (9). There is considerable evidence supporting that miRNA deregulation plays a fundamental role in carcinogenesis. Distorted expression profiles of miRNAs in several cancer types lead to different malignant phenotypes for tumor progression, and this in turn may promote chemo-resistance and eventually result in a poor prognosis (10). Hence, miRNAs have been studied in depth to determine new diagnostic and prognostic cancer biomarkers, and to help development of efficient therapeutic targets. MiRNAs are involved in the pathogenesis of oral cancer and are believed to be potential biomarkers in cancer diagnosis, owing to their aberrant expression and single nucleotide polymorphisms (11). Myriad studies have been performed to establish if miRNAs have an impact on susceptibility to OSCC, and the results have been disputable and discrepant.

Table 2. Genotype and allele frequencies of *miR-146G/C* and *miR-149C/T* variants in groups

miR-146aG/C				
	Patient group	**Control group**	**p**	**OR, % 95 CI**
Genotypes	**n=42 (%)**	**n=100 (%)**		
GG	13 (31.0)	59 (59.0)	**0.000**	-
GC	19 (45.2)	40 (40)		
CC	10 (23.8)	1 (1)		
GG+GC:CC	32 (76.19):10 (23.81)	99 (99):1 (1)	**0.002**	30.9 (3.81-251.1)
GG:GC+CC	13 (30.95):29 (69.05)	59 (59):41 (41)	**0.002**	3.21 (1.49-6.90)
Alleles				
G	45 (53.57)	158 (79)	**0.0001**	3.26 (1.88-5.64)
C	39 (46.43)	42 (21)		
miR-149C/T				
	Patient group	**Control group**	**p**	**OR, % 95 CI**
Genotypes	**n=42 (%)**	**n=100 (%)**		
CC	12 (28.6)	40 (40)	0.425	-
CT	25 (59.5)	51 (51)		
TT	5 (11.9)	9 (9)		
CC+CT:TT	37 (88.09) :5 (11.91)	91 (91):9 (9)	0.596	1.36 (0.42-4.35)
CC:CT+TT	12 (28.57):30 (71.43)	40 (40):60 (60)	0.197	1.66 (0.76-3.63)
Alleles				
C	49 (58.33)	131(65.5)	0.253	1.35(0.80-2.28)
T	35(41.66)	69 (34.5)		

OR: Odds ratio; CI: Confidence interval, n: Number
p values were obtained using chi-square tests and Fisher's exact tests. The results that are statistically significant are typed in bold.

Table 3. Clinical features of patients according to *miR-146aG/C* and *miR-149C/T* genotypes								
Clinical features	***miR-146aG/C***				***miR-149C/T***			
Genotypes	**GG**	**GC**	**CC**	**p**	**CC**	**CT**	**TT**	**p**
	n (%)	**n (%)**	**n (%)**		**n (%)**	**n (%)**	**n (%)**	
Alcohol								
Yes	2 (25.0)	5 (62.5)	1 (12.5)	0.523	4 (50.0)	4 (50.0)	0 (0)	0.233
No	11 (32.4)	14 (41.2)	9 (26.5)		8 (23.5)	21 (61.8)	5 (14.7)	
Smoking								
Yes	1 (20)	3 (60.0)	1 (20.0)	0.861	3 (60.0)	2 (40.0)	0(0)	0.342
No	10 (33.3)	12 (40.0)	8 (26.7)		7 (23.3)	18 (60.0)	5 (16.7)	
Family history								
Yes	2 (28.6)	4 (57.1)	1 (14.3)	0.741	3 (42.9)	4 (57.1)	0 (0)	0.448
No	11 (31.4)	15 (42.9)	9 (25.7)		9 (25.7)	21 (60.0)	5 (14.3)	
Treatment response								
Yes	9 (31.0)	14 (48.3)	6 (20.7)	0.606	8 (27.6)	18 (62.1)	3 (10.3)	0.746
No	4 (33.3)	4 (33.3)	4 (33.3)		4 (33.3)	6 (50)	2 (16.7)	
Living status								
Living	11 (31.4)	14 (40.0)	10 (28.6)	0.606	10 (28.6)	22 (62.9)	3 (8.6)	0.309
Exitus	2 (28.6)	5 (71.4)	0 (0)		2 (28.6)	3 (42.9)	2 (28.6)	
Job								
Farmer	4 (36.4)	6 (54.5)	1 (9.1)	0.316	2 (18.2)	7 (63.6)	2 (18.2)	0.641
Housewife	2 (16.7)	4 (33.3)	6 (50)		2 (16.7)	9 (75.0)	1 (8.3)	
Employee	5 (33.3)	7 (46.7)	3 (20.0)		6 (40.0)	7 (46.7)	2 (13.3)	
Officer	2 (66.7)	1(33.3)	0 (0)		1 (33.3)	2 (66.7)	0 (0)	
Unemployed	0 (0)	1 (100)	0 (0)		1 (100)	0 (0)	0 (0)	

Table 4. Comparative analysis of combined genotypes of gene variants between groups			
miR-146aG/C-miR149C/T	**Patient group**	**Control group**	**p**
	n (%)	**n (%)**	
GG-CC	5 (11.9)	19 (19.0)	0.305
GC-CC	5 (11.9)	21 (21.0)	0.201
CC-CC	2 (4.8)	0 (0)	0.172
GG-CT	7 (16.7)	35 (35.0)	**0.028**
GC-CT	11 (26.2)	16 (16.0)	0.158
CC-CT	7 (16.7)	0 (0)	**0.002**
GG-TT	1 (2.4)	5 (5.0)	0.851
GC-TT	3 (7.1)	3 (3.0)	0.488
CC-TT	1 (2.4)	1 (1.0)	0.999
n: Number The results that are statistically significant are typed in bold			

