Journal of
Babol University of Medical Sciences
JBUMS
e-ISSN: 2251-7170 p-ISSN: 1561-4107

Association between ICB-1 Gene Polymorphism rs1467465 (A>G) and Thyroid Cancer Susceptibility

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Article Type

Research Paper

Received:
May 1st 2021
Revised:
Sep 14th 2021
Accepted:
Feb 4th 2022

ABSTRACT

Background and Objective: Studies indicate that single nucleotide polymorphism of ICB-1 gene rs1467465 is associated with breast cancer and ovarian cancer. This study was conducted with the aim of investigating the association between ICB-1 gene polymorphism rs1467465 (A>G) and thyroid cancer susceptibility.

Methods: In this case-control study, the peripheral blood of 92 thyroid cancer patients referred to Tabriz hospitals and 203 healthy individuals were prepared. After DNA extraction by saturated salt solution, single nucleotide polymorphism rs1467465 of ICB-1 gene was investigated by TETRA-ARMS-PCR method. Then, allelic and genotypic frequencies of patient and control groups were evaluated and compared.

Findings: In this study, the genotypic frequency of AA, AG and GG in the patient group was 4.34%, 68.47% and 27.17%, respectively, and in the control group was 4.92%, 55.66% and 39.40% respectively. The frequencies of A and G alleles were calculated as 38.58% and 61.41% in the patient group and 32.75% and 67.24% in the control group, respectively. In this study, the relationship between this polymorphism and some clinical and pathological characteristics of thyroid cancer, including gender, age, type, grade and size of tumor, involvement of lymph nodes and involved side, was investigated.

Conclusion: This study showed that AG and GG genotypes have a significant relationship with the risk of developing thyroid tumors in the studied population, and this polymorphism may contribute to determining the relative risk of developing thyroid tumors.

Keywords: *Thyroid Cancer, ICB-1 Gene, Polymerase Chain Reaction.*

Cite this article: Alizadeh M, Pouladi N, Hoseinpour Feizi MA, Madadi Rad R, Emami S. Association between ICB-1 Gene Polymorphism rs1467465 (A>G) and Thyroid Cancer Susceptibility. *Journal of Babol University of Medical Sciences*. 2022; 24(1): 463-71.

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Publisher: Babol University of Medical Sciences

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[DOR: 20.1001.1.15614107.1401.24.1.55.1]
[DOI: 10.22088/jbums.24.1.463]

Introduction

Thyroid cancer is the most common malignancy of the endocrine system, the prevalence of which is continuously increasing. The term thyroid cancer includes a range of different types called papillary thyroid cancer, follicular thyroid cancer, anaplastic thyroid cancer and medullary thyroid cancer (1). The human ICB-1 gene, also known as THEMIS-2 (Thymocyte Selection Associated Family Member 2) and C1orf38, is located at the 1p35.3 position which was introduced and cloned in an analysis of gene expression changes during in vitro differentiation of endometrial tumor cells (2). Recent studies showed that ICB-1 acts as a tumor suppressor in ovarian cancer (3). In a study, the interaction between ERα (Estrogen Receptor α) and ICB-1 gene was reported in breast cancer cells. Moreover, an estrogen response element (ERE) has been identified in the promoter of this gene, which has proven estrogen-induced expression in breast and ovarian cancer cells (4). Stable silencing of ICB-1 in breast and ovarian cancer cells increased the cellular response to estrogen in terms of proliferation and gene expression, suggesting that ICB-1 may exert an antagonistic behavior on the cellular response to estrogen (5). Therefore, the individual level of ICB-1 expression and its splicing, which may be the result of different epigenetic factors, in addition to genetic factors, can influence the intensity of ERα expression and affect both the response to estrogen and the risk of breast cancer (6). One of the researched cases in determining the prognosis of thyroid cancer is the investigation of single nucleotide polymorphisms, which can help to find new treatments and prognosis. rs1467465 (A>G) is an intronic polymorphism and is located between exon 4 and 5 of ICB-1 gene (7). The association between this polymorphism and susceptibility to two types of breast and ovarian cancers has been respectively reported by Springwald et al. and Schüler et al. in the population of Caucasian women (6, 7).

