J Genet Resour2025; 11(2): 249-259 *Homepage: http://sc.journals.umz.ac.ir/*

RESEARCH ARTICLE *DOI: 10.22080/jgr.2025.30090.1448*

Association of *VDR* Gene Polymorphisms with Thyroid Cancer in the Population of Markazi Province

Shadi Sheikhol Eslami Borghani* and Ahmad Hamta

[1] Department of Biology, Faculty of Basic Sciences, Arak University, Arak, Iran

ARTICLE INFO

Article history:
Received 23 May 2025
Accepted 13 July 2025
Available 26 July 2025

Keywords:
Genotyping
Polymorphism
Thyroid cancer
Vitamin D receptor gene

Corresponding authors:
✉ S. Sheikhol Eslami Borghani
shadisheikh1377@gmail.com

p-ISSN 2423-4257
e-ISSN 2588-2589

ABSTRACT

Thyroid cancer is a common endocrine malignancy, especially among young women, and is often curable with early detection. Genetic mutations in cell proliferation pathways, including the vitamin D receptor (*VDR*) gene, may influence cancer risk. Given the role of the *VDR* in cell proliferation and differentiation, any mutation in this gene could be associated with the occurrence of various cancers. Therefore, the aim of this study was to investigate polymorphisms of the *VDR* gene, including the three SNPs rs7975232, rs1544410, and rs2228570, and their relationship with thyroid cancer. In this laboratory study, tissue samples were collected from 40 patients with various types of thyroid cancer as the patient group and 40 healthy individuals as the control group from Ghods Hospital in Markazi Province. Tissue blocks were sectioned and used for DNA extraction. Then, using the Tetra-Arms PCR technique, the genotypes of each individual for all three SNPs were examined. SPSS software, the Chi-square test, and logistic regression were used to analyze the data and determine significance levels. A p-value < 0.05 was considered statistically significant. Based on the obtained results, in the patient group, the frequency of genotypes for SNP rs7975232 in individuals with genotypes AA ($p< 0.001$) and AC ($p= 0.003$), and genotypes for SNP rs1544410 in individuals with genotypes TT and CC ($p< 0.001$), showed a significant increase compared to the control group. Also, the frequency of genotypes for SNP rs2228570 in the patient group with genotypes CC, TC, and TT showed a significant increase compared to the control group, with significance levels of $p= 0.001$, $p= 0.007$, and $p< 0.001$, respectively. Our findings indicate that the rs2228570 is associated with increased risk and warrants further investigation as a potential genetic marker for risk assessment in this population.

Please cite this paper as: Sheikhol Eslami Borghani, S., & Hamta, A. (2025). Association of *VDR* gene polymorphisms with thyroid cancer in the population of markazi province *Journal of Genetic Resources,* 11(2), 249-259. doi: *10.22080/jgr.2025.30090.1448*

Introduction

Cancer is a leading cause of mortality worldwide, and its incidence is increasing globally. Although endocrine cancers are relatively rare, they may be among the most important and potentially treatable cancers. In fact, thyroid cancer is the most common type of endocrine cancer, accounting for 1% of all new cancers (Cocolos *et al.*, 2022). According to global cancer statistics, thyroid cancer is the fifth most common cancer among women worldwide and the fourth most common cancer in Iran. The incidence of this cancer is higher in women than in men, with approximately 1-5% of cases occurring in women and less than 2% in men (Mehta and Mehta, 2002). Children and young adults are more affected than the elderly population. The incidence of thyroid cancer in the female population of Markazi Province is higher than in men, ranking ninth among common cancers for women. Abnormal and excessive growth of the thyroid can originate from any of the cell types present in the thyroid gland, including thyroid

follicular cells, calcitonin-producing C cells, lymphocytes, stromal and vascular elements, as well as metastatic tumors from other parts of the body. Cancer is the result of accumulated genetic changes (Hajizadeh *et al.*, 2015). Understanding how these changes affect the disease status provides hope for developing new treatments and may offer better prognostic information for each patient. Molecular analysis of thyroid tumors has shown that many genetic changes play a role in carcinogenesis. The most commonly affected genes are those involved in DNA repair, signal transduction, and control of the cell cycle and proliferation. Vitamin D is an anti-proliferative agent against cancer cells and regulates cell differentiation. It acts through the vitamin D receptor. The active metabolite of vitamin D, 1,25(OH)2D3, is a hormone that, in addition to calcium (Sinha, 2018) and phosphorus metabolism, possesses anti-cancer properties. The biological effects of 1,25-dihydroxyvitamin D3 are mediated through binding to the vitamin D receptor (VDR) by a complex network of genomic and non-genomic mechanisms. The study by Beysel *et al.* (2018) on the VDR gene showed that the FokI polymorphism could be considered a prognostic factor for papillary thyroid carcinoma. Also, the study by Carvalho *et al.* (2019) on the association between genetic variation in the vitamin D pathway and thyroid cancer concluded that VDR gene polymorphisms, due to their influence on circulating vitamin D levels, may be associated with an increased risk of thyroid cancer (Patel, 2020). Many genetic changes, such as mutations and single-nucleotide polymorphisms (SNPs), have been identified to be associated with the risk of thyroid cancer. SNPs are popular molecular genetic markers in disease genetics studies and pharmacogenomic research. It is a single base change in a DNA sequence with a natural variation of two possible nucleotides at a given position (Beysel *et al.*, 2018). This change occurs at a specific position in the genome and has an allele frequency of 1% or more. Approximately 325 million SNPs have been identified in the human genome, with 15 million of them present at frequencies of 1% or higher in different populations worldwide. Given the important anti-proliferative properties of vitamin D, the crucial of the *VDR* gene in the metabolism of this vitamin, and also the key role of various SNPs in the occurrence of different cancers, especially thyroid cancer, our study focused on genotyping three SNPs rs7975232, rs1544410, and rs2228570 related to the *VDR* gene in association with thyroid cancer in the population of Markazi Province using the tetra-ARMS PCR technique.

