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Association of vitamin D receptor gene polymorphisms with osteosarcoma risk and prognosis

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ABSTRACT

Objective: Through its receptor (VDR), vitamin D_3 plays an important role in a wide variety of cellular processes. Polymorphisms in VDR gene have been linked to risk of various cancers and their prognoses. We conducted a case-control study to analyze he relationship of VDR gene polymorphisms with the occurrence and prognosis of osteosarcoma.

Methods: Fifty-eight osteosarcoma patients and 75 healthy controls were included in the study. Single nucleotide change polymorphisms (SNPs) in *Cdx2, FokI, BsmI, ApaI* and *TaqI* regions of VDR gene were examined with SNaPshot mini-sequencing technique. Allele and genotype frequencies in patients and controls were compared. The association of polymorphic genotypes with osteosarcoma was evaluated. The relationship of the presence of polymorphism to prognostic parameters and survival rates were also analyzed.

Results: Allele and genotype frequencies of *Cdx2, FokI, BsmI, ApaI* and *TaqI* regions in VDR gene were found to be similar in patients and controls. Polymorphisms in these regions were not associated with osteosarcoma risk. In patients having *Cdx*2 polymorphic allele, tumor volume was greater (p:0.041), metastasis was more common (p:0.042) and histopathological response to chemotherapy was worse (p:0.044). Good histopathological response was significantly higher in patients with *BsmI* homozygous polymorphism (p:0.037). In the presence of heterozygous *Cdx2* and homozygous a higher three-year overall survival rate was found, while there was a higher event-free survival rate in patients with *ApaI* polymorphisms.

Conclusion: Our results suggested that although polymorphisms of VDR gene are not related to the development of osteosarcoma, they may be important for prognosis. Understanding the effect of VDR polymorphisms on osteosarcoma will be important in identifying new prognostic parameters and new targets for treatment.

1. Introduction

The active form of vitamin D, 1,25 dihydroxy vitamin D_3 (vitamin D_3), plays an important role in calcium homeostasis and bone metabolism [1]. It is clear that it also has effects on a wide range of fundamental biological functions such as cell cycle regulation, differentiation and immunomodulation. The molecular mechanisms of these effects are complex. The influence of vitamin D_3 upon the expression of several transcription factors, cell cycle arrest proteins and growth factors may explain these complex mechanisms [1,2]. Most of the biological activity of vitamin D_3 is mediated through its nuclear receptor (VDR). VDR binds to vitamin D_3 with high affinity and controls the expression of target genes by acting as a ligand activated transcription factor [2].

Changes in level of vitamin D_3 and variations in VDR have been linked to a variety of diseases such as recurrent infections, osteoarthritis, osteoporosis, diabetes mellitus, and cardiovascular and autoimmune diseases [3,4]. Moreover, strong epidemiological evidence showed a relationship between vitamin D_3 deficiency and several types of cancer [5,6]. Numerous genetic and biological studies established that alterations in VDR gene are also associated with cancer. Single nucleotide changes causing variations in DNA sequence, referred to as polymorphism, occur in more than 1% of the population. Single nucleotide change polymorphisms (SNPs) in VDR gene have been connected to the risk of various cancers such as breast, prostate and colon carcinomas and their prognoses [4,7]. However, there are only a few studies evaluating the relationship between malignant bone tumors and the polymorphisms in VDR gene which is a key regulator of bone

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Table 1
Allel frequency in *Cdx2, FokI, BsmI, ApaI, TaqI* region of VDR gene in patients and controls.

	FokI		*BsmI*		*ApaI*		*TaqI*		*Cdx2*	
	F	f	B	b	A	a	T	t	G	g
Patients	77.5%	2.5%	57.8%	42.2%	79.3%	20.7%	40.3%	59.4%	33.6%	66.4%
Control	72%	28%	62.6%	37.4%	80%	20%	43.5%	56.6%	34%	66%
P	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05

health. We conducted a case-control study to analyze the relationship of SNPs in VDR gene with the risk of occurrence and the prognosis of osteosarcoma.

2. Material and methods

Fifty-eight pediatric osteosarcoma patients diagnosed in the Ankara Oncology Research and Training Hospital were included in the study. The age and gender of patients, the location, size and histopathological subtype of the tumor, response to treatment and survival times were recorded. As a control group, 75 age- and sex-matched children, who were admitted to hospital for various reasons other than chronic diseases or malignancies, were also included in the study. After informed consent had been obtained from all the families, a 2 ml venous blood sample was drawn from patients and controls. Samples were stored at −20 °C in EDTA-containing tubes for future analysis.

