

Association of lncRNA ANRIL rs10757278 A>G Variant, Tumor Size, Grading, Tumor Site, and Tumor Stage in Oral Squamous Cell Carcinoma Patients

Maryam Salah¹, Mostafa Rezaee², Mohamad Javad Fattahi³, Abbas Ghaderi³, Bijan Khademi⁴, Mohammad Javad Mokhtari*⁵

Abstract

Background: Oral Squamous Cell Carcinoma (OSCC) is a pressing global health challenge. Long non-coding RNAs (lncRNAs) have emerged as pivotal regulators. Among these, the lncRNA ANRIL (antisense non-coding RNA in the INK4 locus) has a role in cancer progression. The aim of this study was to look into possible links between a certain genetic variant of lncRNA ANRIL, rs10757278 A/G, and OSCC risk and tumor features in the Iranian population.

Methods: We conducted a case-control study, enrolling 101 OSCC patients and 115 healthy controls. We took out the genomic DNA and used the tetra-primer ARMS-PCR (tetra-primer amplification refractory mutation system-polymerase chain reaction) method to find the rs10757278 genotype. We evaluated the associations between genotypes and both OSCC susceptibility and various tumor characteristics.

Results: Although we did not observe significant differences in allele and genotype frequencies between cases and controls, we revealed compelling associations between genotypes and tumor characteristics. Genotypes AG and GG were linked to smaller tumor sizes, while genotypes with at least one wild-type allele (A) were linked to well differentiated OSCC. Specific genotypes exhibited significant associations with tumor sites, with the tongue demonstrating the strongest correlation.

Conclusions: The rs10757278 A/G variant did not show a direct link with OSCC risk, but its complex effect on tumor behavior suggests that it may play a bigger role in the development of OSCC. These findings open avenues for future investigations to uncover hidden genetic interactions, and potentially inform more targeted therapeutic strategies.

Keywords: Carcinoma, Squamous Cell, Long non-coding RNA, Polymorphism.

Introduction

Head and neck squamous cell carcinomas (HNSCC) pose a formidable global health challenge, ranking as the sixth most prevalent cancer worldwide (1, 2). While modifiable behaviors like tobacco and alcohol consumption, along with environmental

exposures, contribute to HNSCC, emerging evidence underscores the role of genetic polymorphisms in genes governing carcinogen metabolism and immune responses in shaping susceptibility to HNSCC (3-8).

Recent advances in genomic research have

1: Department of Orthodontics, School of Dentistry, Shiraz University of Medical Sciences, Shiraz, Iran.

2: Department of Oral Medicine, School of Dentistry, Shiraz University of Medical Sciences, Shiraz, Iran.

3: Shiraz Institute for Cancer Research, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran.

4: Department of Otolaryngology, Shiraz University of Medical Sciences, Shiraz, Iran.

5: Department of Biology, Zarghan Branch, Islamic Azad University, Zarghan, Iran.

*Corresponding author: Mohammad Javad Mokhtari; Tel: +98 71 32626911; E-mail: mj.mokhtari@iau.ac.ir.

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illuminated the genetic underpinnings of HNSCC. Intriguingly, non-coding RNAs are emerging as crucial players in the symphony of cancer biology (9). Among them, long non-coding RNAs (lncRNAs) stand out for their intricate regulatory roles. These molecules, which can't code for proteins, control epigenetics, the cell cycle, differentiation, and the stress response in a very complex way (10). Within this intricate landscape, one particular non-coding RNA, ANRIL (antisense non-coding RNA in the INK4 locus), beckons further exploration. ANRIL's complex role in the TGF- β /Smad signaling pathway has been linked to controlling cell growth and death in oral squamous cell carcinoma (11, 12).

Our study starts to look into the connections between some ANRIL genetic variants, mainly the rs10757278 single nucleotide polymorphism (SNP), and how OSCC started in the Iranian population. Our thorough allelic and genotypic analysis aims to reveal the intricate web of connections between tumor features and genetic makeup, which has the potential to change how OSCC is diagnosed and treated.