The aim of this research is to compare the allelic and genotypic frequencies of ICB-1 gene polymorphism rs1467465 (A>G) in both control and patient groups to investigate the possibility of an association between thyroid cancer susceptibility and this polymorphism in East Azerbaijan province.

Methods

This case-control study was approved by the ethics committee of Tabriz University of Medical Sciences with the code IR.TBZMED.REC.1399.1162. After obtaining written consent, this research was conducted on 92 people with thyroid cancer from the population of East Azerbaijan province who referred to Noor-e Nejat and Imam Reza hospitals in Tabriz and 203 control samples who had no history of thyroid cancer themselves and their first- and second-degree relatives. The type and grading of the tumor was diagnosed by a pathologist.

Extraction of genomic DNA from peripheral blood: In this research, 4 cc of peripheral blood was collected from patients and transferred to test tubes containing blood anticoagulant (EDTA). Then, DNA extraction was done by salting out method (8). The extracted DNA was analyzed both qualitatively and quantitatively by electrophoresis on 1.5% agarose gel in the radiobiology laboratory of Tabriz University Faculty of Natural Sciences.

Polymerase chain reaction technique: TETRA-ARMS-PCR (Tetra Primer-Amplification Refractory Mutation System-Polymerase Chain Reaction) technique was used in this study to investigate A>G polymorphisms. Primers were synthesized based on previous studies (for single nucleotide polymorphism rs1467465) (7). The outer forward and reverse primers with a product length of 511 bp, which had the sequence of 5'-CGACAGACCTTAGCCCGTGT-3' and 5'-GGAGACAGGGTGTTCCTGG-3', respectively, and the inner forward primer with a product length of bp198 with the sequence of 5'-CGTCATGTAGCATCTGGCACA-3' and inner reverse primer with product length of bp356 with the

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sequence 5´-GGACTTCCATTGCGTTCCCCCAAC-3´ were used. PCR was performed based on a total volume of 10 microliters, containing 1 microliter of genomic DNA, 0.3 microliters of all four primers with a concentration of 10 Mm (manufactured by Genfanavaran Company, Iran), 2.8 microliters of distilled water and 5 microliters of PCR Mastermix (Taq DNA Polymerase Mix Red-Mgcl2 1.5 mM/2 mM) (manufactured by AMPLIQON, Iran) in a thermocycler (manufactured by Techne Biometra, UK-Germany). PCR program was performed for 5 min at 95 °C, followed by 33 PCR cycles of 95 °C (30 s), 62 °C (35 s), and 72 °C (1 min), followed by a final extension for one 10-minute step at 72°C. Finally, the results of TETRA-ARMS-PCR technique were observed on 3% agarose gel (agarose LE powder of SinaClon, Iran).

The relationship between genotypes and alleles in the control and the patient groups using Pearson's chi-square test and in cases where the number of observed data was less than 5, Fisher's exact test with an odds ratio (OR) and a confidence coefficient (CI) of 95% were calculated. Javastat online statistics package (www.statpages.info/ctab2x2.html) was used to calculate the mentioned statistical tests for genotypes and alleles and SPSS software version 25 was used to check the relationship between genotypes and clinical characteristics of patients as well as the mean index and $p<0.05$ was considered significant.

Insilico analysis: Insilico analysis of this study was conducted using the website and online software such as Alibaba, HaploReg, rSNPBase, RNAsnp and 2 Splice Aid. Alibaba website version 2.1 was used to determine the presence of putative transcription factor binding sites in polymorphism. Determining the propensity of transcription factors and proteins for the desired polymorphism was done using HaploReg website version 4.1. rSNPBase was used to determine the regulatory role of proximal and distal polymorphism. RNAsnp, which determines the effect of polymorphism on the second structure of RNA. Finally, Splice Aid 2 was used, which actually contains information about various factors related to the pre-mRNA sequence during the splicing process and leads to interactions with mature transcripts.