After the thawing of blood samples, nucleic acid isolation was performed via the spin-colon method (Qiagen®, QIAamp DNA Mini Kit). *Cdx2, FokI, BsmI, ApaI* and *TaqI* regions of VDR gene were amplified. Multiplex PCR was performed with the in-house designed primers. PCR mixture; 10 ng of genomic DNA was set to be 0.1 mmol/L of each primer (forward and reverse), 0.4 mmol / L dNTP, 3.0 mmol/L MgCl2, 10x PCR buffer, and 1 U Taq polymerase and final volume of 20 μL. Post-PCR purification was performed with 5 μl SNaPshot ready reaction mix (Applied Biosystems, Inc.), 2 μl purification product, 0.4 μM primer (for each parameter) in 10 μl reaction volume. After the Minisequencing reaction, 10 μl of final volume 1 was treated with SAP for 60 min at 37 °C. Enzyme inactivation was then performed at 75 °C for 15 min. Minisequencing products (0.5 μl) were mixed with 9 μl of Hi-Di formamide and 0.5 μl GeneScan-120 LIZ size standard (Applied Biosystems) and denatured at 95 °C for 5 min. Fluorescent labeled products were analyzed with an ABI 3130 Genetic Analyzer (Applied Biosystems). The obtained data were evaluated with GeneMapper 4.0 software (Applied Biosystems Co. Ltd., USA).

Allele and genotype frequencies of patients and control groups were identified and compared with the chi-square test. Association of polymorphic genotypes with osteosarcoma was evaluated by using Mantel–Haenszel test and odds ratios were calculated with a 95% confidence interval. The relationship between the prognostic parameters and the presence of polymorphisms were analyzed with chi-square test. Survival rates were calculated with Kaplan–Meier test. The relationship of the presence to polymorphisms and survival rates were analyzed with log-rank test.

3. Results

In the osteosarcoma group, there were 33 boys (56.8%) and 25 girls (43.2%) with a mean age of 12.45 ± 3.48 (4–18) years. Fifty-two percent of the control group was male (*n*:39) and 48% was female (*n*:36) and the mean age was 11.01 ± 3.9(5–19) years. There were no clinical findings of hypovitaminosis D in neither patient nor the control group. Although vitamin D levels were not specifically measured, no laboratory evidence of hypovitaminosis D, such as hypocalcemia, was also not present in the patient group.

Tumor locations were the lower extremities in 48 patients (82.8%), upper extremities in five (8.6%) and flat bones in five (8.6%). Histopathological subtypes were osteoblastic in 41 cases (70.7%), other types (chondroblastic, fibroblastic and small cell type) in seven (13.8%) and un-classified in nine (15.5%). Tumor volume was more than 200 mm^3 in 20 patients (34.5%), less than 200 mm^3 in 36 (62.1%) and un-determined in 2 (3.4%). Twelve patients (20.7%) had metastatic disease (6 lung, 4 lung&bone, 2 others) at diagnosis. Patients were treated with MAP protocol (methotrexate 12 gr/m^2, doxorubicin $2 \times 30\ mg/m^2$, cisplatin $120\ mg/m^2$). After chemotherapy the percentage of necrosis in tumor tissue was higher than 90% in 23 cases (39.7%), less than 90% in 17 (29.3%) and unknown in 18 (31%).

Allele frequency of all studied regions in patients with osteosarcoma and the control group were similar (Table 1). In osteosarcoma patients, the frequency of homozygous and heterozygous polymorhisms were 6.9% and 18%, respectively in *FokI* region, 12.1% and 60.3% in *BsmI* region, 32.8% and 53% in *Apa I* region, 37.9% and 56.9% in *TaqI* region, and 3.4% and 34.5% in *Cdx2* region. The differences in genotype frequencies of the patient and control group were not significant (Table 2). The presence of heterozygous polymorphism in *BsmI* (OR:1.48), *ApaI* (OR:1.46) and *TaqI* (OR:1.35) and *Cdx2* (OR:1.12) regions seemed to increase osteosarcoma risk. However, the increased odds did not reach statistical significance since the 95% confidence interval spanned 1.0 (Table 3).

