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Association between *HOTAIR* rs1899663 G>T Gene Polymorphism and Thyroid Cancer Susceptibility

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ABSTRACT

Background and Objective: Studies show that single nucleotide polymorphisms of the *HOTAIR* gene are associated with a variety of cancers, including colorectal, breast, and thyroid cancers. The product of the *HOTAIR* gene is a long non-coding RNA that is involved in regulating gene expression, cell cycle and apoptosis and is considered an oncogene. The present study was conducted to investigate the association between *HOTAIR* rs1899663 G>T gene polymorphism and thyroid cancer susceptibility in northwestern Iran.

Methods: In this case-control study, peripheral blood was obtained from 90 patients with thyroid cancer referred to Tabriz hospitals as well as 198 healthy individuals. After DNA extraction by saturated salt and proteinase K method, single nucleotide polymorphism of *HOTAIR* rs1899663 gene was examined by tetra-primers ARMS PCR. Then, allelic and genotypic frequencies of control and case groups were calculated and compared.

Findings: In this study, the genotypic frequencies of GG, GT and TT in the case group were 23.3%, 49% and 27.7%, and in the control group were 20.2%, 41% and 38.8%, respectively. Allele frequencies of G and T were 48% and 52.2% in the case group and 41% and 59% in the control group, respectively. Allelic and genotypic comparisons between case and control groups showed no significant relationship.

Conclusion: The results of the study showed that *HOTAIR* rs1899663 gene polymorphism is not associated with any of the clinicopathological features of thyroid cancer.

Keywords: *Cancer, Thyroid, HOTAIR, Polymorphism.*

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Introduction

Thyroid cancer is one of the most common endocrine malignancies (1). According to the GLOBOCAN website, Asia has the highest prevalence of thyroid cancer in both genders and at all ages (2). Studies show that the expression of *HOTAIR* gene significantly increases in many cancers, especially in malignancies. Increased expression of this gene and the degree of lymph node involvement are also related (3).

The *HOTAIR* gene is an oncogene and plays an important role in regulating gene expression as well as chromatin dynamics. This gene, also known as *HOXC-AS4* and *HOXC11-AS1*, is located on the long arm of chromosome 12 in 13q13 region and between clusters of the HOXC gene, which is transcribed in the opposite direction of these genes (4). The transcript of this gene is a long non-coding RNA (lncRNA) that acts as a molecular scaffold (5) binds to the PRC2 protein complex with its domain 2´ and to LSD1 with its domain 3´ (6). PRC2 is a transcriptional inhibitor that trimethylates the *HOX-D* gene. LSD1, with *HOXD* methylation, eventually leads the cell to become cancerous (6, 7).

The aim of this study was to compare the allelic and frequencies of *HOTAIR* rs1899663 gene polymorphism in control and case groups to investigate the possible association between thyroid tumor susceptibility and this gene in the northwestern region of Iran.

Methods

This case-control study was performed on 90 patients referred to Noor-e Nejat Hospital in Tabriz after approval by the Ethics Committee of Tabriz University of Medical Sciences with the code of ethics IR.TBZMED.REC.1399.1163. After obtaining written consent, 4 cc peripheral blood with anticoagulant (EDTA) was collected from people with thyroid cancer and 198 control samples from people who had no history of cancer in first- and second-degree relatives. Tumor diagnosis and grading were performed by a pathologist.

Extraction of genomic DNA from peripheral blood: In this study, the method of saturated salt and proteinase K was used to extract DNA from peripheral blood. The extracted DNA was examined both qualitatively and quantitatively by electrophoresis on 2% agarose gel in the cell and molecular biology laboratory of Azerbaijan Shahid Madani University.

Polymerase chain reaction technique: In this study, TETRA-ARMS-PCR technique with four primers was used simultaneously for one reaction. The sequencing primers, temperature profile and PCR reaction concentration are given in Tables 1 to 3.

PCR reaction cycles: PCR was performed in a total volume of 13 microliters in a thermocycler (Techne Biometra, UK-Germany). Finally, the results were observed on 2% agarose gel (agarose powder LE from SinaClon, Iran).

Statistical analysis: The relationship between genotypes and alleles in control and case groups was calculated using Pearson's chi-squared test and Fisher's exact test with odds ratio (OR) and 95% confidence interval (CI). JavaStat online statistics program was used to calculate the statistical tests of genotypes and alleles. To evaluate the relationship between genotypes and clinical characteristics of patients as well as the mean index, SPSS software version 25 was used and $p<0.05$ was considered significant.