Materials and Methods

Study Design and Participant Selection

This case-control study was approved by the local Ethics Committee of Shiraz University of Medical Science with the code number IR.SUMS.DENTAL.REC.1400.143. The study focused on patients primarily diagnosed with oral squamous cell carcinoma (OSCC) who were admitted to the oral and maxillofacial surgery departments of Rajaie Hospital and the otorhinolaryngology department of Khalili Hospital. Inclusion criteria encompassed patients with confirmed OSCC diagnosis through clinical and histopathological examinations. Exclusion criteria included a history of prior cancer therapy, the presence of other malignancies, remote metastasis, genetic disorders, autoimmune diseases, or pregnancy. To establish a baseline for comparison, a group of healthy control individuals was meticulously selected and matched for age and gender with

the OSCC patients. For all patients, we filled out a questionnaire including demographic information and information related to the tumor, including tumor size, tumor stage, tumor grade, tumor site, and lymph node metastasis. Written informed consent was obtained from all participants prior to their enrollment in the study.

SNP Genotyping

DNA Extraction and Assessment

In this study, the DNA extraction kit (Favorgen, Taiwan) was used. Following extraction, the quality of the obtained DNA was meticulously evaluated using a specialized instrument called the spectrophotometer (Eppendorf Biophotometer, Germany). This device measured the concentration of DNA at a specific wavelength, which is 260 nm, providing an indication of the amount of DNA present. Additionally, the purity of the DNA was assessed by calculating the absorbance ratios at two distinct wavelengths: 260 nm and 280 nm. These measurements offered insights into the quality and potential contamination of the DNA samples.

Genotyping Process

The sequence of lncRN ANRIL was acquired from a reputable source, the National Center for Biotechnology Information (NCBI) database, Gene ID: 100048912, accessible at the web address <http://www.ncbi.nlm.nih.gov>. We used the Tetra-ARMS PCR method; according to previous research, this is the fastest and lowest-cost method for studying SNPs with an accuracy of 99.9% (13, 14). The total volume of the PCR reaction was 20 μ L, including the genomic DNA (50 ng), dNTPs (250 μ M), 1 U Taq of DNA polymerase, 1.5 mM of $MgCl_2$, and 0.5 μ M of each primer. The thermal PCR cycling contained initial denaturation at 95 °C for 5 min, accompanied by 30 s of cycles—denaturing at 95 °C for 30 s, annealing at 54 °C for 45 s, extension at 72 °C for 40 s, and final extension at 72 °C for 10 min. Then the electrophoresis of PCR products was performed on a 2 percent agarose gel. Table 1 presents the SNP and tetra-primer

ARMS-PCR primers' sequences. The size of the products included outer primers (443 bp), the G allele (234 bp), and the A allele (263 bp). To confirm the accuracy of the genotyping results obtained through the PCR process, a

carefully chosen subset of samples underwent random sequencing. This additional step served as a quality control measure, ensuring that the genotyping outcomes were consistent and reliable.

Table 1. Primers Used for Detection of rs10757278 A/G in lncRNA ANRIL.

SNP	Product size (bp)	primers	Sequence (5' to 3')
rs10757278	Outer primers 443	Forward outer	GGGCATTAAGAAATGGATGGGTAGACAAAA
		Reverse outer	GCTGTTCTCAATTAGCCAGGACTACCTCT
	G allele: 234	Forward Inner	AAGTCAGGGTGTGGTCATTACGGGAA
	A allele: 263	Reverse Inner	CTCAGTCTTGATTCTGCATCGCTTCC

Statistical analysis

Using a chi-squared (χ^2)-test to assess the Hardy-Weinberg equilibrium (HWE); we compared the expected and observed genotype frequencies among the controls. We also tested the relationship of each SNP with case/control status by employing unconditional logistic regression analyses. These relationships were tested considering various genetic models, including dominant, co-dominant, recessive, and over-dominant models. Furthermore, we scored the odds ratio (OR), along with 95% confidence intervals (95% CIs), to determine how strongly polymorphisms are related to OSCC risk. We set $p < 0.05$ as the threshold for statistical significance. We employed the SPSS software (version 19.0) for all analyses.

Results

General characteristics

The case group comprised 101 patients diagnosed with OSCC. Out of the total, there were 50 males, accounting for 49.50% of the group, and 51 females, making up 50.49%. The patients' ages ranged from 19 to 90 years. The average age of the patients with OSCC was 61.83 ± 15.63 years, while the mean age of the healthy individuals was 51.38 ± 16.82 years. Out of 115 healthy individuals, there were 54 males (46.95%) and 61 females (53.04%). The comparison of age distribution between the case and control groups showed no statistically

significant difference (p value = 0.18). Furthermore, a meticulous assessment of gender distribution between the two groups indicated no significant disparities (p value = 0.42).