Materials and Methods

Sampling and DNA extraction

In this case-control study, a total of 80 participants were investigated. The case group consisted of 40 patients with histopathologically confirmed thyroid cancer. The control group included 40 individuals who had undergone thyroid surgery for benign conditions (*e.g.*, goiter) and whose adjacent thyroid tissue was confirmed to be histologically normal and free of any malignancy. All samples, in the form of paraffin-embedded tissue blocks, were collected from the archives of Ghods Hospital in Markazi Province. Cases and controls were matched for age and sex to minimize potential confounding effects. All patients signed informed consent forms, and this study was approved by the Ethics Committee of Arak University (Ethical code: IR.ARAKU.REC.1402.071).

A microtome was used to obtain 3-5 µm thick sections from the FFPE tissue blocks. The sections were collected and stored in labeled containers at room temperature. DNA was subsequently extracted from these sections using the FFPESambio DNA extraction kit (Sambio Company, South Korea). The quality and concentration of the extracted DNA were assessed using a Nanodrop spectrophotometer and 1% agarose gel electrophoresis, and samples meeting the quality criteria were used for subsequent experiments.

Tetra-ARMS PCR

Tetra-ARMS PCR, fully named Amplification Refractory Mutation System PCR, also commonly known as the mutation-resistant system, is a PCR-based method for detecting the presence of SNPs in the genome. This technique can distinguish heterozygous, recessive homozygous, and dominant homozygous individuals at a gene locus. This method does not require restriction enzymes. The primers for this technique are very precise. In this research, this

technique was used to investigate rs7975232, rs2228570, and rs1544410 and determine the genotypes of healthy and patient individuals. For designing primers for the Tetra-ARMS PCR techniques, the sequence related to the *VDR* gene from the NCBI database was used. Then, using Primer1 software, Tetra-ARMS PCR primers were designed. Primer BLAST software was used to check the specific amplification of the target fragments. The quality of the designed primers was checked using OLIGO7 software to avoid heterodimer formation and hairpin structures. The sequences related to Tetra-ARMS PCR are shown in Table 1. The DNA sequences amplified by the designed primers were verified by comparing the resulting chromatogram data with published sequencing data from other studies. The amplified fragment showed a perfect match with the reference sequence containing the target SNPs. This validation confirms the accuracy and specificity of our primer design and genotyping methodology. To perform PCR, after the primers reached room temperature, 0.2 ml microtubes were placed according to the number of samples. Then, 3 µl of each DNA sample was added to the microtube, along with 1.6 µl of Nuclease-Free Water, 5 µl of Red MASTER MIX(Sinoheh Co, Iran), and 0.1 µl of each of the 4 relevant primers. The final volume of each microtube was brought to 10 µl. Then, the DNA fragments were amplified for 35 cycles as follows: an initial denaturation at 95°C for 5 minutes; denaturation at 94°C for 30 seconds; annealing at 61.3°C, 66°C, and 68°C for 1 minute for rs1544410, rs2228570, and rs7975232, respectively; extension at 72°C for 1 minute; and a final extension at 72°C for 10 minutes. After PCR, a 2.5% agarose gel and an electrophoresis device were used to examine the bands and determine the genotypes of the individuals. In this study, a 50 bp DNA ladder (SMOBIO, Iran) was used to evaluate the length of the generated bands.

Table 1. Sequences of primers used in Tetra-ARMS PCR.

SNP ID	Primer sequences (5'–3')	Genotypes and Product size (bp)
rs7975232	*Outer- F*: 5'ACAGATGTGAAGGCTGGTGGCCAGAG	*Outer- F*/ *Outer- R*= 447
	Outer- R: 5'GATCATCTTGGCATAGAGCAGGTGGCTG	AA: *Inner- F*/*Outer- R*= 202
	Inner- F: 5'GGGGTGGTGGGATTGAGCAGTGAAGT	CC: *Outer- F*/ *Inner- R*= 296
	Inner- R: 5'AAGGCACAGGAGCTCTCAGCTGGACC	
rs2228570	*Outer- F*: 5'CAGGCAGCTGATTCCAAGCCATGCTCT	*Outer- F*/ *Outer- R*= 409
	Outer- R: 5'GAGAGCCTGGGAGGAGGGCTCACCTGAA	CC: *Inner- F*/*Outer- R*= 197
	Inner- F: 5'CCGTGGCCTGCTTGCTGTTCTTACAGGTAC	TT: *Outer- F*/ *Inner- R*= 267
	Inner- R: 5'GGAAGTGCTGGCCGCCATTGCCTACA	
rs1544410	*Outer- F*: 5'TCCTCTTCGGCCTTTTCTCCCTC	*Outer- F*/ *Outer- R*= 347
	Outer- R: 5'AGAGCCCCTGTGGTGTGTGGAC	CC: *Inner- F*/*Outer- R*= 161
	Inner- F: 5'GCAGAGCCTGAGTATTGGGAAGGC	TT: *Outer- F*/ *Inner- R*= 230
	Inner- R: 5'GGGCCACAGACAGGCCTTCA	