Results

The products of PCR were observed by electrophoresis on 3% agarose gel. Genotypes from left to right are AG, AA and GG, respectively. By amplifying the target sequence of ICB-1 gene by the synthesized primers, three expected products including a 511 bp fragment for the control band, a 356 bp fragment for the G allele and a 198 bp fragment for the A allele were obtained (Figure 1).

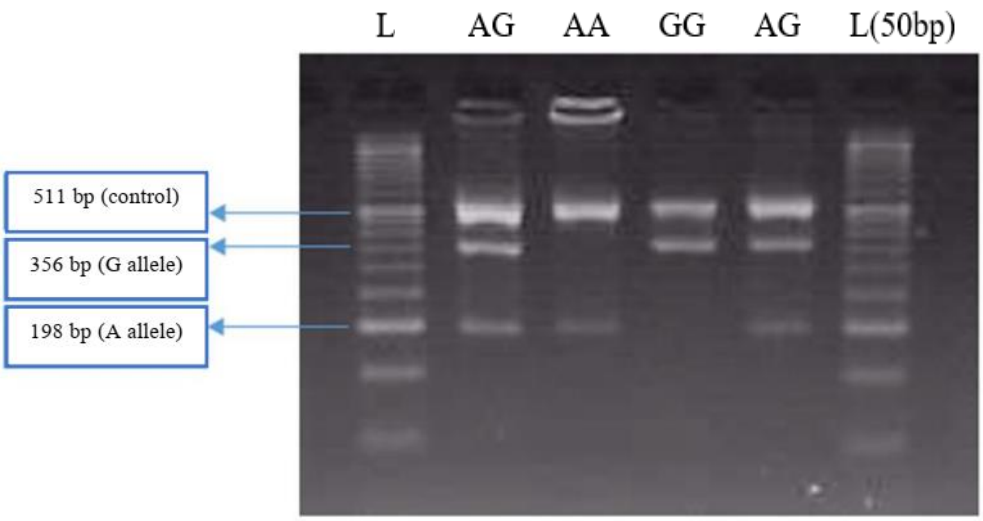


Figure 1. Electrophoresis of a number of PCR products. Patients and 50-bp markers on 3% agarose gel

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Sequencing results: In order to confirm the genotypes obtained by the TETRA-ARMS-PCR method, several samples were sent to Topazgene Co. for sequencing by the Sanger sequencing method, and the results were sent to the website www.ncbi.nlm.gov/blast to be compared with the original sequence. The results of sequencing by Sanger method are given in Figure 2.

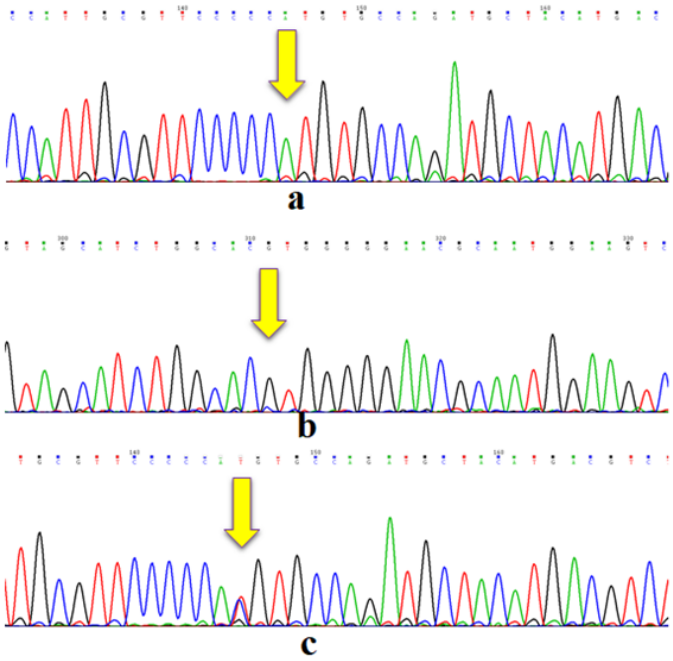


Figure 2. Sequencing results by Sanger method for AA (a), GG (b) and AG (c) genotypes.