Genotyping of ANRIL rs10757278 polymorphism

The genotyping of ANRIL rs10757278 was performed using the tetra-primer-ARMS-PCR method. Examination of the genotypes of the rs10757278 polymorphism showed that the frequency of AA, GG and AG genotypes in the patient group was 14 (13.86%), 30 (29.70%), 57 (56.43%), and 10 (8.69%), 47 (40.86%), 58 (50.43%) in the control group, respectively. Also, the distribution of genotypes revealed that the control group ($\chi^2 = 1.80$, $df = 1$, $P = 0.17$) and case group ($\chi^2 = 2.51$, $df = 1$, $P = 0.11$) is in Hardy-Weinberg equilibrium, respectively.

rs10757278 polymorphism and the risk of OSCC

The study of genotypic analysis in OSCC patients compared to healthy people revealed four interesting inheritance models (Table 2). The results revealed that none of these models demonstrated a significant correlation between genotypes and the presence of OSCC ($p > 0.05$). To fully understand the possible connections between the changed allele G in rs10757278 of lncRNA ANRIL, the study and control groups' allele frequencies of G were carefully

compared. The results revealed no statistically significant difference (p value = 0.08).

In order to fully understand the results, a correlation test was carefully carried out to find any possible links between genotypes and tumor size, grade, site, stage, and lymph node metastasis (Table 3). The findings indicated that genotypes AG and GG were notably associated with a tumor size smaller than 4 cm ($p < 0.05$); however, genotype AA did not exhibit the same correlation. It was clear that genotypes with at least one wild-type allele (A)

were strongly linked to OSCC cells that were well differentiated ($p < 0.05$). This helped us learn more about how OSCC cells change. Interestingly, all genotypes demonstrated substantial associations with tumor sites, with the tongue exhibiting the highest association ($p < 0.05$). Furthermore, genotype GG exclusively displayed a significant association with tumor stage ($p < 0.05$). Despite these observations, no statistically significant outcomes were detected in relation to lymph node metastasis of OSCC cells ($p > 0.05$).

Table 2. Evaluation of rs10757278 Genotypes in Patients with OSCC versus Healthy Individuals under Different Inheritance Models.

SNP	Genotype	Inheritance model	Study group	Control group	OR	P value
rs10757278	AA	Codominant	14	10	1	-
	AG		57	58	0.70 (0.28-1.70)	0.43
	GG		30	47	0.45 (0.17-1.15)	0.09
	AA+AG	Recessive	71	68	1	-
	GG		30	47	0.61 (0.34-1.07)	0.08
	AA+GG	Over-dominant	44	57	1	-
	AG		57	58	1.27 (0.74-2.17)	0.37
	AA	Dominant	14	10	1	-
	GG+AG		87	105	0.59 (0.25-1.39)	0.22
	A	Allele	85	78	1	-
	G		117	152	0.70 (0.47-1.04)	0.08

Table 3. The estimated p values of associations of each genotype with tumor size, tumor grade, tumor site, tumor stage, and lymph node metastasis.

rs10757278	Tumor size	Grading	Tumor site	Stage	Lymph Node metastasis
AA	-	0.02* (Well-differentiated)	0.002* (Prevalent in tongue)	0.44	0.36
AG	0.002* (Less than 4cm)	0.002* (Well-differentiated)	< 0.001* (Prevalent in tongue)	0.08	0.68
GG	0.003* (Less than 4cm)	0.09	< 0.001* (Prevalent in tongue)	0.01* (Stage III)	0.63

*: Statistically significant.

Discussion

Our careful study exhibited significant associations between specific genotypes with tumor site and tumor size. However, we did not show a statistically significant difference between the case and control groups in the frequency of the mutated allele G within rs10757278 of lncRNA ANRIL. The findings indicated that genotypes AG and GG were notably associated with a tumor size smaller than 4 cm. All genotypes demonstrated substantial associations with tumor sites, with the tongue exhibiting the highest association. Also, genotypes with at least one wild-type allele (A) were strongly linked to OSCC cells that were well differentiated. Furthermore, genotype GG exclusively displayed a significant association with tumor stage.