Results

PCR product analysis

To genotype the rs7975232, a PCR reaction was performed with the designed primers. The 447 bp product from the outer primers was observed as a control band in all samples. The 202 bp band from the inner Forward primer and outer Reverse primer was identified in patients, and the 296 bp band from the outer Forward primer and inner Reverse primer was identified in healthy individuals. The simultaneous presence of the 202 and 296 bp bands, alongside the control band, indicated a heterozygous genotype (Fig. 1A). The frequencies of genotypes in the patient and control groups are presented in Table 2.

To genotype the rs2228570 polymorphism, a PCR reaction was performed with the designed primers. The 409 bp product from the outer primers was observed as a control band in all samples. The 197 bp band from the inner Forward primer and outer Reverse primer was identified in patients, and the 267 bp band from the outer Forward primer and inner Reverse primer was identified in healthy individuals. The simultaneous presence of the 197 and 267 bp bands alongside the control band indicated a heterozygous genotype (Fig. 1B). The frequency of genotypes in the patient and control groups is presented in Table 2.

To genotype the rs1544410 polymorphism, a PCR reaction was performed with the designed

primers. The 347 bp product from the outer primers was observed as a control band in all samples. The 161 bp band from the inner Forward primer and outer Reverse primer was identified in patients, and the 230 bp band from the outer Forward primer and inner Reverse primer was identified in healthy individuals. The simultaneous presence of the 161 and 230 bp bands alongside the control band indicated a heterozygous genotype (Fig.1C). The frequency of genotypes in the patient and control groups is presented in Table 2.

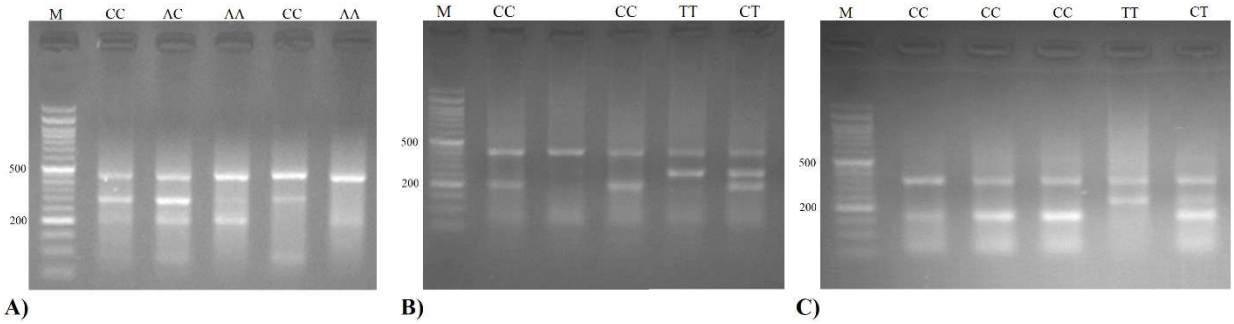

Fig. 1. Agarose gel electrophoresis of PCR amplicons of the *VDR* gene: A) PCR products of the rs7975232 for three genotypes of AA (447 and 202 bp), CC (447 and 296 bp), and AC (447, 202, and 296 bp); B) PCR products of the rs2228570 for three genotypes of CC (409 and 197 bp), TT (409 and 267 bp), and CT (409, 197, and 267 bp); C) PCR products of the rs1544410 for three genotypes of CC (347 and 161bp), TT (347 and 230 bp), and CT (347, 161, and 230 bp). Lane M= 50 bp DNA ladder.

Table 2. Genotype frequency comparison of rs7975232, rs2228570, and rs1544410 between case and control groups.

SNP ID	Genotypes	Control (n= 40)	Case (n= 40)
		Number (Percentage)	Number (Percentage)
rs7975232	AA	0 (0.0%)	14 (100.0%)
	CC	32 (55.2%)	26 (44.8%)
	AC	8 (100.0%)	0 (0.0%)
rs2228570	CC	9 (28.1%)	23 (71.9%)
	TT	25 (100.0%)	0 (0.0%)
	TC	6 (26.1%)	17 (73.9%)
rs1544410	CC	0 (0%)	22 (55%)
	TT	30 (75%)	0 (0%)
	TC	10 (25%)	18 (45%)

Demographics of patients

In this study, 40 patients with thyroid cancer were investigated, of whom 38 were female and 2 were male. The average age of the patients was 45 years, with an age range of 19 to 81 years. Among the patients in the study, papillary thyroid carcinoma was the most frequently observed subtype, representing 77.5% of cases (31 patients). Neoplastic lesions accounted for 12.5% (5 patients), while follicular thyroid carcinoma was identified in 7.5% (3 patients). Medullary thyroid carcinoma was the least common, occurring in only 2.5% of patients (1 case). Among the patients in the study, tumors located in both the right and left lobes were the most common, accounting for 40% of cases (16 patients). Tumors confined to the right lobe were observed in 30% of patients (12 cases), while those in the left lobe represented 25% (10 patients). Tumors restricted to the isthmus were the least frequent, occurring in only 5% of cases (2 patients). The findings of this study are consistent with global statistics, especially regarding gender ratio and cancer type. However, the high percentage of involvement of both thyroid lobes (40%) and the low percentage of isthmus involvement (5%) may require further investigation. These results can serve as a database for future studies on thyroid cancer in the region.

