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ORIGINAL PAPER

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Association of vitamin d receptor genotypes with calcium excretion in nephrolithiatic subjects in northern India

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Abstract Our objective was to investigate the association between the vitamin D receptor (VDR) allelic variants (Bsm I and Fok I) and nephrolithiasis in northern India. A total of 150 nephrolithiatic patients and 100 age and sex matched controls were enrolled for study. A 10 ml blood sample was obtained for biochemical analysis and DNA isolation. In addition, 24 h urine samples were obtained from each patient for the estimation of calcium and creatinine. PCR was performed for the Bsm I and Fok I VDR variants. The association between Bsm I and Fok I VDR polymorphism and nephrolithiasis was investigated after digestion with restriction enzymes (3 U). The product was analysed on 3% agarose gel for Bsm I and 15% polyacrylamide gel for Fok I allelic variants. We did not observe any significant differences in the prevalence of either the Bsm I or Fok I VDR genotypes between stone formers and controls. The B allele was found to be more prevalent in hypercalciuric patients compared to controls and nephrolithiatic subjects. The subjects with the bb genotype exhibited a higher calcium excretion than the BB genotype. Patients with the F allele were also found to excrete higher urinary calcium. VDR genotypes may be associated with increased calcium excretion in hypercalciuric nephrolithiatic subjects.

Keywords Vitamin D receptor · Genotypes · Bsm I · Fok I · Calcium excretion

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Introduction

Epidemiological studies have suggested that genetic predisposition along with environmental factors plays a major role in the etiology of the kidney stones [1, 2]. Hypercalciuria is a common abnormality found in 40% of the patients with nephrolithiasis [3]. Intestinal hyperabsorption is predominantly seen in hypercalciuric subjects and the only hormonal stimulus for the intestinal absorption of calcium is via 1, 25 $(OH)_2$ vitamin D_3 and its receptor. An increase in vitamin D receptor (VDR) number has been observed in hypercalciuric patients [4] and in hypercalciuric rats [5]. Scott and co-workers have recently shown a suggestive linkage between the VDR locus and idiopathic stone formation in a French Canadian population [6]. Moreover, the absorption of calcium is also associated with VDR genotype [7, 8]. Northern India represents an endemic region for kidney stone formation, with 33% of stone formers having hypercalciuria. Since genotype and allele frequencies are known to differ in different ethnic populations, we investigated the association of the VDR gene allelic variants Bsm I and Fok I in calcium stone formers.

Patients and methods

A total of 150 nephrolithiatic patients (105 males and 45 females, mean age 39.38 ± 1.102 years, range 18–65) attending or admitted to the urology clinic of our institute were enrolled for study. The major chemical constituents of the stones were calcium and oxalate. One hundred age and sex matched controls (76 males and 24 females, mean age 43.25 ± 2.05 years) with no evidence of stone disease based on clinical evaluation and the absence of radiopacity in the KUB region on plain abdomen X-ray, and without any family history of stone disease were included in the study. A 10 ml blood sample was obtained from patients and controls for biochemical investigation and DNA extraction. In addition, 24 h urine samples were obtained for the estimation of calcium and creatinine. The study was approved by the institute's ethics committee. Calcium and creatinine were measured spectrophotometrically in a semi-autoanalyzer using their respective kits: calcium was measured using the Calcium ASX kit (Chema Diagnostica, Italy) and creatinine by

the Creatinine Liquicolor kit (Human, Germany). Urinary 24 h calcium levels exceeding 2SD from the mean 24 h urinary calcium excretion (> 252.48 mg/24 h) of the healthy control population were termed hypercalciuria [9].

Genomic DNA was extracted from blood by the method of Sambrook and Russell [10]. Two different restriction fragment length polymorphisms with respect to restriction enzymes Bsm I and Fok I were studied in the VDR gene.

Bsm I genotyping was carried out according to Ruggiero et al. [11]. The reaction mixture contained genomic DNA, 1×PCR buffer, 1.5 mM $MgCl_2$, 200 mM dNTPs, 20 pmol of each primer and 2.5 units of Taq polymerase in a total volume of 25 µl for each polymorphism. Amplified DNA was analysed on 2% agarose gel and a single band corresponding to 850 bp was obtained. The PCR products (10 µl) were digested with the restriction enzyme Bsm I (3 U) for 16 h at 37°C. Digested PCR products was analysed on 3% agarose gel. Three different patterns of bands corresponding to three different genotypes were observed for Bsm I: (1) a single 850 bp band for amplified DNA without restriction (BB genotype), (2) two bands at 640 bp and 190 bp, excisable with enzyme (bb genotype), and (3) heterozygous genotype (Bb) showing three bands at 640 bp, 190 bp and 850 bp (Fig. 1).

