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Medullary thyroid cancer is associated with high serum vitamin D level and polymorphism of vitamin D receptors

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ABSTRACT

Background: Thyroid cancer is the most common endocrine malignancy. Studies have observed an anti-cancer effect for vitamin D and found that polymorphisms of vitamin D receptors can influence the prevalence of various cancers. The present study investigated the serum level of vitamin D and FokI, BsmI and Tru9I polymorphisms of vitamin D receptors. *Methods:* Forty patients with medullary thyroid cancer and 40 healthy controls were investigated. The genomic DNA of the subjects was extracted using saturated salt/proteinase K and investigated by PCR sequencing. Serum levels of vitamin D were evaluated by ELISA. The results were analyzed in SPSS and GraphPad Prism 5 software. *Results:* The genotypic and allelic frequencies of FokI and BsmI polymorphisms showed no significant differences between test and control groups. For Tru9I polymorphism, Tt genotype and t allelic frequency in the test group were significantly different from those of the control group. Also, we found Tt genotype and

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t allelic frequency to be significantly associated with medullary thyroid cancer (MTC) type and the agressiveness of the disease. The average serum vitamin D level was 23.32 ng/mL and 18.95 ng/mL for patients and controls, respectively, and the difference between the two groups was statistically significant. Moreover, we found high serum vitamin D level to be associated with t allelic frequency. *Conclusions:* Unexpectedly, the mean serum vitamin D level of the test group was significantly higher than that of the control group. Tru9I polymorphism was found to be significantly correlated with the prevalence of medullary thyroid carcinoma.

KEYWORDS

medullary thyroid carcinoma, vitamin D, vitamin D receptor, polymorphism

INTRODUCTION

Thyroid cancer is the most common endocrine malignancy and accounts for 1–2% of all cancers. Epidemiological studies have reported a gradual increase in its overall incidence [7, 38]. Thyroid cancers can be divided into four main subtypes including papillary, follicular, medullary and anaplastic [8, 11]. Medullary thyroid cancer (MTC) is a rare form of thyroid cancer that accounts for 5–8% of all thyroid cancers. MTC originates from the parafollicular cells (C cells), whereas the other three types originate from follicular cells.

Several factors are involved in the growth of the thyroid gland. Thyroid-stimulating hormone (TSH) plays a major role in regulating growth and differentiation of thyroid cells. TSH stimulates the production of molecules involved in intracellular signal transduction, including cAMP [44], iodine uptake and cell growth [29]. The active form of vitamin D (1,25-$(OH)_2$ D_3) binds to its receptor (VDR) using genomic and non-genomic mechanisms that inhibit the proliferative effect of TSH on thyroid cells. It is logical to propose that vitamin D plays a role in regulating thyroid gland cell proliferation. Binding of 1,25-$(OH)_2$ D_3 to its receptor results in pleiotropic effects regulating calcium-phosphate metabolism, cell proliferation, differentiation, apoptosis, angiogenesis and metastasis. Vitamin D can thus be described as a very important agent in the anti-cancer response [3]. Accordingly, many studies have shown the anticancer effects of vitamin D on breast, prostate, colon and endocrine cancers [20].

In mammals, the expression of VDR is high in metabolic tissue, including the intestine, kidney, skin and thyroid gland. VDR is also expressed in high levels in tumor tissue [16]. This nuclear receptor function is affected by the presence of various genetic polymorphisms [26]. Several polymorphisms have been identified in the introns and exons of the VDR gene. These include Tru9I and BsmI on intron 8, FokI on exon 2 and *Taq*I on exon 9 [33]. FokI is located on the 5′ end and the other three polymorphisms are located on the 3′ end of the VDR gene [21].

Several studies have found a relationship between VDR gene polymorphisms and the risk of various cancers [30, 35, 46]. Also, we have previously demonstrated an association between RET gene polymorphisms and the risk of medullary thyroid cancer [18]. It could be concluded that changes in the vitamin D system can affect cancer progression. The current study evaluated serum levels of vitamin D and polymorphisms of the VDR gene in patients with MTC and compared the results with those of a healthy control group.