Insilico Analysis: Insilico analysis was performed in this study using website and online applications such as rSNPBase, RNAsnp and SpliceAid 2.

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Table 1. Sequencing primers

Primer name	Sequencing primers	Primer type
forward outer (FO)	TGAAAGCCACGATCATTTAACATAACCA	General Primer
reverse outer (RO)	TATCTACGGAGGACTTACCTTATTCCTG	General Primer
forward inner (FI)	CCATTATTCCAGTTGAGGAGGGTGAA	Primer T
reverse inner (RI)	CCAAAAGCCTCTAATTGTTGTCGCC	Primer G

Table 2. PCR reaction components

Reaction components	Value	Manufacturer
Mastermix	5 µl	Ampliqon, Iran
External primers	0.7 µl	Genfanavaran, Iran
Internal primers	0.3 µl	Genfanavaran, Iran
ddH_2O	5 µl	Exir Pharmaceutical Company
DNA	1 µl	Extracted from people's blood

Table 3. Temperature and time specifications

Cycle stage	Temperature (Celsius)	Time (seconds)	Number of cycles
Initial denaturation	94	300	1
denaturation	94	30	40
binding	59	45	40
Expansion	72	30	40
final expansion	72	240	1
Maintenance	4	30	1

Results

Products amplified by PCR were observed by 2% agarose gel electrophoresis and individuals were classified into three genotypes GG, TT and GT based on the created sections (Figure 1).

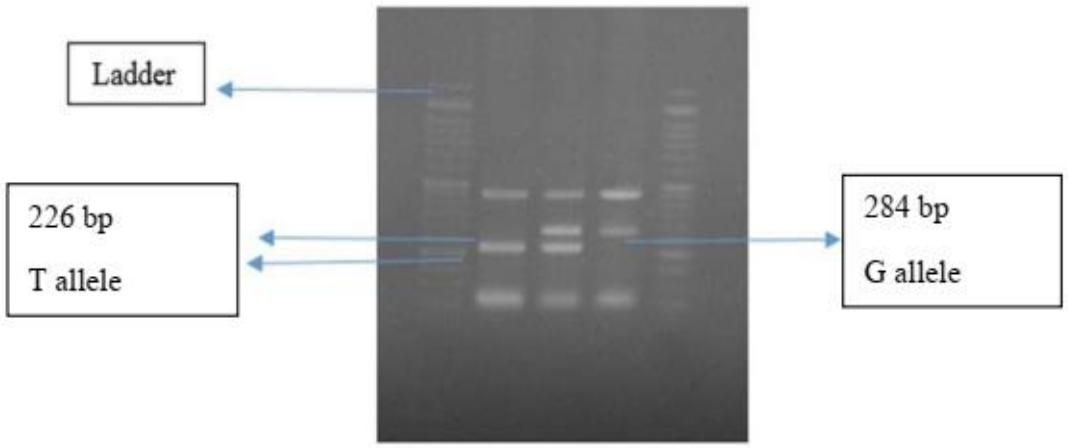

Figure 1. Electrophoresis of PCR products of patients and 50 bp markers on 2% agarose gel.
The genotypes are TT, GT and GG from left to right, respectively.

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Genotypic Relationship and Clinical Pathological Characteristics of Patients: Patients were evaluated for clinical characteristics such as gender, age, tumor type, tumor grade, tumor size, lymph node involvement and involved side (Table 4) and no significant relationship was observed between genotypes and clinicopathological features of patients. Frequency of genotype and allele: Frequency of G allele was detected in 48% of patients and 41% of healthy individuals and T allele in 52.2% of patients and 59.3% of healthy individuals. GT genotype had the highest frequency in both patient and control groups. Allelic and genotypic frequencies in the two groups were not significantly different between case and control groups in the study population (Table 5).

