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Association of Vitamin D Receptor Gene Polymorphism with Renal Cell Carcinoma in Japanese

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Abstract. Molecular epidemiologic studies have reported a relationship between 1α,25 dihydroxyvitamin D3 ($1,25(OH)_2D_3$) and the development and progression of malignant tumors. ($1,25(OH)_2D_3$) exerts its biological activity by binding the vitamin D receptor (VDR), while recent studies have demonstrated that VDR gene polymorphisms affect serum levels of ($1,25(OH)_2D_3$). Serum levels of ($1,25(OH)_2D_3$) are reported to be significantly lower in patients with renal cell carcinoma (RCC) compared to non-cancer control patients. The purpose of this study was to investigate the TaqI VDR polymorphism in Japanese RCC patients and non-cancer controls in order to determine if an association exists between VDR genotype and the risk of developing RCC as well as clinical risk factors. A total of 102 RCC patients and 204 controls were genotyped for a previously described TaqI restriction fragment length polymorphism (RFLP) of the VDR gene. Products were digested into T allele or the t allele according to the absence or presence of a TaqI restriction site. Individuals were classified as TT, Tt or tt. The genotype TT was statistically more frequent among RCC patients (80.4%) compared to controls (61.8%) (OR = 2.54; 95% CI, 1.44–4.46; p = 0.0006). In addition, the occurrence of the genotype TT was significantly higher in patients with rapid-growth-type group (92.1%) compared to slow-growth-type group (73.4%) (OR = 4.22; 95% CI, 1.15–15.53; p = 0.0175). These data demonstrate that VDR genotype plays an important role in determining the risk of developing more aggressive RCC in Japanese.

Key words: Vitamin D receptor, Gene polymorphism, RCC, Japanese

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VITAMIN D is ingested in the diet and generated in the form of vitamin D3 in the skin by UV irradiation. Vitamin D3 subsequently undergoes sequential hydroxylation in the liver and kidney to form 1α,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$) or 24,25-dihydroxyvitamin D3 ($24,25(OH)_2D_3$) [1, 2]. $1,25(OH)_2D_3$ is called active vitamin D3 and exhibits the most prominent biological activity in the regulation of calcium homeostasis [1, 3].

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However, active vitamin D3 ($1,25(OH)_2D_3$) also has other important roles including the regulation of immunological function as well as modulating the processes of cell differentiation and carcinogenesis with documented effects upon oncogene expression [4]. A recent study has demonstrated that serum levels of active vitamin D3 were significantly lower in patients with renal cell carcinoma (RCC) compared to controls [4, 5]. Active vitamin D3 exerts its multiple biological effects by binding the vitamin D receptor (VDR), a nuclear hormone receptor [6]. The VDR gene is known to have polymorphisms that may result in variation in VDR expression as well as in the circulating levels of active vitamin D3 [7, 8]. The VDR alleles can be analyzed using restriction fragment length polymorphisms (TaqI, BsmI, ApaI) that

lie in the region from exon 8 to the 3′ untranslated region (3′UTR). The 2 most common VDR alleles are BAt and baT. In the BAt allele, the restriction sites for BsmI and ApaI are absent but the restriction site for TaqI is present, whereas the opposite is true for the baT allele (BsmI and ApaI are present but TaqI is absent). These alleles are in linkage disequilibrium and occur together in 97% of cases [7]. Therefore, we have developed an assay for one of the candidate sequences viz. the TaqI polymorphism. In this study, we tested the hypothesis that the genotype TT, which is associated with lower circulating levels of active vitamin D3 [7, 8], would be associated with an increased risk of RCC in Japanese compared to the Tt and tt genotype. In addition, we examined possible associations between the VDR polymorphisms and tumor growth type in RCC patients.