Frequencies and logistic regression analysis

The genotypic distribution of the rs2228570 polymorphism differed significantly between

patients and controls (p< 0.001). Among the participants, 32 individuals were homozygous for the C allele (CC genotype), 25 were homozygous for the T allele (TT genotype), and 23 were heterozygous (TC genotype). Post-hoc analysis revealed a significantly higher frequency of the CC genotype in the patient group compared to controls (p< 0.001), suggesting its association with an increased risk of thyroid cancer. A significant difference was also observed in the genotypic frequencies of the rs1544410 polymorphism between cases and controls (p< 0.001). The distribution was as follows: 22 individuals with the CC genotype, 30 with the TT genotype, and 28 with the TC genotype. Subsequent analysis indicated that the TT and CC genotypes were significantly more prevalent in the patient (p< 0.001 for both), implicating them as potential risk factors. Similarly, the genotypic distribution of the rs7975232 polymorphism showed a significant association with thyroid cancer status (p< 0.001). The group comprised 14 AA homozygotes, 58 CC homozygotes, and 8 AC heterozygotes. Further analysis demonstrated that the AA and AC genotypes were significantly overrepresented in the patient group (p< 0.001 and p= 0.003, respectively), highlighting their potential role in disease susceptibility. Allelic analysis showed that in rs2228570, the C allele was associated with an increased risk of disease (OR=8.647), while the T allele was more common in the control group. In rs7975232, the A allele was more commonly observed in patients (p< 0.001). In rs1544410, the C allele was also more frequent in patients, although its definitive effect on increasing risk was not confirmed (Table 3).

Table 3. Allele frequencies and logistic regression analysis of SNPs between the patient and control groups

SNP	Allele	Frequency in patients (n=40)	Frequency in controls (n= 40)	χ^2	p-value	OR	95% CI
rs7975232	A	28 (35.0%)	8 (10.0%)	-	-	-	-
	C	52 (65.0%)	72 (90.0%)	14.337	0.000	0.206	0.087 – 0.489
rs2228570	C	63 (78.8%)	24 (30.0%)	-	-	-	-
	T	17 (21.3%)	56 (70.0%)	68.283	0.000	0.041	0.018 – 0.097
rs1544410	C	62 (77.5%)	10 (12.5%)	-	-	-	-
	T	18 (22.5%)	70 (87.5%)	36.386	0.000	8.647	4.217 – 17.73

OR= Odds ratio; 95% CI = 95% confidence interval (Lower–Upper).

Analysis of dominant, recessive, and co-dominant models

In the rs1544410 polymorphism, the C allele represents the variant. To elucidate its association with thyroid cancer risk, we evaluated its effect under recessive, dominant, and co-dominant genetic models. A Chi-square test for the homozygous CC genotype revealed a significantly increased risk in patients compared to controls, indicating that the CC genotype elevates the risk of thyroid cancer (OR= 3.222, 95% CI= 2.196-4.729, p< 0.001). Analysis under a dominant genetic model (combined CC + TC genotypes versus TT) was also associated with increased risk (OR= 0.200, 95% CI= 0.115-0.348, p< 0.001). However, analysis of the heterozygous TC genotype under a co-dominant model (TC versus CC+ TT) showed no significant association with increased thyroid cancer risk with OR= 0.061, 95% CI= 0.950-6.339, p= 0.061 (Table 4). These results suggest that the risk effect of the C allele in this population operates primarily through a recessive model, where the homozygous CC genotype confers significant risk. In the rs7975232 polymorphism, the A allele represents the variant. To determine its association with thyroid cancer risk, we analyzed its effect under recessive, dominant, and co-dominant genetic models. A Chi-square test for the homozygous AA genotype revealed a significantly increased risk in patients compared to controls, indicating that the AA genotype elevates the risk of thyroid cancer (OR= 2.538, 95% CI= 1.882-3.424, p< 0.001). However, analysis under a dominant genetic model (combined AA + AC genotypes versus CC) showed no significant association with increased risk (OR= 2.154, 95% CI= 0.784-5.920, p= 0.133). In contrast, analysis of the heterozygous AC genotype under a co-dominant model (AC versus AA+ CC) demonstrated a significant association with increased thyroid cancer risk with OR= 1.250, 95% CI= 1.071-1.459, p= 0.003 (Table 4). These results suggest that for the rs7975232 polymorphism, the risk effect is primarily observed in the homozygous AA

genotype under a recessive model, while the heterozygous AC genotype also contributes to risk under a co-dominant model. The lack of significance in the dominant model indicates that the presence of a single A allele (AC genotype) alone, when grouped with AA, does not confer a significantly different risk compared to the wild-type CC genotype in this model. In the rs2228570 polymorphism, the C allele represents the variant. To elucidate its association with thyroid cancer risk, we evaluated its effect under recessive, dominant, and co-dominant genetic models. A Chi-square test for the homozygous CC genotype revealed a significantly increased risk in patients compared to controls, indicating that the CC genotype elevates the risk of thyroid cancer (OR= 4.660, 95% CI= 1.764-12.311, p= 0.001). Analysis under a dominant genetic model (combined CC + TC genotypes versus TT) was also associated with an increased risk (OR= 0.273, 95% CI= 2.381-0.420, p< 0.001). Furthermore, analysis of the heterozygous TC genotype under a co-dominant model (TC versus CC + TT) showed a significant association with increased thyroid cancer risk with OR= 4.188, 95% CI= 1.436-12.218, p= 0.007 (Table 4). These results indicate that for the rs1544410 polymorphism, the risk effect was observed across multiple genetic models. Both the homozygous variant (CC) and heterozygous (TC) genotypes are associated with significantly increased risk under recessive and co-dominant models, respectively. The significant association under the dominant model further supports the role of the C allele in increasing susceptibility to thyroid cancer (Table 4).