MATERIALS AND METHODS

Sample preparation

The current study was carried out after obtaining prior permission from the Institutional Ethical Committee of the Cellular and Molecular Endocrinology Research Center at Shahid Beheshti University of Medical Sciences (No: IR.ARUMS.REC.93.66; dated: 28/2/2015). Written informed consent was received from all participants.

Forty patients with MTC including, 30 hereditary (29 FMTC and 1 MEN2B) and 10 sporadic cases were enrolled in the present study. The diagnoses were confirmed by pathological examination of thyroid tissue samples. Study controls were 40 age- and gender-matched normal healthy persons without a family history of thyroid carcinoma and with no autoimmune diseases, selected from the healthy staff members of Cellular and Molecular Endocrinology Research Center at Shahid Beheshti University of Medical Sciences.

About 10 mL of peripheral blood was obtained from each participant. Genomic DNA was extracted from nuclear cells of the peripheral blood using a standard salting-out procedure [31]. The concentration of the DNA sample was evaluated by UV spectrophotometry.

Polymerase chain reaction and genotyping

The polymerase chain reaction (PCR) assay was performed in a final volume of 35 μL in premixed microtubes (Bioneer; South Korea) containing 1 U Taq polymerase, dNTP (250 mM), Tris-HCl (10 mM), KCl (30 mM) and $MgCl_2$ (1.5 mM). The following were added to each microtube: 1 μL (5–10 pmol) of each amplification primer, 1 μL (5–50 ng) of DNA and 32 μL of sterile distilled water. PCR was carried out for 30 cycles (each cycle consisted of 35 s at 94 °C for denaturation, 1 min at 60.5 °C for annealing and 45 s at 72 °C for extension) with an initial denaturation at 95 °C and final extension at 72 °C for 10 min. The 237 bp fragment encompassing the FokI-rs2228570 polymorphic site and the 339 bp fragment encompassing the BsmI-rs1544410 and Tru9I-rs757343 polymorphic sites were amplified using the specific primers (Table 1).

The purified PCR products were sequenced using forward primers by Bioneer (South Korea). Sequences were analyzed using Chromas Lite software (Technelysium) and verified against the sequence at the NCBI GenBank (National Center for Biotechnology Information) (Fig. 1).

Measurement of 25-(OH) D_3

The concentration of 25-(OH) D_3 was measured in the plasma of patients with MTC (n = 40) and those in the control group (n = 40) using an Elisa kit according to instructions by the manufacturer (Calbiotec; USA).

Table 1. The primers used in VDR gene PCR

	Primer
FokI	F:5-GATGCCAGCTGGCCCTGGCA-3 R:5-ATGGAAACACCTTGCTTCTT-3
BsmI & Tru9I[a]	F:5-CAGAGTGTGCAGGCG-3 R:5-CCCTCTTTGGACCTCATCAC-3

[a]BsmI and Tru9I are located on intron 8, therefore both could be amplified using a single pair of primers.