Fok I genotyping was carried out according to Chen et al. [12]. PCR was carried out in 25 µl reaction mixture containing genomic DNA, 1×PCR buffer, 1.5 mM $MgCl_2$, 200 mM dNTPs, 20 pmol of each primer and 2.5 units of Taq polymerase for each polymorphism. Amplified DNA products were analysed on 2% agarose gel and a single band of 265 bp was obtained. The PCR products (10 µl) were subjected to digestion with restriction enzyme Fok I (3 U) for 16 h at 56°C. Digested PCR products were separated on 15% polyacrylamide gel. Three different patterns of bands corresponding to three different genotypes were observed for Fok I: (1) a single 265 bp band for amplified DNA without restriction (FF genotype), (2) two bands at 169 bp and 96 bp, excisable with enzyme, (ff genotype), and (3) heterozygotes (Ff) showing three bands at 265 bp, 169 bp and 96 bp (Fig. 2).

Results were expressed as mean ± SE The χ^2-test was performed to assess the significance of differences in the frequencies of the VDR genotype between nephrolithiatic patients and controls. All statistical analyses were performed using SPSS (SPSS, Chicago III., USA).

Results

Genotype and allele frequencies for Bsm I

The percent prevalence of the VDR-Bsm I genotypes and allele frequencies in renal stone formers and control subjects are shown in Fig. 3. In the control group, 26/100 subjects were homozygous bb, 28/100 were heterozygous Bb and 46/100 were BB homozygotes. In nephrolithiatic subjects, 62/150 (41.3%) had the heterozygous Bb genotype and 48/150 (32%) were BB homozygotes and 40/150 were bb (26.7%). No significant difference in bb genotype frequency was observed between nephrolithiatic subjects and the controls. There was also no significant difference in allele frequencies between the controls and nephrolithiatic subjects (Table 1). In 47 hypercalciuric nephrolithiatic subjects, the prevalence of the bb genotype was higher compared to controls ($P < 0.05$). The prevalence of the heterozygous genotype Bb was 18/47 (38.3%) in hypercalciuric subjects and 28% in control subjects (Fig. 3). The b allele was more prevalent in hypercalciuric subjects compared to control subjects (Table 1).

Genotype and allele frequencies for Fok I

The prevalence of genotype and allele frequencies for Fok I in kidney stone patients and controls are shown in Fig. 4. No significant difference in percent genotype prevalence was observed between patients and controls. The percent allele frequencies for F and f alleles were also not statistically different between the two groups. In nephrolithiatic and hypercalciuric nephrolithiatic subjects, the heterozygous (Ff) genotype was more prevalent ($P < 0.05$) than the FF or ff genotypes. In hypercalciuric nephrolithiatic subjects, the percent frequency of the homozygous (ff) genotype was found to be less ($P < 0.05$) than the controls and nephrolithiatic subjects (Fig. 4). The percent frequency of the F allele was found to be higher in hypercalciuric nephrolithiatic subjects as compared to the f allele (Table 2).

Urinary calcium and VDR genotypes

Table 3 shows the correlation between serum calcium and 24 h urinary calcium excretion in nephrolithiatic