Materials and Methods

Subjects

In the present case-control study, a total of 102 patients (77 men, 25 women) with RCC and 204 controls (162 men, 42 women) were enrolled from our urology clinic. There was no statistical difference in age at diagnosis between the two groups (59.1 ± 10.1 vs 60.6 ± 14.3, RCC patients vs controls, $p = 0.210$; unpaired Student's t test) (Table 1). All cases and controls gave their informed consent to participate in this study. The diagnosis of RCC was confirmed histologically following nephrectomy while lymph node or distant metastatic lesion was diagnosed by histological or radiological findings. All the controls consisted of urology clinic patients with benign retroperitoneal and genitourinary diseases. Physical, serological and radiological examinations were performed in all controls in order to exclude the possibility of RCC or other malignant disease. Blood samples were obtained between March 1996 and September 2001 from both patient groups, and patient data were obtained from the medical records.

Table 1. Characteristics of renal cell carcinoma patients and control patients.

Parameter	RCC[a] patients	Control patients
No. of patients	102	204
Gender		
Male	77	162
Female	25	42
Age (mean ± SD[b])	59.1 ± 10.1[c]	60.6 ± 14.3[c]

[a] RCC; renal cell carcinoma, [b] SD; standard deviation
[c] $p = 0.210$; unpaired student's t test

Genotyping

Genomic DNA was isolated from peripheral leukocytes by proteinase K digestion and phenol/chloroform extraction. The VDR TaqI genotype was determined by a polymerase chain reaction (PCR) method described by Riggs *et al.* [9]. A 740 base pair (bp) fragment was generated by PCR with primers located within intron 8 and exon 9. The primer sequences were 5′cag agc atg gac agg gag caa 3′(forward) and 5′gca act cct cat ggc tga ggt ctc 3′(reverse). The 35 cycles were performed using Taq polymerase (Perkin Elmer Co., Ltd., NJ, USA) at the following conditions: denaturation (94°C, 1 min), annealing (57°C, 1 min) and extension (72°C, 1 min). Following completion of the PCR, 10 μL of PCR products were removed, subjected to restriction digestion with TaqI (Takara Shuzo Co., Ltd., Kyoto, Japan) at 65°C for 3 h and run on 3% Nusieve agarose gels [10]. The presence of a C>T change at the third position of codon 352 in exon 9, which is the code for isoleucine, is associated with the loss of a TaqI restriction site. The resulting alleles are designated T (TaqI site absent; 2 fragments of 495 bp and 245 bp) or t (TaqI site present; 3 fragments of 290 bp, 245 bp and 205 bp). Individuals were therefore classified as TT, Tt or tt (Fig. 1) [10].

Examination of tumor growth type, stage of disease and pathological grade of tumor in RCC patients

The patients with RCC were determined using prognostic items; exacerbated erythrocyte sedimentation rate (ESR), elevated C-reative protein (CRP), elevated serum α2-globulin, and pyrexia. Patients with three or more positive items was classified as having a rapid-growth-type tumor that were susceptible to early recurrence or metastasis. The other patients were classified as having a slow-growth-type tumor [5, 11]. Stage of disease and grade of tumor of patients with RCC were evaluated at diagnosis using the General Rules for Clinical and Pathological Studies on Renal

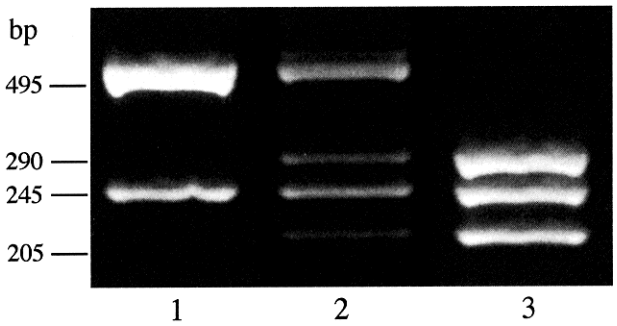

Fig. 1. PCR fragment patterns obtained for the three possible genotypes after TaqI digestion of the 740 bp amplified region of the VDR gene. The 245 bp fragment is constant in all genotypes, which is a control cut created by a nonpolymorphic TaqI site within amplification. Lane 1: genotype TT (2 fragments of 495 bp and 245 bp), Lane 2: genotype Tt (4 fragments of 495 bp, 290 bp, 245 bp and 205 bp), Lane 3: genotype tt (3 fragments of 290 bp, 245 bp and 205 bp.).