The relationship of SNPs in VDR gene to the prognosis of disease were also analyzed. Tumor volume was more than 200 mm^3 in 81.8% of patients who carried the polymorphic *Cdx*2 allele while this ratio was 55.5% in patients with wild type genotype (*p*:0.041). Frequency of metastatic disease was more common in patients who had the *Cdx*2 polymorphic allele than those having the wild type genotype (36.6% vs 11.1%, *p*:0.042). Furthermore, good histopathological response to chemotherapy (>90%) was obtained in 43.8% of patients with polymorphic *Cdx2*, while this ratio reached 76.2% in patients with wild type *Cdx2* (*p*: 0.044). More than 90% necrosis was determined in all patients with *BsmI* homozygous polymorphic genotype, but this ratio was 54.8% in patients with wild type *BsmI* (*p*:0.037). Patients were followed for a median of 49.5 months after treatment. The three-year overall survival rate was lower in patients with heterozygous polymorphic *Cdx2* than in those with wild type although the difference was not statistically significant (73.3% and 92.6%, *p*: 0.06). The three-year event-free survival rate was significantly higher in the presence of homozygous polymorphic *ApaI* than others (87.2% vs. 51.2% *p*: 0.015). The other polymorphic genotypes had no effect on survival.

4. Discussion

In-vitro and in-vivo studies have shown that vitamin D_3 exerts an inhibitory effect on the development and progression of cancer. Various cellular functions of vitamin D_3, including inhibition of proliferation, induction of apoptosis and differentiation, modulation of growth factor-mediated signaling and inhibition of angiogenesis play a role in its anticancer effects [2,5,6]. Binding of the vitamin to VDR and its nuclear activation are necessary for the biological effects to occur. VDR, which can be categorized as a ligand activated transcription factor, is a member of the nuclear receptors superfamily of steroid hormones and is present in more than 30 tissues and organs [3,4,8]. VDR expression has also been determined in many types of cancer cells such as breast, prostate, pancreas, colon, bladder, cervix, melanoma, leukemia and

Table 2
Polymorphism frequency in *Cdx2, FokI, BsmI, ApaI, TaqI* region of VDR gene in patients and controls.

	FokI polymorphism			*BsmI* polymorphism			*ApaI* polymorphism			*TaqI* polymorphism			*Cdx2* polymorphism		
	Homozygous (ff)	Heterozygous (Ff)		Homozygous (bb)	Heterozygous (Bb)		Homozygous (aa)	Heterozygous (Aa)		Homozygous (tt)	Heterozygous (Tt)		Homozygous (Gg)	Heterozygous (gg)	
Patients	31.0%	6.9%		60.3%	12.1%		34.5%	3.4%		13.8%	53.4%		5.2%	56.9%	
Control	42.7%	6.7%		50.7%	12.0%		32%	4.0%		21.3%	44.0%		9.3%	49.3%	
P	> 0.05	> 0.05		> 0.05	> 0.05		> 0.05	> 0.05		> 0.05	> 0.05		> 0.05	> 0.05	

lymphoma [7,9].

It was shown that when exposed with carcinogenic agents, VDR knockout mice exhibit an enhanced susceptibility to development of cancer. An increased risk of development and progression of epithelial tumors such as colon and skin carcinoma has been reported in VDR knockout mice [10]. However, there were no such studies on osteosarcoma.

Single nucleotide polymorphisms causing a subtle sequence variation in VDR gene, which is located in chromosome 12q13.1, usually have only a minimal or modest effect. But sometimes they may influence protein synthesis and function as well as receptor affinity and binding to nuclear DNA [3,4]. Such polymorphisms, which are supposed to modulate the activity of the VDR gene, may potentially exert significant effects on the whole of the VDR-mediated signaling mechanism and alter the response to vitamin D_3 and its biological functions [3,4].

Although a large number of polymorphic area have been identified in VDR gene, *FokI, BsmI, TaqI, ApaI* and *Cdx2*, which may have functional importance, are the most frequently studied. It is for this reason that we chose to analyze the polymorphisms of these regions. *FokI* is located at the 5′ end in exon 2, the coding region of the gene. The *FokI* polymorphism that alters the start codon results in the production of an elongated protein which is less active [3,4,8,9]. Several studies showed that the ff and Ff genotypes of the *FokI* are associated with decreased transcriptional activity [3,4,8]. *BsmI and ApaI* (in intron 8) and *TaqI* (in exon 9) are located in the 3′ untranslated region of the VDR gene. Polymorphisms in these regions may influence messenger RNA stability and protein translation efficiency [3,4,8]. The *Cdx2* is located in the 1e promoter region of the gene and its polymorphism may affect the binding of specific transcription factors [3,4,8].