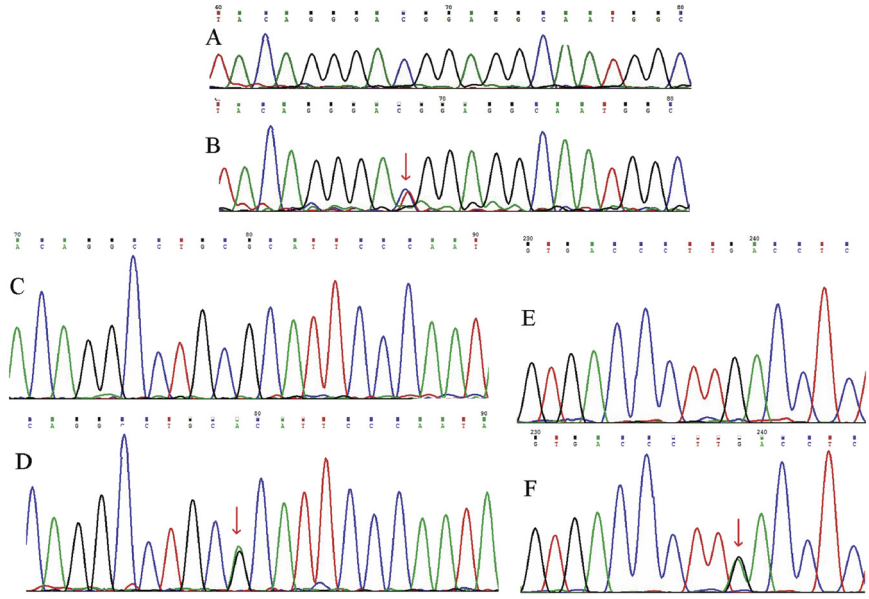

Fig. 1. VDR DNA sequencing results: (A) and (B) show results in exon 2. Peaks in (A) show the natural sequence of DNA and peaks in (B) show the expected heterozygous mutation (Fok-rs2228570 T > C). (C), (D), (E) and (F) show results in intron 8. (C) and (E) show natural sequence of DNA, respectively. (D) and (F) show expected heterozygous mutations (BsmI-rs1544410 A > G) and (Tru9I-rs757343 A > G), respectively

Statistical analysis

The association between MTC and genotypes was evaluated by the Chi-square test using SPSS (version 25). Fisher's Exact probability test was applied for analyzing the Tru9I polymorphism since the tt allelic frequency for control group was zero. The odds ratios and their 95% confidence intervals were estimated. The frequency distributions of the VDR polymorphisms were tested for Hardy–Weinberg equilibrium using the Chi-square test (df = 1). A subgroup analysis was performed to evaluate the association between VDR polymorphism (Tru9I) and MTC type, pathological findings and gender of patients using Chi-square test. Also, Chi-square test was used to investigate the association between vitamin D level and gender of study subjects, VDR polymorphism (Tru9I), MTC type and pathological findings. Since the vitamin D levels were not normally distributed, the comparison of vitamin D between the test and control groups was estimated by the Mann–Whitney test using GraphPad Prism 5. A *P*-value of < 0.05 was considered significant.

RESULTS

A total of 40 patients with MTC (mean age 36 ± 7.57) and 40 healthy controls (mean age 33 ± 6.02) were included in this study. Analysis showed no statistically significant difference in mean age between the test and control groups. Distribution of patients by gender, age of disease onset and MTC type are shown in Table 2.

Table 2. Baseline characteristics of patients/kindreds with medullary thyroid carcinoma indexed by age of disease onset and gender

Family/Patient No.	Gender	MTC type	Age of onset	Family/Patient No.	Gender	MTC type	Age of onset
1	M	FMTC	17	21	M	FMTC	32
2	M	FMTC	33	22	F	FMTC	26
3	M	Sporadic	54	23	F	FMTC	28
4	M	Sporadic	61	24	F	FMTC	43
5	M	Sporadic	48	25	F	MEN2B	11
6	F	FMTC	25	26	F	FMTC	32
7	F	FMTC	46	27	M	FMTC	13
8	M	Sporadic	57	28	M	Sporadic	47
9	F	FMTC	35	29	F	Sporadic	50
10	M	FMTC	48	30	F	FMTC	48
11	M	FMTC	24	31/1	F	FMTC	25
12	F	FMTC	21	31/2	F	FMTC	25
13	F	FMTC	22	31/3	F	FMTC	27
14	F	FMTC	29	32	F	FMTC	37
15	F	FMTC	37	33	M	FMTC	28
16	F	FMTC	20	34	F	FMTC	38
17	M	FMTC	37	35	F	Sporadic	61
18	F	FMTC	36	36	M	Sporadic	32
19	F	Sporadic	44	37	F	Sporadic	49
20	F	FMTC	40	38	F	FMTC	34

M: Male, F: Female, MTC: Medullary Thyroid Cancer, FMTC: Familial Medullary Thyroid Cancer, MEN2B: Multiple Endocrine Neoplasia 2B.