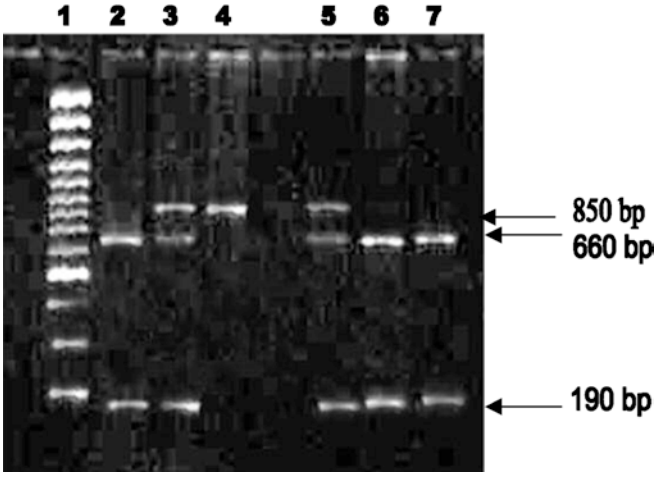


Fig. 1

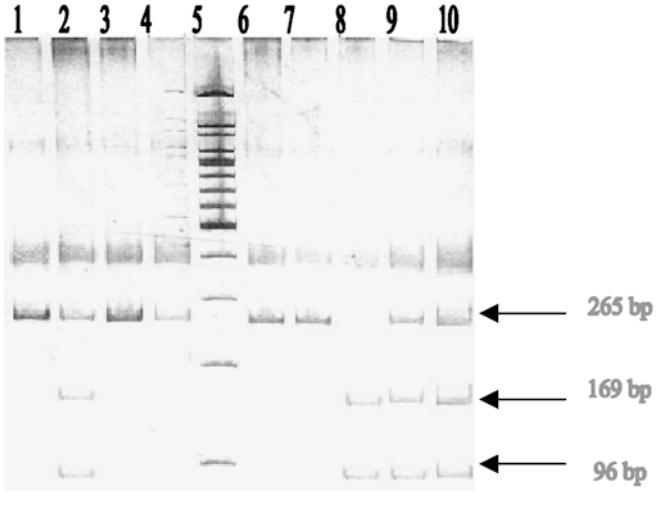
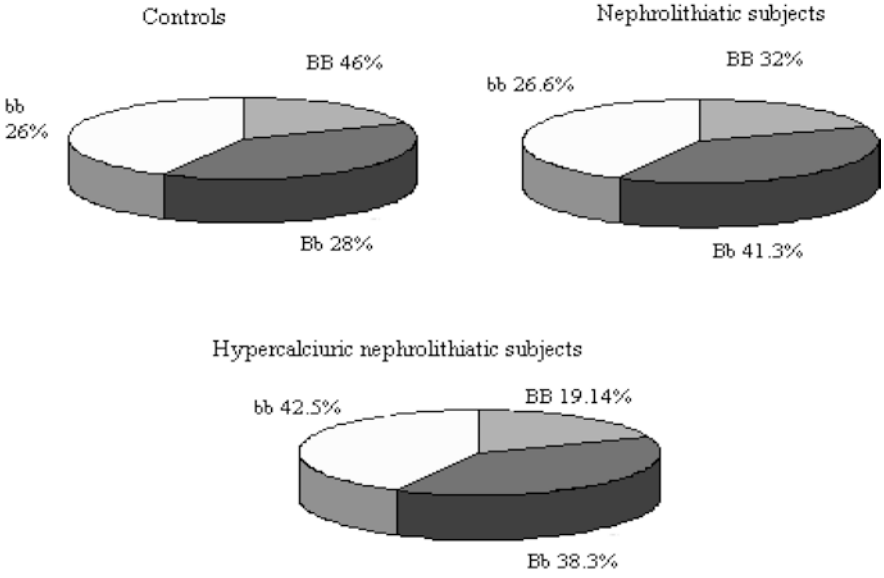


Fig. 2

Fig. 3



subjects and hypercalciuric nephrolithiatic subjects with different genotypes of the Bsm I site. Serum calcium levels were comparable in all genotypes in both nephrolithiatic and hypercalciuric nephrolithiatic subjects. However, there was a significantly higher 24 h urinary calcium excretion ($P = 0.001$) in the nephrolithiatic subjects with the bb homozygous genotype (262.61 ± 24.28 mg/24 h) compared to the Bb (165.76 ± 17.26 mg/24 h) and homozygous BB (205.68 ± 14.29 mg/24 h) genotypes. In hypercalciuric nephrolithiatic subjects, the 24 h urinary calcium excretion was also significantly higher in the bb genotype ($P < 0.05$) compared to the Bb and BB genotypes.

The correlation between serum calcium and 24 h urinary calcium excretion in nephrolithiatic subjects and hypercalciuric nephrolithiatic subjects with different genotypes for the Fok I site is shown in Table 4. Nephrolithiatic subjects with homozygous (FF) or heterozygous (Ff) genotypes showed significantly higher ($P < 0.05$) calcium excretion compared to the ff genotype. In the hypercalciuric nephrolithiatic group, heterozygotes (Ff) were found to excrete more calcium than the ff genotype. Serum calcium was significantly higher ($P < 0.05$) in hypercalciuric nephrolithiatic subjects with the ff genotype compared to the FF or Ff genotypes.

Table 1 Allele frequency of the VDR gene for *Bsm*I restriction site in controls, nephrolithiatic subjects and hypercalciuric nephrolithiatic subjects

	Alleles	
	B	b
Controls ($n = 100$)	60%	40%
Nephrolithiatic subjects ($n = 150$)	52.7%	47.3%
Hypercalciuric nephrolithiatic subjects ($n = 47$)	38.3%	61.7%

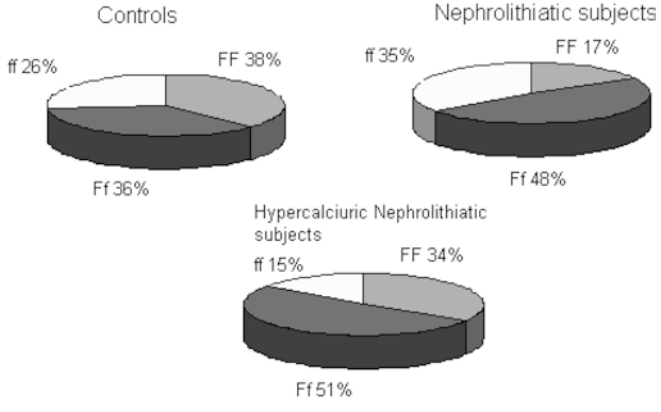


Fig. 4

Discussion

Hypercalciuria is an important metabolic condition seen in patients with renal urinary stones. The genesis of hypercalciuria appears to have a varied etiology such as intestinal hyperabsorption, renal leak or resorptive hypercalciuria due to hyperparathyroidism. Hypercalciuria is also known to be familial, and genetic factors along with environmental factors have been proposed to