Table 4. Logistic regression analysis of dominant, recessive, and co-dominant models for rs2228570, rs7975232, and rs1544410.

SNP ID	Genetic model	Compared genotypes (Test/Reference)	OR	95% CI (Lower)	95% CI (Upper)	p-value	χ^2 (df=1)
rs2228570	Recessive	CC / (TT+TC)	4.66	1.764	12.311	0.001	10.208a
	Dominant	(CC+TC) / TT	0.23	0.100*	0.528*	0.000**	36.364a
	Co-dominant	TC / (CC+TT)	4.19	1.436	12.218	0.007	7.384a
rs7975232	Recessive	AA / (CC+AC)	2.54	1.882	3.424	0.000**	16.970a
	Dominant	(AA+AC) / CC	2.15	0.784	5.920	0.133	2.257a
	Co-dominant	AC / (CC+AA)	1.25	1.071	1.459	0.003	8.889a
rs1544410	Recessive	CC / (TT+TC)	3.22	2.196	4.729	<0.001	30.345a
	Dominant	(CC+TC) / TT	0.20	0.115	0.348	<0.001	48.000a
	Co-dominant	TC / (CC+TT)	2.46	0.950	6.339	0.061	3.516a

OR= Odds ratio; 95% CI= 95% confidence interval.

Analysis of inheritance models

In rs2228570, genotype CC posed the highest risk compared to TT (OR= 4.660, p< 0.001). Genotype TC also significantly increased risk (OR= 4.188, p= 0.007).In rs7 975232, genotype AA was associated with increased risk (OR= 2.538, p< 0.001), and also, genotype AC showed a similar effect (OR= 1.250, p= 0.003). In rs1544410, genotype CC was associated with increased risk (OR= 3.222, p< 0.001), while genotype TC did not show a significant association.

Examination of Hardy-Weinberg Equilibrium showed that the genotype distributions for rs2228570 and rs7975232 deviated significantly from expectation (p< 0.05), while rs1544410 was in equilibrium. This observed disequilibrium for rs2228570 and rs7975232 (p= 0.027 and p< 0.001, respectively) may be attributed to the limited sample size of the study population, which increases susceptibility to genetic drift, along with the potential influence of selection or other evolutionary forces acting on these functional loci.

Statistical analysis of genotype combinations

In the comparative analysis of the distribution of combined genotypes for the three studied SNPs using the chi-square test, the obtained p-value was less than the significance level of 0.05. This finding indicates that there is a statistically significant association between some combined genotypes at these three loci and the risk of thyroid cancer, and certain specific genotypic arrangements increase the likelihood of developing the disease. Certain specific combined genotypes, such as CCCCCC, TCTCCC,

TCCCCC, TCTCAA, and TCCCAA, had OR > 1, indicating an increased risk of disease. Therefore, it is necessary to examine the frequency of genotypes individually in the two groups of patients and healthy individuals. Therefore, it is necessary to examine the frequency of each of these combined genotypes separately in the patient and healthy control groups. Due to the very low frequency of some combined genotypes in the study samples, meaningful statistical analysis for them is not feasible, and these cases were excluded from the final analysis. The combined genotypes of the three studied polymorphisms are shown in Table 5.

Table 5. Association of combined genotypes of rs2228570, rs7975232, and rs1544410 with Thyroid cancer risk.

Genotype	OR	95% CI (Lower)	95% CI (Upper)	χ^2	Df	p-value
CCCCCCC	1.444	1.172	1.781	14.512[a]	1	0.000
CCTCCC	0.121	0.014	1.034	5.000[a]	1	0.025
TCTCCC	1.000	0.134	7.470	0.000[a]	1	1.000
TCCCCC	1.143	1.017	1.285	5.333[a]	1	0.021
TCTCAA	1.212	1.051	1.398	7.671[a]	1	0.006
TCCCACA	1.081	0.990	1.181	3.117[a]	1	0.077
TTTTCC	0.625	0.492	0.795	18.462[a]	1	0.000

OR= Odds ratio; 95% CI = 95% confidence interval

Analysis of haplotypes

According to the statistical analysis of data using the SNP Analyzer 2 software, there was a significant difference in the distribution of haplotypes of the three studied SNPs between the patient and control groups (Fig. 2). The green line in the figure indicates the threshold of significance. If the column representing the haplotypes derived from the three mutations crosses. The results are presented in the following order of haplotype combinations: rs1544410, rs2228570, and rs7975232. The green line, combining haplotypes of the three mutations, is considered statistically significant (Figure 8). Among the haplotypes, the sequences TTC and CCC were observed with the highest frequency (26%) in individuals.