Table 3. Frequencies, *P*-values and Odds ratios of VDR polymorphisms in patients with medullary thyroid cancer and controls

Polymorphism	Genotype	Carcinoma	Control	*P*-value	OR (95% CI)
FokI	FF	1 (2.5%)	3 (7.5%)	Reference Genotype	
	Ff	12 (30.0%)	12 (30.0%)	0.876	1.08 (0.41–2.84)
	Ff	27 (67.5%)	25 (62.5%)	0.611	3.24 (0.32–33.22)
BsmI	BB	23 (57.5%)	16 (40.0%)	Reference Genotype	
	Bb	11 (27.5%)	20 (50.0%)	0.502	2.61 (0.98–6.92)
	bb	6 (15.0%)	4 (10.0%)	0.953	0.96 (0.23–3.95)
Tru9I	TT	21 (52. 5%)	33 (82. 5%)	Reference Genotype	
	Tt	18 (45.0%)	7 (17. 5%)	0.006	0.24 (0.09–0.69)
	tt	1 (2. 5%)	0 (0%)	1.00	(1.62–2.53)

OR: Odds Ratio; CI: Confidence Interval

Genotypes

All genotypes in the FokI and BsmI polymorphisms displayed similar frequencies in the test and control groups and no association between these polymorphisms and the risk of MTC was observed ($P > 0.05$). For the Tru9I polymorphism, the Tt genotype frequency in the test group

Table 4. Allele frequencies, *P*-values and Odds ratios of VDR polymorphisms in patients with medullary thyroid cancer and controls

Polymorphism	Allele	Carcinoma	Control	*P*-value	OR (95% CI)
FokI	F	14 (17.5%)	18 (22.5%)	Reference Allele	
	F	66 (82.5%)	62 (77.5%)	0.429	1.37 (0.63–2.98)
BsmI	B	57 (71.25%)	52 (65.0%)	Reference Allele	
	b	23 (28.75%)	28 (35.0%)	0.396	1.33 (0.68–2.60)
Tru9I	T	60 (75.0%)	73 (91.25%)	Reference Genotype	
	T	20 (25.0%)	7 (8.75%)	0.006	0.29 (0.11–0.73)

OR: Odds Ratio; CI: Confidence Interval.

Table 5. Analysis of the association of Tru9I polymorphism with type of disease and gender of MTC patients

Related to	**Tru9I Genotype (for MTC patients)**					***P*-value**	**OR (95% CI)**
Gender		Male		Female			
		N	% (within gender)	N	% (within gender)		
	TT	8	57.1	13	50.0	0.242	0.556 (0.20–1.49)
	Tt+tt[a]	6	42.9	13	50.0		
MTC type		FMTC		Sporadic			
		N	% (within MTC type)	N	% (within MTC type)		
	TT	13	43.3	8	80.0	0.044	5.23 (0.947–28.90)
	Tt+tt	16	54.3	2	20.0		
Pathological[b] findings		Aggressive		Non-aggressive			
		N	% (within pathological findings)	N	% (within pathological findings)		
	TT	3	21.4	18	69.2	0.004	0.121 (0.026–0.557)
	Tt+tt	11	78.6	8	30.8		

[a]There was only one patient with "tt" genotype in our population (a female with non-aggressive FMTC). None of the controls showed "tt" genotype. [b]Criteria for aggressive disease include: patients with stage III and IV (A, B or C) MTC or those patients who developed metastasis during the study period.