Genotype and allele frequency: This study showed that the highest frequency was related to AG genotype, which was observed in 55.66% of healthy people and 68.47% of sick people. GG genotype was seen in 39.40% of healthy people and 27.17% of sick people, and finally 4.92% of healthy people and 4.34% of sick people showed AA genotype. By obtaining $p<0.05$ for AG ($p=0.038$) and GG ($p=0.042$) genotypes, it can be concluded that there is a significant relationship between these genotypes between healthy and sick people (Table 1). The highest allele frequency was related to G allele, which was observed in 67.24% in healthy people and 61.41% in sick people, and the frequency of A allele in healthy and sick people was 32.75% and 38.58%, respectively. There was no significant difference in the frequency of A and G alleles for single nucleotide polymorphism rs1467465 between the control and patient groups (Table 1).

Table 1. Genotype and allele frequency distribution in healthy and sick people

Genotype/allele	Patient (n=92) Number(%)	Healthy (n=203) Number (%)	p-value (Chi-square)	p-value (Fisher)	OR (95% CI)
AA	4(4.34)	10(4.93)	0.829	1.000	0.877 (0.268-2.874)
AG	63(68.47)	113(55.66)	0.038	0.041	1.730 (1.029-2.910)
GG	25(27.17)	80(39.4)	0.042	0.049	0.574 (0.335-0.983)
A	71(38.59)	133(32.76)	0.168	0.191	1.290 (0.898-1.852)
G	113(61.41)	273(67.24)	0.168	0.191	0.775 (0.540-1.114)

OR: Odds Ratio, CI: Confidence Interval

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Patients were also evaluated in terms of clinical characteristics such as gender, age, tumor type, tumor grade, tumor size, lymph node involvement, involved side. The only significant relationship was observed between the type of genotypes and the characteristic of tumor size (p=0.023) (Table 2).

Table 2. Relationship between ICB-1 gene polymorphism rs1467465 (A>G) and clinical and pathological characteristics of thyroid cancer patients

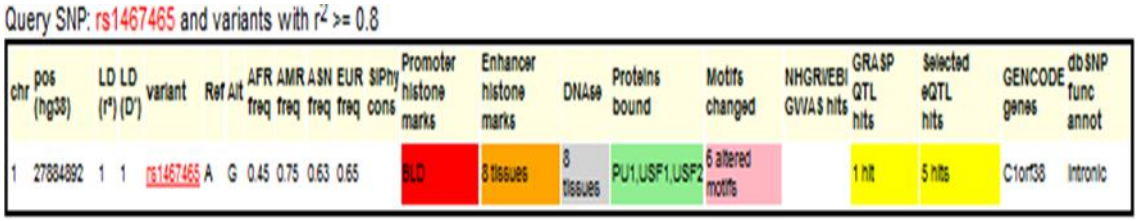
Clinical and pathological features	Genotypes			
	AA	AG	GG	p-value*
Gender				
Woman	3	44	23	0.125
Man	1	19	2	
Age				
>38	3	28	12	0.527
≤38	1	35	13	
Tumor type				
Follicular adenoma	1	13	6	
Follicular carcinoma	1	0	1	
Hürthle cell adenoma	0	1	0	0.233
Hürthle cell carcinoma	0	1	0	
Medullary carcinoma	0	1	1	
Papillary carcinoma	2	44	14	
Tumor grade				
I	4	40	12	
II	0	3	2	0.782
III	0	5	3	
IV	0	1	1	
Tumor size				
>2.5	0	11	11	0.023
≤2.5	3	33	7	
Involvement of lymph nodes				
N0	2	21	7	
N1	1	10	4	
N1a	0	6	5	0.774
N1b	0	3	0	
Nx	0	10	4	
Involved side				
Right	2	25	7	
Left	1	15	6	0.914
Both sides	0	6	2	

*p<0.05 is acceptable

Insilico analysis: The results of this study using Alibaba software showed that by changing the wild – type allele to the mutant allele, transcription factors GR, NF-1, USF and USF, NF-1, SP-1 can bind respectively.