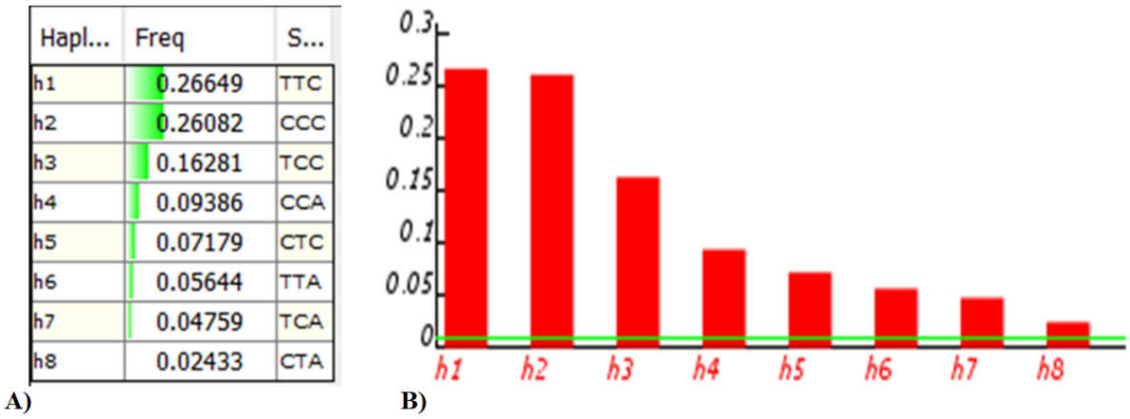

Fig. 2. Haplotype frequency distribution of rs2228570, rs7975232, and rs1544410 SNPs that were calculated using SNP Analyzer 2 software: A) Tabular representation of haplotype names, their corresponding allele combinations, and estimated frequencies; B) Graphical bar plot illustrating the relative frequencies of each haplotype.

Discussion

This case-control study provides evidence for a significant association between specific polymorphisms in the vitamin D receptor (*VDR*) gene and the risk of thyroid cancer in a population from central Iran. Our findings highlight the potential role of genetic variation in the vitamin D pathway in thyroid carcinogenesis, with particularly strong signals observed for the functional rs2228570 polymorphism. The most striking finding of our study was the complete

absence of the TT (Wild-type) genotype of the rs2228570 polymorphism in the patient cohort, with all 25 carriers of this genotype belonging to the control group (Table 5). This distribution strongly implies a protective role for the T allele against thyroid cancer in this population. Conversely, both variant-carrying genotypes (CC and TC) were significantly overrepresented in patients. Logistic regression analysis reinforced this observation, showing that the homozygous CC genotype conferred the highest risk (OR= 4.66, p< 0.001), while the heterozygous TC genotype also significantly elevated risk (OR= 4.19, p= 0.007) compared to the TT reference (Table 4). This pattern is consistent with a co-dominant or additive model of inheritance for the risk C allele at this locus. The rs2228570 polymorphism results in a shorter, purportedly more transcriptionally active VDR protein. Our results suggest that this altered protein function may dysregulate vitamin D-mediated pathways involved in cell cycle control, proliferation, or apoptosis in thyroid tissue, thereby increasing susceptibility to malignancy. This aligns with the study by Beysel *et al*. (2018), which identified the rs2228570 polymorphism as a poor prognostic factor in papillary thyroid carcinoma. For the rs7975232 polymorphism, we observed an exclusive presence of the homozygous variant AA genotype in the patient group (14 patients vs. 0 controls), indicating a pronounced risk association under a recessive model (OR= 2.54, p< 0.001). Interestingly, the heterozygous AC genotype was found only in controls in our sample, preventing its classification as a risk genotype in this specific cohort and hinting at a potential heterozygote advantage or complex allele interaction that merits exploration in larger studies. Regarding the rs1544410 polymorphism, a clear stratification was also evident: the homozygous CC genotype was found exclusively in patients, while the homozygous TT genotype was exclusive to controls. This suggests that the C allele may be a risk factor and the T allele a protective factor at this locus, operating primarily through a recessive mechanism for risk, as indicated by the significant OR for the CC genotype (OR= 3.222, p< 0.001). The genotypic findings of this study were compared with other relevant studies, revealing a complex pattern of both alignment and discrepancy, largely attributable to differences in population genetics, disease subtype, and sample size. Regarding the functional *FokI* polymorphism (rs2228570), our finding of a strong protective effect for the TT genotype contrasts with a study on medullary thyroid carcinoma (MTC) by Ramezani *et al*. (2020), suggesting that VDR associations may be histology-specific (Ramezani *et al*., 2020). However, our risk association for the C allele is consistent with studies on other cancers, such as breast cancer (Hadi, 2022; Lafi *et al*., 2024), and with findings by Penna-Martinez *et al*. (2009) and Sharma *et al*. (2010) in thyroid cancer, supporting a broader oncogenic role for this variant. The discrepancy with Zarrin *et al*. (2018) may stem from population and sample size differences. For the *ApaI* polymorphism (rs7975232), the pronounced risk associated with the AA genotype in our cohort aligns with findings from a study on periodontitis in Iran, which used a similar genotyping technique (Torbati *et al*., 2021). This suggests a potential shared pathway in immune-inflammatory dysregulation. Conversely, our results for *BsmI* (rs1544410) and the overall pattern for all three SNPs differ from those reported by Maciejewski *et al*. (2019) in a different ethnic population, underscoring the critical impact of ethnic background on genetic associations. The alignment with Gunes *et al*. (2020) and Jabir *et al*. (2023) on specific loci further highlights context-dependent effects (Gunes *et al*., 2020; Jabir *et al*., 2023). In terms of demographic factors, our study found no significant association between age and thyroid cancer risk. This contrasts with studies reporting increased thyroid nodule prevalence or MTC risk with age (Kwong *et al*., 2015; Sahli *et al*., 2021) but aligns with other cohorts (Haymart *et al*., 2009), possibly reflecting the specific age distribution of our papillary thyroid cancer-dominated sample (Haymart *et al*., 2009; Kwong *et al*., 2015; Sahli *et al*., 2021). While a higher proportion of females was observed