Table 6. Analysis of the association of 25-(OH) D level with gender and Tru9I genotype in study subjects

Related to	25 (OH) D level[a]	Controls						Patients					
		Male		Female		*P*	OR (95% CI)	Male		Female		*P*	OR (95% CI)
		N	% (within gender)	N	% (within gender)			n	% (within gender)	N	% (within gender)		
Gender	High (≥18.2 ng/mL)	11	61.1	6	27.3	0.031	4.19 (1.10–15.9)	10	71.4	11	42.3	0.079	3.40 (0.84–13.77)
	Low (≤18.2 ng/mL)	7	38.9	16	72.3			4	28.6	15	57.7		
Genotype	25 (OH) D level	Controls						Patients					
		TT		Tt+tt[b]		*P*	OR (95% CI)	TT		Tt+tt		*P*	OR (95% CI)
		N	% (within genotype)	n	% (within genotype)			n	% (within genotype)	n	% (within genotype)		
	High (≥18.2 ng/mL)	13	39.4	4	57.1	0.38	2.05 (0.39–10.7)	7	33.3	14	73.7	0.011	5.6 (1.42–21.94)
	Low (≤18.2 ng/mL)	20	60.6	3	42.9			14	66.7	5	26.3		

[a]Sampling was performed in winter and the cutoff point was determined based on the median vitamin D level of control samples.
[b]There was only one patient with "tt" genotype in our population (a female with non-aggressive FMTC and a vitamin D level of more than 18.2 ng/ml). None of the controls showed "tt" genotype.

was significantly different from control group ($P = 0.006$). The frequencies of FokI, BsmI and Tru9 genotypes, *P*-values and odds ratio (OR) for the test and control groups are shown in Table 3.

The allelic frequencies in the polymorphisms in the test group were also determined. Except for Tru9I, there were no notable differences in allelic prevalence between groups (Table 4). For the Tru9I polymorphism, the frequency of the t allele was significantly different between groups ($P = 0.006$). Statistical analysis showed that the frequency distribution of VDR polymorphisms in the study population attained Hardy-Weinberg equilibrium ($P > 0.05$).

Subgroup analysis of Tru9I genotype

The associations between the Tru9I polymorphism and gender, MTC type and disease aggressiveness were analyzed within the MTC patient group. According to the cross-table (Table 5), no significant association is observable between Tru9I genotype and the gender of patients. However, there is a strong association between disease aggressiveness and Tru9I genotype. According to our results the frequency of Tt and tt genotypes is significantly higher in patients with aggressive disease compared to patients with non-aggressive tumors ($P = 0.004$). It should be noted that the

Table 7. Analysis of the association of 25-(OH) D level with MTC type and disease aggressiveness

Related to	25 (OH) D[a] level (for MTC patients)					*P*-value	OR (95% CI)
MTC type[b]		**FMTC**		**Sporadic**			
		N	% (within MTC type)	N	% (within MTC type)		
	High (≥ 18.2 ng/mL)	18	60.0	2	20.0	0.017	6.90 (1.23–38.51)
	Low (≤ 18.2 ng/mL)	11	36.7	8	80.0		
Pathological[c] **findings**		**Aggressive**		**Non-aggressive**			
		N	% (within pathological findings)	N	% (within pathological findings)		
	High (≥ 18.2 ng/mL)	8	57.1	13	50.0	0.666	0.750 (203–2.77)
	Low (≤ 18.2 ng/mL)	6	42.9	13	50.0		

[a]Sampling was performed in winter and the cutoff point was determined based on the median vitamin D level of control samples. [b]There was only one patient with MEN2B disease in our population who was excluded from this analysis. [c]Criteria for aggressive disease include: patients with stage III and IV (A, B or C) MTC or those patients who developed metastasis during the study period.

majority of t alleles were present in Tt genotype, and there was only one patient with tt genotype who was a female with non-aggressive FMTC. Also, the Tt and tt genotypes were more frequent in patients with FMTC compared to those with sporadic tumors ($P = 0.044$).