Significant association between various VDR polymorphisms and the development and outcome of various adult cancers such as breast (*FokI, BsmI, ApaI*), prostate (*FokI, BsmI, TaqI*), skin (*FokI, BsmI, TaqI*), colorectal (*FokI, BsmI*), ovary (*FokI, ApaI*) and renal cell carcinomas (*FokI, ApaI*) have been reported [7,9,11,12]. However, different results were observed in some other case-control and cohort studies [9,12]. Several meta-analyses which were conducted to clarify the relationship between these polymorphisms and cancer showed that there is a significant increase in the malignant melanoma and breast cancer risk in *FokI* ff genotype, a significant increase in colorectal carcinoma risk in *TaqI* tt genotype. A significant increase in the risk of all cancers types in *Cdx2* gg genotype, while a significant decrease was found in the development of cancer at any site [11–15]. Data indicating an association between VDR polymorphisms and cancer prognosis are strongest for breast cancer (*BsmI, TaqI*), prostate cancer (*FokI*), malignant melanoma (*BsmI*) and renal cell carcinoma (*TaqI*) [11–13].

There are several studies on the role of VDR gene polymorphisms in the occurrence and prognosis of pediatric cancers. Bienertova–Vasku et al. [16] investigated *FokI, BsmI, TaqI, ApaI and Cdx2* polymorphisms of VDR gene in 111 pediatric cases with different types of malignant solid tumors. They observed that the polymorphic genotype in *FoxI* was weakly associated with a reduced risk of susceptibility to cancer. They found that the survival rate of patients carrying heterozygous SNPs in *BsmI* and *TaqI* regions were significantly worse than patients with homozygous genotypes [16]. Yılmaz et al. [17] reported that there was no relationship between polymorphisms in *Taq1, Fok1* and *Bsm1* regions and the occurrence of brain tumors in 32 children. Tekgündüz et al. [18] found no significant difference between the 95 pediatric Hodgkin's lymphoma cases and controls in terms of *Cdx2, Fok1, Bsm1, Apo1* and *Taq1* polymorphisms. They concluded that VDR polymorphisms do not play a role in Hodgkin's lymphoma development. Dawidowska et al. [19] observed that polymorphic variants of *BsmI* were significantly implicated in minimal residual disease on day 15 in pre-B cell ALL.

There is limited data linking VDR gene polymorphisms with osteosarcoma risk and prognosis. However, several experimental studies

Table 3
Polymorphism frequency in *Cdx2, FokI, BsmI, ApaI, TaqI* region of VDR gene in patients and controls and association with osteosarcoma risk.

	VDR aleli	Hasta	Kontrol	OR	%95 CI	p
FoxI	Wild	36 (%62.0)	37 (%49.3)	1.68	0.83–3.37	0.14
	Heterozygous polimorphic	18 (%31)	32 (%42.7)	0.60	0.29–1.24	0.17
	Homozygous polimorphic	4 (%6.9)	6 (%8.0)	0.85	0.22–3.17	0.81
Bsm1	Wild	16 (%27.6)	28 (%37.3)	0.63	0.30–1.34	0.23
	Heterozygous polimorphic	35 (%60.3)	38 (%50.7)	1.48	0.74–2.96	0.26
	Homozygous polimorphic	7 (%12.1)	9 (%1.02)	1.07	0.35–2.88	0.99
Apa1	Wild	19 (%32.8)	26 (%34.7)	0.97	0.47–2.01	0.94
	Heterozygous polimorphic	31 (%53.4)	33 (%44.0)	1.46	0.73–2.90	0.28
	Homozygous polimorphic	8 (%13.8)	16 (%21.3)	0.59	2.23–1.49	0.26
Taq1	Wild	22 (%37.9)	31 (%41.3)	0.86	0.43–1.75	0.69
	Heterozygous polimorphic	33 (%56.9)	37 (%49.3)	1.35	0.38–2.70	0.38
	Homozygous polimorphic	3 (%5.2)	7 (%9.3)	0.53	0.13–2.14	0.36
Cdx2	Wild	36 (%62.1)	48 (%64.0)	0.92	0.45–1.87	0.82
	Heterozygous polimorphic	20 (%34.5)	24 (%32.0)	1.12	0.54–2.31	0.76
	Homozygous polimorphic	2 (%3.4)	3 (%4.0)	0.85	0.13–2.31	0.87