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The result of HaploReg software, which is a tool to discover interpretations of the non-coding genome in a variety of haplotype blocks such as candidate regulatory SNPs in disease-related locations, is shown in Figure 3. rSNPBase also showed that our target polymorphism has only a remote regulatory role. Changes in the spatial structure caused by polymorphism were analyzed by RNAsnp online software and considering that in this analysis, p<0.2 is considered as a significant level, the obtained p-value was more than 0.2 and according to the prediction of the second structure of RNA by RNAsnp online software, no change was observed in the second structure of RNA of ICB-1 gene A>G rs1467465 (p=0.8523). Furthermore, based on the minimum energies obtained for wild – type and mutant sequences, it can be concluded that there was no significant change in the stability of wild – type and mutant RNAs (Figure 4). The results of Splice Aid online software 2 showed that there is no binding site either in the presence of the wild – type allele or in the presence of the mutant allele for various factors that bind to the pre-mRNA sequence.


Query SNP: rs1467465 and variants with r^2 >= 0.8

chr	pos (hg38)	LD (r^2)	LD (D')	variant	Ref	Alt	AFR freq	AMR freq	ASN freq	EUR freq	SiPhy cons	Promoter histone marks	Enhancer histone marks	DNAse	Proteins bound	Motifs changed	NHGRI/EBI GWAS hits	GRASP QTL hits	Selected eQTL hits	GENCODE genes	dbSNP func annot
1	27884892	1	1	rs1467465	A	G	0.45	0.75	0.63	0.65		BLD	8 tissues	8 tissues	PU1,USF1,USF2	6 altered motifs		1 hit	5 hits	C1orf38	intronic

Figure 3. Result of HaploReg software: by converting allele A to G in rs1467465 polymorphism, PU1, USF1 and USF2 proteins become able to bind

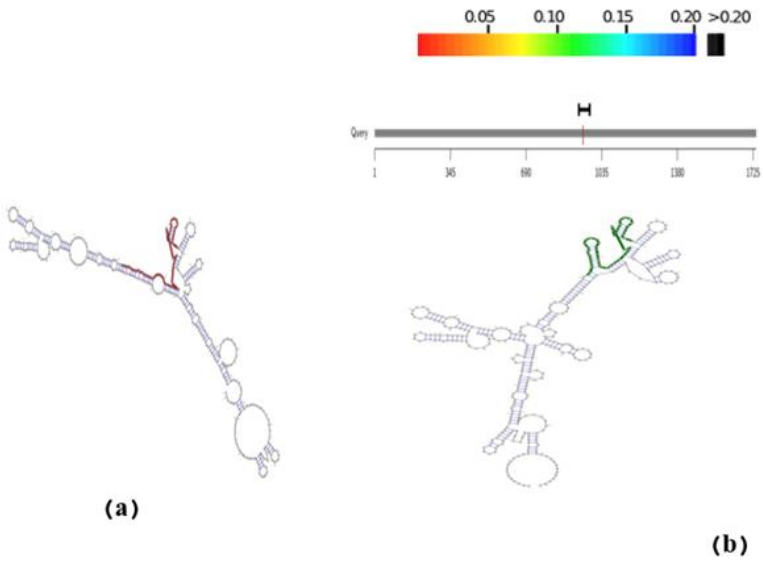


Figure 4. Prediction of RNA secondary structure change by RNAsnp online software: schematic comparison of RNA secondary structure in the wild – type state (Minimum free energy= -116.50 kcal/mol) (a) and the polymorph state (Minimum free energy= -116.60 kcal/mol) (b)

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Discussion

In this study, a significant relationship was observed for AG and GG genotypes among healthy and sick people, and a significant relationship between genotypes and tumor size was also observed. The frequency of tumors was 3 times higher in women than in men, and papillary thyroid cancer was the most common among patients. By comparing the tumor grade of the studied subjects, the frequency of first-grade tumors was higher than other grades, which indicates the early and timely diagnosis of thyroid tumors.