Cell Carcinoma [12].

Statistical analysis

The relative associations between RCC patients and controls were assessed by calculating odds ratios (OR) from contingency tables. The OR and 95% confidence intervals (CI) were calculated by multiple regression analysis using the JMP program package (Version 3, SAS Institute Inc., NC, USA). Statistical significance was defined as a p value of less than 0.05.

Results

Frequency of the VDR alleles in RCC patients and controls

The first major finding of this study was that the occurrence of the TT genotype, which is associated with lower circulating levels of active vitamin D3 [7, 8], was significantly higher among RCC patients (80.4%) than non-cancer controls (61.8%) (OR = 2.54; 95% CI, 1.44–4.46; p = 0.0006) (Table 2). These data suggest that the TT genotype is associated with an increased risk of RCC in Japanese compared to Tt and tt genotype.

Frequency of the VDR alleles of RCC patients categorized by tumor growth type

We also examined the association between the VDR genotypes and tumor growth type in RCC patients. The occurrence of the genotype TT was significantly higher in patients with rapid-growth-type group (92.1%) compared to slow-growth type group (73.4%) (OR = 4.22; 95% CI, 1.15–15.53; p = 0.0175) (Table 2). All 38 patients of rapid-growth type group were locally advanced or metastatic disease (extrarenal disease: pT3a–c/pT4/N1–2/M1) and high grade tumor (grade 3). In 64 patients with slow-growth type group, locally advanced or metastatic disease and high grade tumor were only 5 patients and 2 patients, respectively. Therefore, the second major finding of this study is that the genotype TT is associated with a rapid-growth-type tumor in RCC patients.

Table 2. Frequency of the vitamin D receptor alleles using the TaqI restriction fragment length polymorphism in renal cell carcinoma patients and controls, and in patients categorized by tumor growth type.

	Genotype; n (%)			TT vs (Tt + tt) OR[a] (95% CI[b])	*p*
	TT	Tt	tt		
Controls (n = 204)	126 (61.8)	70 (34.3)	8 (3.9)	1	
[c]RCC (n = 102)	82 (80.4)	19 (18.7)	1 (0.9)	2.54 (1.44–4.46)	0.0006
Between growth types of RCC					
Slow growth type (n = 64)	47 (73.4)	16 (25.0)	1 (1.6)	1	
Rapid growth type (n = 38)	35 (92.1)	3 (7.9)	0 (0.0)	4.22 (1.15–15.53)	0.0175

[a] OR; Odds ratio, [b] 95% CI; 95% confidence interval, [c] RCC; Renal cell carcinoma.

Discussion

The incidence of breast cancer and colon cancer in various geographic populations exhibits a negative correlation with exposure to UV irradiation [13, 14]. Also, recent reports have demonstrated a significant north-south trend of prostate cancer mortality rates in the USA with higher mortality rates being found in the northern USA, possibly implicating the lack of active vitamin D3 [15, 16]. These epidemiological studies suggested that exposure to UV irradiation may partially protect against the development and progression of cancer of the breast, colon and prostate. A possible mechanism for the proposed protective effect of UV irradiation has been suggested by recent studies that have documented an association between VDR polymorphisms and the development of prostate cancer [17–19]. Recent study demonstrated clinical and pathological significance of VDR gene polymorphism for Japanese prostate cancer. In that study, the genotype TT, which is associated with lower circulating levels of active vitamin D3, was significantly associated with a more advanced clinical stage of disease and a higher pathological grade of tumor in prostate cancer patients. VDR polymorphisms do play an important role in determining the clinical and pathological risk factor of prostate cancer in Japanese [10].