showed the effect of vitamin D_3 and VDR interaction on various cellular processes in osteoblasts whose malignant transformation results in osteosarcoma. Antiproliferative, differentiating and proapoptotic effects of vitamin D_3 on osteoblasts have been reported. Vitamin D_3 has been shown to inhibit cell cycle progression at the G1 phase and to promote osteoblast differentiation [19,20]. It was shown that VDR was expressed in osteosarcoma cells derived from cell lines or animal tumors, which indicates that osteosarcoma cells recognize and potentially respond to vitamin D_3 [21,22]. There are experimental studies which demonstrated that vitamin D_3 inhibits proliferation and enhances differentiation in osteosarcoma cells as it does in osteoblasts. For instance, Thomson et al. [21] demonstrated that human osteosarcoma cell lines respond to vitamin D_3 by undergoing differentiation and apoptosis. Barroga et al. [23] reported that the stimulation of canine osteosarcoma cells with vitamin D_3 results in their differentiation to more mature cells that exhibit properties of functionally mature osteoblastic bone cells. Davies et al. [22] observed a negative correlation between VDR expression and Ki-67 proliferation indexes in canine osteosarcoma cells although there was no relationship with tumor grade, type or location. Gallagher et al. [24] showed the expression of VDR in human osteosarcoma tissues obtained from the tumors of primary or metastatic osteosarcoma. They did not find any relationship between VDR expression and tumor grade, type, location, patients' age or sex. Wu et al. [25] observed that vitamin D_3 treatment reduced cell proliferation only in human osteosarcoma cells that express native levels of VDR protein. They also showed that anti-proliferative actions of vitamin D_3 involve VDR mediated activation of the MAPK pathway. Yang et al. [26] found that inhibition of Snail-1, which is overexpressed in osteosarcomas, could enhance anti-proliferative activity of vitamin D_3 by increasing the expression of VDR.

In the light of these data, we investigated the role of polymorphisms in *FokI, BsmI, TaqI, ApaI* and *Cdx2* regions of VDR gene in the development, prognostic parameters and outcomes of osteosarcoma. We could not find a significant association between these polymorphisms and osteosarcoma occurrence. In the literature, there were only two clinical studies evaluating the association between the occurrence of osteosarcoma and variations in VDR gene. Miller et al. [27] investigated the role of deletions, rearrangements and point mutations of VDR gene in a variety of cancers including 68 osteosarcoma cases. Only two alterations, one silent mutation in codon 79, and a base change in intron 3, were detected in two osteosarcoma samples. They concluded that changes in VDR gene do not play a role in the development of either osteosarcoma or other cancers studied. Ruza et al. [28] analyzed the frequency of the genotypes for the *FokI, ApaI* and *TaqI* polymorphisms of VDR gene in Spanish osteosarcoma patients. They showed a significantly higher frequency of the Ff genotype for the *Fok I* polymorphism compared to controls. The odds ratio for this genotype was found to be 1.78 with an increased relative risk of 78% for heterozygous Ff carriers. However, in our study, no significant correlation was found between the risk of osteosarcoma and *FokI* polymorphism.

We also analyzed the relationship between the polymorphisms in VDR gene and prognostic parameters and survival rates. Larger than 200 mm^3 tumor volume, presence of metastasis, and less than 90% necrosis in tumor tissue after chemotherapy are known to be related with worse prognoses in osteosarcoma [27]. We found that patients carrying the *Cdx*2 polymorphic allele have all three bad prognostic factors significantly more frequently. Furthermore, the three-year overall survival rate was lower in patients with the heterozygous polymorphic *Cdx2* genotype. Although there were no studies in the literature which showed the effect of Cdx2 on the prognosis and outcome of cancers, meta-analyses suggested that the Cdx2 polymorphism significantly increased overall cancer risk [14,15].

In conclusion, our results suggested that polymorphisms of VDR gene are not related with the development of osteosarcoma, but that they, especially in Cdx2 region, may have a prognostic importance. Osteosarcoma is the most common malignant bone tumor in children and adolescents. At present, the survival rate of the disease has not exceeded 60–70% with the current managements [29]. Therefore, there is a need to identify novel therapeutic approaches to improve the outcome of disease. Understanding the effect of VDR polymorphisms on osteosarcoma prognosis will be important in this aspect, which may make it possible to determine new targets for treatment.

Conflict of interest statement

Authors have no conflicts of interest or financial support to declare.

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