Table 4. Genotypic relationship between rs1899663 and clinicopathological features of patients

Clinical and pathological features	Genotypes			p-value
	TT	GT	GG	
Gender				
Female	18	34	16	0.939
Male	7	10	16	
Age				
>38	13	24	8	0.533
≤38	12	20	13	
Tumor type				
Follicular adenoma	6	11	6	
Follicular carcinoma	1	1	0	0.867
Medullary carcinoma	1	0	1	
Papillary carcinoma	17	29	13	
Tumor grade				
I	11	14	5	
II	6	13	9	0.633
III	1	2	0	
IV	0	1	1	
Tumor size				
>2.5	10	23	10	0.439
≤2.5	8	9	8	
Lymph node involvement				
N0	11	12	4	0.551
N1	3	7	2	
Nx	11	12	10	
Involved side				
Right	10	13	8	0.989
Left	7	11	4	
Both sides	2	4	2	

Table 5. Allelic and genotypic distribution in control and case groups in *HOTAIR* gene polymorphism

Genotype/ allele	Control (n=198) Number(%)	Patient (n=90) Number(%)	*Pearson´s p-value	OR (CI 95%)
GG	40(20.2%)	21(23.3%)	0.547	1.202 (0.660-2.189)
GT	81(41%)	44(49%)	0.205	1.382 (0.837-2.280)
TT	77(38.8%)	25(27.7%)	0.068	0.604 (0.351-1.040)
G	161(41%)	86(48%)	0.109	1.335 (0.937-1.904)
T	235(59%)	94(52.2%)	0.109	0.749 (0.525-1.068)

***p<0.05 is acceptable.**

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Insillico results: RNAsnp online software shows that if the wild-type G allele is changed to T, there is a significant change in the second structure of RNA ($p<0.2$) (Figure 2).

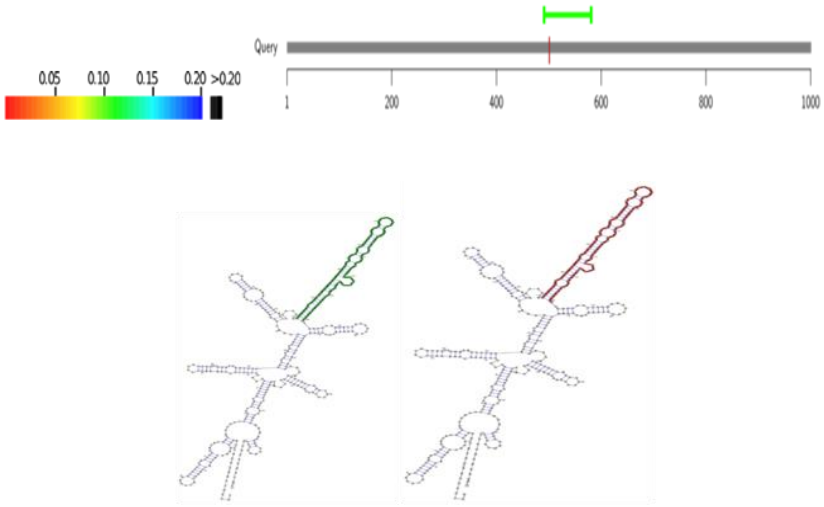

Figure 2. DNAsp software result: Schematic comparison of the second structure of RNA in the wild type (a) and polymorphic type (b)

Another analysis with SpliceAid 2 software shows that in the polymorphic type, the two proteins SRP30C and ETR-3 bind to the SNP region, whereas in the presence of the wild-type allele (G), the two proteins are not able to bind (Figure 3).