In this context it is interesting to note the recent work by Fujioka *et al.* [5] which documented that the serum levels of active vitamin D3 were significantly lower in RCC patients compared to controls in Japanese. Furthermore, they demonstrated that the serum levels of active vitamin D3 were significantly lower in RCC patients with T3 and T4 disease (tumor invasive from outside the kidney) compared to those with T1 and T2 disease (tumor located inside the kidney). Lastly, they demonstrated that the serum levels of active vitamin D3 were significantly lower in RCC patients with rapid-growth tumor compared to those with slow-growth tumor [5]. Currently, there is a scarcity of data regarding the associations of VDR genotype with RCC patients. In this study, the genotype TT, which is associated with lower circulating levels of active vitamin D3, was significantly higher among RCC patients compared to controls. Moreover, the occurrence of the TT genotype was significantly associated with a rapid-growth tumor in RCC patients. Active vitamin D3 exerts its biological effect by binding to the VDR within the nucleus of the target cell [6]. Morrison *et al.* [7] demonstrated that the BAt allele had 140% greater receptor activity than the baT allele with this difference being proposed to be secondary to either enhanced gene transcription or increased mRNA stability thereby resulting in individual variation in VDR expression [7, 17].

In 1981, active vitamin D3 was noted to suppress the growth of murine myelogenic leukemia (M1 cells) and to induce their differentiation into macrophage-like cells [20]. Since then, the effects of active vitamin D3 action upon cell growth and differentiation have attracted considerable attention. The VDR has been found in both normal and malignant tissue [21, 22]. Recent reports demonstrated that active vitamin D3 stimulated fibronectin synthesis in several human cell lines and inhibited angiogenesis in chick chorioallantoic membranes [1, 23, 24]. Since tumor growth and metastasis necessitates both penetration of extracellular matrix and new blood vessel formation it may be appreciated that these biological action of active vitamin D3 may well play a role in retardation of cancer growth. Fujioka *et al.* [1] demonstrated that active vitamin D3 exerted dose-dependent antitumor activity against Renca tumor in mice *in vivo*, inhibiting tumor growth and prolonging survival.

In the clinical setting of prostate cancer, active vitamin D3 may retard the progression of indolent prostate cancer to more active disease [15, 17]. Indeed, oral administration of active vitamin D3 delays the recurrence of prostate cancer following primary therapy [25]. This evidence indicates that active vitamin D3 can be effective in slowing the progression of prostate cancer in patients. It is therefore intriguing that a recent case report described a patient with RCC with multiple bone metastases who developed almost complete resolution of the metastatic bone lesions following treatment with active vitamin D3 and interferon-a although it is unclear at this stage which agent was predominantly responsible for the impressive clinical response [26].

Many cancer cells have been associated with defective gap junctional intercellular communication (GJIC), which can occur at the level of connexin (Cx) expression and transport or gap junction formation [27]. Furthermore, a number of tumor-promoting agents inhibit GJIC during the transformation of

normal cells while inhibition of GJIC and decreased connexin expression is evident in malignant tissue thereby indicating that modulation of GJIC is an important player in the process of carcinogenesis [5, 28, 29]. Tumor cells transfected with Cx genes exhibit normal cell characteristics and a delayed growth rate indicating that Cx genes may act as tumor-suppressor genes [5, 30]. Fujioka *et al.* [5] studied the influence of active vitamin D3 upon GJIC and Cx43 mRNA expression in cultured human renal proximal tubular epithelial cells (RPTEC) during carcinogenesis. The expression of the gap junctional protein connexin Cx43 was detected in human kidney [5, 31]. Active vitamin D3 significantly enhanced GJIC function of human RPTEC, whereas the tumor promoters N-nitrosodimethylamine (NDMA) and N-ethyl-N-hydroxyethylnitrosamine (EHEN) were suppressive. Furthermore, simultaneous treatment of RPTECs with the tumor promoters NDMA and EHEN together with active vitamin D3 resulted in the maintenance of GJIC function and Cx43 mRNA expression at pretreatment levels [5]. These data suggest that a decrease in the serum level of active vitamin D3 is a risk factor for the development and progression of RCC, and that active vitamin D3 may act to prevent or retard RCC by preserving GJIC and Cx43 expression.

In conclusion, this study indicates that the TaqI polymorphism exhibited a significant association with the risk of developing RCC and is a risk factor for clinical and pathological advanced disease in Japanese.

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