The results of SNP analysis with Alibaba software showed that by changing the wild – type allele to the mutant allele, transcription factors GR, NF-1, USF and USF, NF-1, SP-1 can bind, respectively. One of the researched cases in determining the prognosis of thyroid cancer is the investigation of single nucleotide polymorphisms, which can help to find new treatments and prognosis. rs1467465 (A<G) is an intronic polymorphism and is located between exon 4 and 5 of ICB-1 gene (7).

In a study, Springwald et al. showed that the single nucleotide polymorphism rs1467465 of the human ICB-1 gene may affect the susceptibility to breast cancer (6). The results of their study showed that the frequency of A allele was higher in breast cancer patients compared to healthy people. Thus, the frequency of A allele of this polymorphism can be associated with an increased risk of breast cancer, and the frequency of homozygous AA genotype of this polymorphism was significantly higher in the patient group compared to the control group, which shows that the AA homozygous genotype is associated with an increased incidence of breast cancer (6). Schüler et al. also showed that the single nucleotide polymorphism rs1467465 of the human ICB-1 gene may affect the susceptibility to ovarian cancer (7). By comparing the frequency of genotypes among patients with ovarian cancer, they showed the highest frequency was related to the AG heterozygous genotype of single nucleotide polymorphism rs1467465 of the ICB-1 gene and the genotype-phenotype relationship showed that the heterozygous rs1467465 genotype can be a risk factor for ovarian cancer and the frequency of homozygous genotypes in this study showed no significant difference between the two groups and by analyzing the frequency of alleles of this polymorphism, they concluded that in women with ovarian cancer, the A allele of rs1467465 was significantly higher than in women without ovarian malignancy (7). In the present study, in line with the two mentioned studies, a significant relationship was found between the studied polymorphism and the risk of thyroid cancer.

In general, by comparing all three types of studies, we come to the conclusion that the frequency of A allele in patients with breast and ovarian cancer is higher than in healthy people, while the frequency of G allele is higher in patients with thyroid cancer than in healthy people. The highest genotypic frequency in two ovarian and thyroid cancer studies was related to the AG genotype, which can be a risk factor for cancer. Another difference that we can point out is that in the two studies related to breast and ovarian cancer, all patients and control subjects were women, while for the present study there was no limitation in terms of gender and both men and women, and healthy and sick people participated in this study, and it should also be noted that in each of these studies, the number of participants, matching (in terms of age, gender), mean age and geographical region were also different. In addition, it is worth mentioning that the types of cancers are different and genetic and epigenetic factors should also be considered.

In this study, we investigated intronic polymorphisms without clear functional association, because the ICB-1 gene does not show SNPs in exons or in transcription factor binding sites. Several studies have reported the association between intronic SNPs and disease risk (9-11). Intronic SNPs can act on amplifying or silencing signals of intronic splicing and thus modulate the expression of different splice types (12). ICB-1 lacks any predicted enzymatic activity or protein interaction domains, but has a number of predicted tyrosine phosphorylation sites (13).

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In general, the allelic frequency and distribution of different genotypes of the ICB-1 rs1467465 polymorphism in Azeri-Iranian population is reported in the current research project. According to the results, the frequency of AG and GG genotypes of this polymorphism in the studied population is significantly associated with the risk of thyroid cancer. However, the allelic frequency of this polymorphism in the studied population has no significant association with the risk of thyroid cancer. Therefore, this polymorphism can be considered as a predisposing factor for thyroid cancer. In examining the relationship between clinical symptoms and genotype, there is only a significant relationship between this polymorphism and tumor size.

Suggestion: It is recommended to study the association between this polymorphism and other polymorphisms of this gene in thyroid cancer and other common cancers in other regions of Iran and the world.

Conflict of interest: This research has no conflict of interest with individuals or organizations.

Acknowledgment

The support of the Research Vice-Chancellor of Shahid Madani University of Azerbaijan is hereby acknowledged.

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