25-(OH) D_3 plasma level

Plasma levels of 25-(OH) D were measured for the participants of both groups. A cutoff point was established to be used for categorizing study subjects into groups with high and low serum vitamin D levels. The cutoff point was determined based on the median of vitamin D levels obtained from control samples (18.2 ng/mL). Low vitamin D level (≤18.2 ng/mL) was more frequent in female controls ($P = 0.031$). Presumably, this is the result of clothing habits in the Iranian population. However, no significant difference in the distribution of high and low vitamin D levels was observable between male and female patients (Table 6). Our results show no significant association between serum vitamin D level and Tru9I polymorphism within the control group, whereas high vitamin D level (≥18.2 ng/mL) is more frequent in patients with Tt

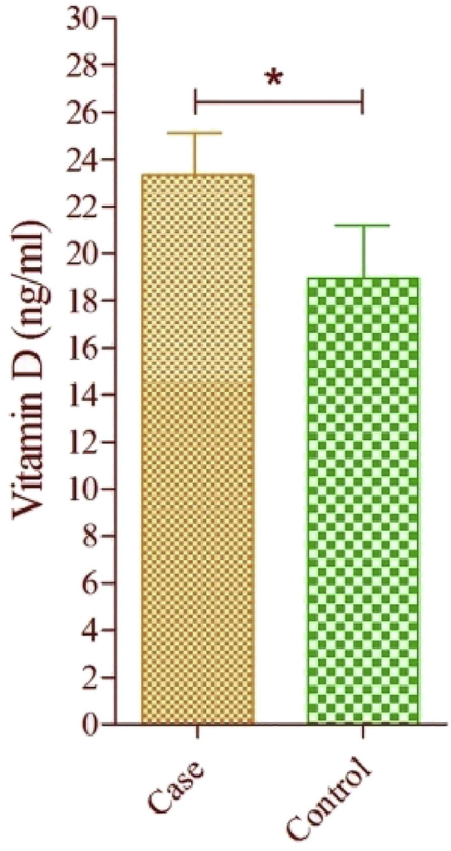

Fig. 2. Mean serum vitamin D level in test and control group ($P = 0.02$)

and tt genotypes ($P = 0.011$) (Table 6). We also performed a subgroup analysis to evaluate the association of serum vitamin D level with MTC type and pathological findings (Table 7). According to our results there is no significant association between serum vitamin D level and disease aggressiveness, whereas the frequency of high vitamin D level in patients with FMTC is significantly higher than patients with sporadic MTC ($P = 0.017$). The average serum level of vitamin D was 23.32 ng/mL for the test group and 18.95 ng/mL for the control group, and this difference was statistically significant ($P = 0.02$) (Fig. 2).

DISCUSSION

Previous studies have shown an association between the serum levels of leptin, vaspin and medullary thyroid cancer [17, 19]. To our knowledge, the present work is the first study evaluating the serum level of vitamin D and FokI, BsmI and Tru9I polymorphisms in patients with MTC compared to a healthy control group. Vitamin D has a role in different pathways, including those involved in anticancer activity, inhibition of cell proliferation and invasion, angiogenesis and metastasis of cancer cells [20, 26]. Previous findings indicate that dysfunction of VDR or vitamin D deficiency can increase the risk of chronic diseases including cancer [47]. The antitumoral effect of vitamin D through its nuclear receptor (VDR) is well-documented. Previous findings indicate that increased local action of vitamin D is associated with differentiation, reduced proliferation and favorable prognosis in papillary thyroid carcinoma (PTC) [25]. Moreover, the antiproliferative effect of vitamin D on different thyroid cell lines – especially on papillary, follicular and anaplastic cells – has been investigated, mostly attributing an anti-proliferative activity to vitamin D analogs, which is most likely mediated through overexpression of the tumor suppressor p27 [5, 32]. However, the results by the studies concerning the antitumoral effect of vitamin D on C cells are controversial. There is almost consensus upon a decreasing effect of 1,25-(OH)2 D3 (calcitriol) on calcitonin secretion [36, 48]. However, the data about proliferative effect of vitamin D on C cells are very inconsistent. In contrast to antitumorigenic activity of vitamin D on most cancer cells, some findings indicate that vitamin D treatment results in enhanced cell proliferation in C cells (TT cell line) through increased gene expression of the c-myc oncogene [2, 6, 48, 49, 50]. These findings may partly explain the inconsistency of our results on vitamin D level with other studies. Based on the literature, the association between low vitamin D level and increased risk of most cancers is evident. However, for thyroid cancers, there are few studies evaluating the mentioned relationship. The data from clinical studies provide inconsistent information regarding the relationship between vitamin D status and risk of thyroid cancers [23, 27, 28, 41, 42, 45]. To our knowledge, there is only one isolated study reporting results consistent with our data on on vitamin status and MTC. This study introduces vitamin D supplementation as a risk factor for medullary thyroid cancer (OR: 1.8) [40]. Referring to our results, it is clear that although MTC patients exhibit higher vitamin D levels in comparison to control subjects, the mean vitamin D level is lower than the normal range for both MTC patients and the control group. It is very important to note that the vitamin D level in the Iranian population is significantly lower than what is established as the normal range by the world health authorities, and this is possibly due to the clothing, cultural and social norms.