Numerous studies have highlighted the significance of lncRNA ANRIL in diverse cancers and diseases, including hepatocellular carcinoma, prostate cancer, lung cancer, bladder cancer, cervical cancer, colorectal cancer, esophageal SCC, and oral SCC (15-25). These findings show how complicated this lncRNA's effects are on tumorigenesis and disease progression. Findings of our study were not concordant with them. It seems that even though some genetic differences may not directly affect OSCC risk, they still have big effects on how cancer starts and spreads. The complex nature of lncRNA ANRIL's role in cancer growth calls for more research.

It's interesting that our results agree with those of Khorshidi et al., who found a strong link between lncRNA ANRIL SNPs rs10757278 and the risk of breast cancer in Iranian patients (26). Furthermore, Maruei-Milan et al. looked into lncRNA ANRIL polymorphisms in papillary thyroid cancer, showing how these genetic differences affect different types of cancer (27, 28). Additionally, our study did not find a strong direct link between the rs10757278 mutation and OSCC susceptibility. However, it is important to understand that genetic variations within lncRNAs have complex effects. lncRNAs, like ANRIL, control many things in cells, such as proliferation, cycle dynamics,

apoptosis, and differentiation. All of these things help tumors grow (29, 30). Varying forms of long noncoding RNAs can change their structure, stability, and how they interact with microRNAs, which could help cancer grow (31).

Moreover, our meticulous exploration of genotypic associations with tumor characteristics unearthed intriguing insights. Genotypes AG and GG were notably linked to smaller tumor sizes, while genotypes AA and AG were associated with lower-grade OSCC. This suggests that the rs10757278 mutation plays an indirect role in the development of OSCC, most likely by affecting not only susceptibility but also how the tumor grows and how cells differentiate. Comparable trends were identified in haplotype analyses by Maruei-Milan et al., underscoring the intricate interplay between genetic variations and tumor behavior (27, 32). The results suggest that the changed allele G in the lncRNA ANRIL may play a secondary role in the development of cancer, affecting certain aspects of tumor growth.

The intricate genetics of lncRNA ANRIL and its connections to cancer susceptibility and progression transcend the influence of a single genetic variation. The bigger picture of lncRNA ANRIL polymorphisms might connect with complicated molecular pathways, which could help us learn more about how cancer grows. The comprehensive roles of lncRNAs in pivotal cellular processes underscore their multifaceted influence. John R. Prensner and his coworkers found an important role for tissue-specific ncRNAs in prostate cancer and suggested that cancer-specific functions of these ncRNAs in tumorigenesis (37). Even though our study did not directly find a link between the rs10757278 A/G mutations and OSCC risk, this doesn't mean that lncRNA ANRIL does not play a role in shaping cancer landscapes. Genetics, cellular behavior, and disease all interact in complex ways that show how important it is to look into everything. This makes lncRNA ANRIL an important player in the complex world of cancer biology.

Our study has several limitations that should be acknowledged. First, the relatively modest sample size of our study cohort may have limited our ability to detect more nuanced and subtle genetic associations. Larger cohorts might provide more statistical power to uncover additional correlations. Second, the etiology of OSCC is known to be complex, influenced by a myriad of genetic, environmental, and lifestyle factors. These multifaceted contributors could have introduced confounding effects, potentially masking the true impact of genetic variations. Comprehensive studies considering these factors might provide a clearer understanding of the interplay between genetics and OSCC. Despite these problems, our study gives us useful information about the possible links between lncRNA ANRIL genetic variants and OSCC risk and tumor features. We need to do more research with larger and more diverse groups of people in the future to fully understand how lncRNA ANRIL SNPs affect the development and risk of OSCC.

Our study provides insights into the complex interplay between lncRNA ANRIL genetic variation, OSCC susceptibility, and tumor characteristics. The rs10757278 A/G variant did not show a direct link with OSCC

risk, but its complex effect on tumor behavior suggests that it may play a bigger role in the development of OSCC. Future investigations could uncover hidden genetic interactions, shedding light on the intricate mechanisms underlying cancer development.

Ethics Approval

All participants in this study signed an informed consent after being informed about all steps of the study including publication step. This study was approved by an ethics committee, Faculty of medicine, Shiraz University of Medical Sciences with the following code (IR.SUMS.DENTAL.REC.1400.143).

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Conflicts of Interest

No conflict of interest.

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