among cases, the association was not statistically significant. This nuanced finding partially aligns with Jonklaas *et al*. (2012), who noted a convergence of risk in older age groups, and reflects the complex, population-variable sexual dimorphism discussed by Rahbari *et al*. (2010), Jonklaas *et al*. (2012), and Rahbari *et al*. (2010). A notable observation was the significant deviation from the Hardy-Weinberg Equilibrium (HWE) for the rs2228570 and rs7975232 polymorphisms in the control group. While HWE assumes a large, randomly mating population in the absence of evolutionary forces, our study setting inherently violates several of these assumptions. First, the limited sample size of our cohort increases the influence of genetic drift, leading to stochastic fluctuations in allele frequencies. Second, the functional relevance of these SNPs-rs2228570 alters protein structure, and rs7975232 is in a regulatory region, making them plausible targets for natural selection related to vitamin D metabolism, which could distort genotype distributions. Third, the population of Markazi Province may have demographic characteristics, such as limited gene flow or a founder effect, that contribute to unique allele frequencies. Therefore, this HWE deviation does not invalidate our findings but rather underscores that these loci may be subject to demographic and potential selective pressures in this specific population, adding a layer of complexity to their association with disease. The haplotype analysis revealed that specific combinations of alleles across the three loci (*e.g.*, CCC) were more frequent in patients, suggesting potential epistatic interactions between these polymorphisms that could modulate cancer risk beyond the effect of single SNPs. Furthermore, the analysis of combined genotypes identified several multi-locus genotypes (*e.g.,* CCCCCC and TCTCCC) with OR> 1.0, reinforcing the notion that the overall genetic architecture of the VDR gene, rather than isolated variants, influences disease susceptibility. The interpretation of our results must consider several limitations. The relatively small sample size, though sufficient to detect strong genetic effects, may limit the statistical power for subgroup analyses and the generalizability of findings. The use of archival FFPE tissue for DNA extraction, while practical and disease-relevant, can yield fragmented DNA and potentially introduce bias compared to blood-derived DNA. Furthermore, we did not measure circulating levels of 25-hydroxyvitamin D; thus, we could not explore crucial gene-environment interactions between *VDR* genotypes and vitamin D status. Despite these limitations, our study has notable strengths. It is the first to focus on the *VDR*-thyroid cancer association in the population of Markazi Province, Iran, contributing to the understanding of ethnic-specific genetic risk factors. The use of the tetra-ARMS PCR technique provided a reliable and cost-effective method for accurate genotyping. The careful matching of cases and controls and the histopathological confirmation of all samples strengthen the internal validity of the case-control comparison.

Conclusion

Our study demonstrates that polymorphisms in the *VDR* gene, particularly the functional rs2228570 variant, are significantly associated with an increased risk of thyroid cancer in our study population from central Iran. The protective effect of the rs2228570 TT genotype and the risk associated with the CC genotype are clear and compelling findings. These results add to the growing body of evidence implicating vitamin D signaling in thyroid cancer pathogenesis. Future research should aim to validate these associations in larger and multi-center Iranian cohorts. Incorporating measurements of serum vitamin D levels is crucial to disentangle the interplay between genetic predisposition and nutritional status. Functional studies are needed to elucidate the precise molecular mechanisms by which the rs2228570 polymorphism alters VDR function in thyroid cells. Ultimately, understanding these pathways may open avenues for personalized risk assessment and novel preventive or therapeutic strategies targeting the vitamin D pathway in thyroid cancer.

Conflict of interests

The authors declare no conflict of interest related to this publication.

Funding

No funding was dedicated to this study.

References

Cocolos, A.-M., Muresan, A., Caragheorgheopol, A., Ghemigian, M., Ioachim, D., & Poiana, C. (2022). Vitamin D status and VDR polymorphisms as prognostic factors in differentiated thyroid carcinoma. *In Vivo*, 36(5):2434-2441. https://doi.org/10.21873/invivo.12977

Mehta, R. G., & Mehta, R. R. (2002). Vitamin D and cancer. *The Journal of Nutritional Biochemistry*, 13(5), 252-264. https://doi.org/10.1016/S0955-2863(02)00183-3

Hajizadeh, N., Pourhoseingholi, M. A., & Baghestani, A. (2015). Incidence rate of thyroid cancer in Iranian population, trend analysis from 2003 to 2009. *Epidemiology and Health System Journal*, 2(1), 12-17. https://ehsj.skums.ac.ir/

Sinha, T. (2018). Tumors: benign and malignant. *Cancer Therapy and Oncology International Journal*, 10(3), 52-54. https://doi.org/10.19080/CTOIJ.2018.10.555790