Human *miR-146a* is located in the *LOC285628* gene on human chromosome 5 (12). miR-146a is induced by the toll-like receptor 4 found in the NF-κB-dependent signaling pathway, resulting in the downregulation of IL-1 receptor-associated kinase 1 and TNF receptor-associated factor 6 (13). The *miR-146a* variant bears a G/C nucleotide substitution which cause a modification from G:U pair to C:U mismatch in the stem structure of miR-146a precursor. This variant in miR-146a changed the mature miR-146a expression level that played a role in the regulation of cell differentiation and cancer development (14). Wang et al. (15) conducted a meta-analysis and reported that *miR-146aG/C* variant enhanced the risk of cancer in dominant model when all studies were considered in the meta-analysis. Stratified analysis showed a significant link between this variant and cancer susceptibility in Asians but not in Caucasian populations. Numerous studies demonstrate the crucial association between *miR-146aG/C* variant and risk of cancer, such as breast cancer (16), lung cancer (17), bladder cancer (18), and hepatacellular carcinoma (19). Miao et al. (20) and Liu et al. (21) showed that miR-146aG/C did not affect the risk of head and neck cancers. Also, Zhang et al. (22) and Palmieri et al. (23) reported that there was no significant association between *miR-146aG/C* variant and OSCC in Chinese and Italian populations. Another study showed that these variants may modify the risk of HPV16-associated OSCC, particularly in never smokers (24). Hung et al. (25) found that *miR-146aG/C* variant was associated with advanced nodal metastasis of OSCC and higher miR-146 expression in tumors. Chen et al. (26) reported that *miR-146aG/C* variant G/G genotype was associated with decreased risk of OSCC ($p<0.05$). A meta-analysis of 66 case-control studies found that *miR-146aG/C* was

a risk factor for head and neck squamosus cell carcinoma (HNSCC). This meta-analysis included four studies from a Caucasian population and one study from a Chinese population (27). Also, in stratified analysis, the authors found that the *miR-146aG/C* C allele or the CC genotype was a risk factor for HNSCC. Niu et al. (28) observed that *miR-146aG/C* increased significantly head and neck cancer risk in Caucasian population. The reasons for these conflicting results may be different tumour sites, small sample sizes, and lack of information such as other risk factors or confounders. Our study revealed an association between *miR-146aG/C* variant and the risk of OSCC in our population. *miR-146aG/C* CC genotype and C allele were associated with increased risk of OSCC (p=0.000 and p=0.0001, respectively) (Table 2). Furthermore, there was a statistically significant difference when the patients and the controls were compared according to CC vs GG+GC and GG vs GC+CC (p=0.0002 and p=0.002, respectively). These results are compatible with the results of the study by Niu et al. (28)

The single nucleotide polymorphism of *miR-149* gene was first identified by Hu et al. (29) and colleagues. A C/T genetic variant was reported in the *miR-149* gene and was located in the stem region adjacent to the mature *miR-149C/T* sequence. *miR-149C/T* is a proapoptotic miRNA and it represses the expression of Akt1 and E2F1. Silencing of Akt1 and E2F1 can generate apoptosis in human tumour cell lines (30). Recent studies reported that *miR-149C/T* can alter the expression of mature miRNAs or the binding activities to target mRNA, and therefore affect cancer risk via several pathways (30). To date, numerous genetic association studies have been conducted to examine the relationship between the *miR-149C/T* and the risk of several cancers, including colorectal cancer (31), and hepatocellular carcinoma (32). Wei et al. (33) found that the CC genotype of *miR-149C/T* was significantly associated with an increased risk of papillary thyroid cancer compared with TT homozygote. But, Li et al. (34) reported that *miR-149C/T* variant could decrease digestive cancer susceptibility in a meta-analysis study. Tu et al. (35) showed that miR-149 expression was downregulated in HNSCC compared to normal mucosa and this was associated with a poorer patient survival. Additionally, HNSCC patients with the *miR-149C/T T/T* genotype have more advanced tumours and a worse prognosis. In addition, it was found that the CT and TT genotypes of *miR-149C/T* variant were significantly associated with OSCC (30). In the present study, there was no evidence of association between *miR-149C/T* polymorphism and the risk of OSCC.

The association between miRNAs genotypes and clinicopathological parameters of OSCC was also analysed in our study. Genotype distributions of *miR-146aG/C* and *miR-149C/T* were not related to clinical features (p>0.05). But there was a statistically significant difference in the combined analysis. We found that GG/CT combined genotype was associated with decreased risk of OSCC (p=0.028) while CC-CT combined genotype was associated with increased risk of OSCC (p=0.002) (Table 4).

Study Limitations

In this study, several limitations need to be addressed. First, a relatively small sample size may limit the statistical power of our study, especially in the stratification analysis. Second, we selected only OSCC patients as our study population and excluded other head and neck cancer patients in order to control some confounding bias.

Conclusion

In this case-control study, we examined associations between two common variants in miRNAs (*miR-146aG/C* and *miR-149C/T*) and OSCC risk. To the best of our knowledge, this is the first study that correlates the genotype and allele frequencies of these variants with OSCC, in a Turkish population. Our results demonstrate that *miR-146aG/C* gene may play an important role in the development of OSCC in a Turkish population. But it is necessary to conduct further well-designed studies based on larger sample size and homogeneous cancer patients to validate our findings.

Authorship Contributions

Concept: S.Y., Ö.G. Design: S.Y., K.Y. Data Collection or Processing: Ö.G., K.Y. Analysis or Interpretation: S.Y., K.Y. Literature Search: A.F.N., N.K. Writing: A.F.N., N.K.

Conflict of Interest: This study was conducted as a master's thesis. The authors confirm that this article's content has no conflicts of interest.

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