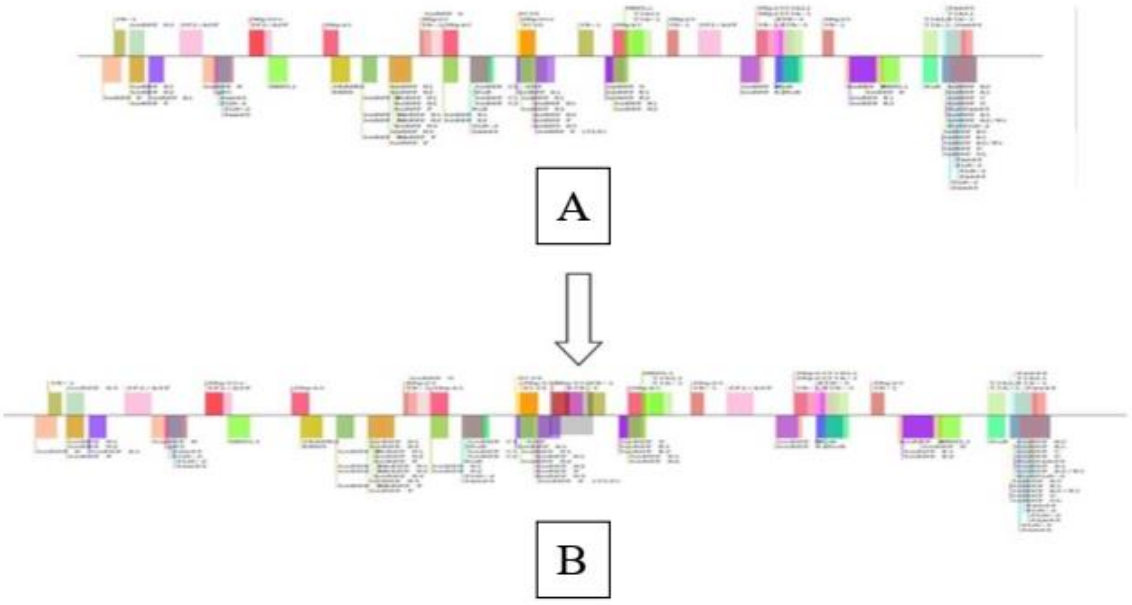

Figure 3. Results of SpliceAid 2 software: A: Binding of different proteins and transcription factors in the presence of wild-type allele (G). B: Binding of two proteins SRP30C and ETR-3 in polymorphic type and SNP expression.

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The rsSNOBase software shows that this polymorphism has only a proximal regulatory role in regulating the expression of the *HOTAIR* gene and has no activity in the distal regulation of this gene (Figure 4).

SNP_ID	rSNP	LD-proxy of rSNP(r^2>0.8)	Proximal regulation	Distal regulation	miRNA regulation	RNA binding protein mediated regulation	eQTL
rs1899663	yes	yes	yes	no	no	no	yes

Figure 4. Results of rsSNOBase software: rs1899663 is close to the *HOTAIR* gene promoter and has no distal regulatory role on the upper and lower points.

Discussion

In this study, no allelic and genotypic relationship was observed between *HOTAIR* rs1899663 gene and thyroid tumor susceptibility in the population of northwestern Iran, but the results of SNP evaluation in RNAsnp online software, in which the significance level is less than 0.2, shows that if the wild-type G allele changes to T, there will be a significant change in the second structure of RNA. There are many polymorphisms on *HOTAIR* gene which are effective in changing the expression of this gene (8). The rs1899663 polymorphism discussed in this study is located in the intron 2 of the *HOTAIR* gene and has an aggravating role (9). The results of a meta-analysis conducted in 2019 show that there is a significant relationship between the risk of cancer and the *HOTAIR* gene, but it does not address the importance of prognosis of this gene in susceptibility to cancer (10).

In another study conducted in 2018, the association between *HOTAIR* gene polymorphisms rs1899663, rs920778, and rs4759314 and cancer was evaluated (11). Examination of 7151 cancer samples and 8740 controls showed no significant relationship between *HOTAIR* gene polymorphism and the risk of cancer, but their supplementary analyses showed that *HOTAIR* rs920778 may be associated with the risk of various cancers (12). According to studies, increased expression of *HOTAIR* gene in cancer cells compared to normal thyroid cells causes an association between this gene and papillary carcinoma (13). Some polymorphisms in this gene have been shown to be moderately associated with papillary carcinoma (14) but it was interesting to note that in rs920778, this association was only significant in women but not in men (15). In another study, various polymorphisms of the *HOTAIR* gene were examined, according to which Rs1899663 G>T was positively associated with an increased risk of breast cancer but was not significantly associated with other cancers (16).

Except for two of the mentioned studies, in most of the performed studies, there is no significant relationship between different diseases and different polymorphisms of *HOTAIR* gene. In the present study, in line with most previous studies, no significant relationship was found between the studied polymorphism and the risk of thyroid cancer. The reason for the contradictory results in different studies in this field can be explained by differences in the genetic background of the studied populations. In addition, the type of cancer studied is different in different studies. Racial differences and environmental factors such as differences in geographical area and consequently differences in the type of nutrition can also be influential factors in this issue. In general, in the present research project, the allelic abundance and distribution of different genotypes of *HOTAIR* rs1899663 polymorphism are reported in the Azeri-Iranian population. According to the results, the frequency of different genotypes and allelic frequency of this polymorphism in the study population is not significantly associated with the risk of thyroid cancer, so this polymorphism cannot be considered as a predisposing factor for thyroid cancer. There is no significant relationship between

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this polymorphism and tumor in examining the relationship between clinical symptoms and genotype. Other studies with a larger number of samples can complement the results of this study.

Due to the detection of this gene in other cancers, the study of this gene in larger statistical communities and comparison of the results of polymorphisms of this gene with other regions of Iran and the world is recommended for future studies.

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