Patel, A. (2020). Benign vs malignant tumors. *JAMA Oncology*, 6(9), 1488-1488. https://doi.org/10.1001/jamaoncol.2020.2592

Beysel, S., Eyerci, N., Pinarli, F. A., Apaydin, M., Kizilgul, M., Caliskan, M., ... & Cakal, E. (2018). VDR gene FokI polymorphism as a poor prognostic factor for papillary thyroid cancer. *Tumor Biology*, 40(11), 1010428318811766. https://doi.org/10.1177/1010428318811766

Carvalho, I. S., Gonçalves, C. I., Almeida, J. T., Azevedo, T., Martins, T., Rodrigues, F. J., & Lemos, M. C. (2019). Association of vitamin D pathway genetic variation and thyroid cancer. *Genes*, 10(8), 572. https://doi.org/10.3390/genes10080572

Sharma, V., Fretwell, D., Crees, Z., Kerege, A., & Klopper, J. P. (2010). Thyroid cancer resistance to vitamin D receptor activation is associated with 24-hydroxylase levels but not the ff FokI polymorphism. *Thyroid*, 20(10), 1103-1111. https://doi.org/10.1146/annurev.med.56.082103.104540

Penna-Martinez, M., Ramos-Lopez, E., Stern, J., Hinsch, N., Hansmann, M. L., Selkinski, I., ... & Badenhoop, K. (2009). Vitamin D receptor polymorphisms in differentiated thyroid carcinoma. *Thyroid*, 19(6), 623-628. https://doi.org/10.3390/ijms232113661

Gunes, A., Yazicioglu, M. B., Tiryaki, C., Uren, N., Ergul, E., Simsek, T., & Cubukcu, A. (2020). Evaluation of vitamin D receptor gene polymorphisms in patients with differentiated thyroid carcinomas and nodular goiter. Minerva Endocrinology, 46(3), 317-324. https://doi.org/10.1186/s12887-023-04487-z

Lafi Z. K., & Mohammed, B. J. (2024). Relationship between vitamin D receptor genotypes (FOK1rs2228570) and IL18 gene expression in sample of multiple sclerosis Iraqi patients. *Human antibodies*, 32(1), 1-8. https://doi.org/10.23736/S0391-1977.20.03200-9

Torbati, E. S., Tavakkoli, N., & Amini, K. (2021). Study of association betweenprs7975232 polymorphism in vitamin D receptor gene and periodontitis by Tetra Arms-PCR. *Journal of Dental Medicine*, 33(4): 227-239. https://jdm.tums.ac.ir/en

Maciejewski, A., Kowalczyk, M. J., Herman, W., Czyżyk, A., Kowalska, M., Żaba, R., & Łącka, K. (2019). Vitamin D receptor gene polymorphisms and autoimmune thyroiditis: are they associated with disease occurrence and its features? *BioMed Research International*, 2019(1), 8197580. https://doi.org/10.1155/2019/8197580

Hadi, S. M. (2022). The impact of vitamin D receptor gene polymorphism (rs2228570) in osteoarthritis in Iraqi women. *Gene Reports*, 27, 101561 https://doi.org/10.1016/j.generep.2022.101561

Zarrin, R., Bagheri, M., Mehdizadeh, A., Ayremlou, P., & Faghfouri, A. H. (2018). The association of FokI and ApaI polymorphisms in vitamin D receptor gene with autoimmune thyroid diseases in the northwest of Iran. *Medical Journal of the Islamic Republic of Iran*, 32, 4. https://doi.org/10.14196/mjiri.32.4

Ramezani, M., Mazani, M., Tabatabaei, M., Rahimian, A., Mosaferi, E., & Hedayati, M. (2020). Medullary thyroid cancer is associated with high serum vitamin D level and polymorphism of vitamin D receptors. *Physiology International*, 107(1), 120-133. https://doi.org/10.1556/2060.2020.00010

Kwong, N., Medici, M., Angell, T. E., Liu, X., Marqusee, E., Cibas, E. S., ... & Alexander, E. K. (2015). The influence of patient age on thyroid nodule formation, multinodularity, and

thyroid cancer risk. *The Journal of Clinical Endocrinology and Metabolism*, 100(12), 4434-4440. https://doi.org/10.1210/jc.2015-3100

Sahli, Z. T., Canner, J. K., Zeiger, M. A., & Mathur, A. (2021). Association between age and disease specific mortality in medullary thyroid cancer. *The American Journal of Surgery*, 221(2), 478-484. https://doi.org/10.1210/jc.2015-3100

Haymart, M. R. (2009). Understanding the relationship between age and thyroid cancer. *Oncologist*, 14(3), 216-221. https://doi.org/10.1634/theoncologist.2008-0194

Jonklaas, J., Nogueras-Gonzalez, G., Munsell, M., Litofsky, D., Ain, K. B., Bigos, S. T., ... & from the National Thyroid Cancer Treatment Cooperative Study Group. (2012). The impact of age and gender on papillary thyroid cancer survival. *The Journal of Clinical Endocrinology and Metabolism*, 97(6), E878-E887. https://doi.org/10.1210/jc.2011-2864

Rahbari, R., Zhang, L., & Kebebew, E. (2010). Thyroid cancer gender disparity. Future oncology, 6(11), 1771-1779. https://doi.org/10.2217/fon.10.127