VDR genes are located on the long arm of chromosome 12 and comprise 8 protein coding exons (exons 2–9) and 6 non-translated exons (exons a1-f1). Studies have shown that single

nucleotide polymorphisms on exon 2 (FokI), intron 8 (BsmI and Tru9I) and exon 9 are associated with increased risk of ovarian [14], breast [43], colon [35], prostate [22], skin [9], follicular and papillary thyroid [37] cancers. The FokI polymorphism, located on the 5′ end, is a T to C substitution in the initial codon and increases the transcriptional activity of the VDR. The resulting difference of three amino acids in VDR length may affect the function of the protein. The polymorphisms located on the 3′ end of the VDR gene do not alter the amino acid sequence; however, it has been reported that the BsmI and Tru9I polymorphisms on the 3′ end may alter transcriptional activity and mRNA degradation [1].

In the present study, the genotypes and alleles of the FokI and BsmI polymorphisms showed no significant association with the risk of MTC. For the Tru9I polymorphism, the Tt genotype and t allele frequencies in the test group were significantly different from the control group, indicating a considerable increased risk for MTC.

A similar study that evaluated vitamin D receptor polymorphism in patients with follicular and papillary thyroid cancer showed a significant association between FokI polymorphism and follicular cancer, but not papillary thyroid cancer. *Taq*I and BsmI polymorphisms showed no significant relationship [37]. Another similar study found no significant association between TaqI, Tru9I and BsmI polymorphisms with follicular and papillary thyroid cancers in the Iranian population; however, there was a significant difference in allele frequencies of the Tru9I polymorphisms between the test and control groups [15].

Significant associations between *Taq*I, Tru9I and BsmI polymorphisms and increased risk of melanoma, prostate, breast and ovarian cancers have been documented in previous studies [10, 24, 46]. Controversially, other studies have observed no significant association between these polymorphisms and prostate and ovarian cancer [4, 34].

Unexpectedly, in our study population, the plasma level of 25–(OH) D3 in the MTC group was significantly higher than in control subjects, whereas many studies reported an inverse relationship between sera levels of vitamin D and colon [13], breast [39], prostate [12] and follicular thyroid (37) cancer. Overall, the epidemiological evidence suggests limited and controversial results about this relationship [12, 39].

The results of the present study demonstrated that the Tru9I polymorphism of the VDR gene may commonly contribute to the risk of medullary thyroid cancer. Since, the present study is the first one focusing on patients diagnosed with MTC, and the sample size was relatively small due to the low rate of prevalence of this cancer, more studies with larger sample sizes are needed to confirm the results.

Conflict of interest: The authors declare that they have no conflict of interest.

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