Table 2 Allele frequency of the VDR gene for *Fok*I restriction site in controls, nephrolithiatic subjects and hypercalciuric nephrolithiatic subjects

	Alleles	
	F	f
Controls ($n = 100$)	55.4%	44.5%
Nephrolithiatic subjects ($n = 150$)	40.9%	59.1%
Hypercalciuric nephrolithiatic subjects ($n = 47$)	59.6%	40.4%

Table 3 Correlation of serum calcium and 24 h urinary calcium excretion with different genotypes for *Bsm*I in nephrolithiatic subjects and hypercalciuric nephrolithiatic subjects. Values are mean ± SE. * $P < 0.05$ as compared to BB genotype; ** $P < 0.05$ as compared to Bb genotype

Nephrolithiatic subjects		
Genotypes	Serum calcium (mg/dl)	Urinary calcium excretion (mg/24 h)
Hypercalciuric nephrolithiatic subjects		
BB	9.13 ± 0.139	165.76 ± 17.26
Bb	9.32 ± 0.157	205.68 ± 14.29*
bb	9.11 ± 0.144	262.61 ± 24.286*,**
BB	9.36 ± 0.384	366.64 ± 46.58
Bb	9.813 ± 0.213	340.66 ± 23.83
bb	9.45 ± 0.144	387.34 ± 24.26*,**

Table 4 Correlation of serum calcium and 24 h urinary calcium excretion with different genotypes for *Fok*I in nephrolithiatic subjects and hypercalciuric nephrolithiatic subjects. Values are mean ± SE. * $P < 0.05$ as compared to ff genotype; ** $P < 0.05$ as compared to FF or Ff genotype

Nephrolithiatic subjects		
Genotypes	Serum calcium (mg/dl)	Urinary calcium excretion (mg/24 h)
Hypercalciuric nephrolithiatic subjects		
FF	9.6 ± 0.19	263.478 ± 26.53*
Ff	9.05 ± 0.126	231.33 ± 17.48*
ff	9.2 ± 0.1466	149.82 ± 10.43
FF	9.8 ± 0.20	350.28 ± 24.05*
Ff	9.28 ± 0.19	391.173 ± 26.53*
ff	10.29 ± 0.45**	291.04 ± 17.06

contribute significantly to its pathogenesis. A linkage has been detected between the vitamin D receptor (VDR) locus and calcium urolithiasis [6]. Common allelic variations in VDR, detected by restriction fragment length polymorphism using Bsm I, Taq I, Apa I and Fok I restriction enzymes may or may not be associated with hypercalciuric stone disease [13, 14, 15, 16, 17]. Vitamin D receptor (VDR) gene polymorphism has also been proposed to be associated with calcium absorption and bone resorption [18]. A genetic association between VDR polymorphisms and idiopathic hypocitraturia, which is also a risk factor for calcium oxalate nephrolithiasis, has recently been reported in calcium stone forming patients [19]. These findings suggest that allelic variations of the VDR gene may partially represent the genetic component of urolithiasis. In the present study, we investigated the association of two polymorphisms of the VDR gene in renal stone patients and controls. We did not observe any significant difference in either Bsm I or Fok I genotype prevalence between stone formers and controls, indicating that VDR genotypes may not be associated with nephrolithiasis. However, when the VDR Bsm I allele frequencies were analysed in hypercalciuric patients, the b allele was found to be more prevalent in these patients compared to controls or nephrolithiatic subjects, suggesting that this allele may be associated with hypercalciuria in these patients. However, since the number of patients with hypercalciuria was relatively small ($n = 47$), we did not observe the percent b allele frequency to be significant. Several other studies have also not found any significant distribution in VDR allele frequencies in renal stone formers compared to the general population [11, 16, 20]. However, it is noteworthy that we found a marked difference in the VDR genotype distribution in our population compared to Caucasians.

We observed a higher prevalence of the Ff genotype in the nephrolithiatic group and the F allele to be more prevalent in hypercalciuric nephrolithiatic subjects, which is in accordance with the hypothesis that the VDR Fok I polymorphism may result in two variants of VDR proteins [21]. The ff and Ff forms of the VDR gene are associated with a decreased VDR efficiency [22]; in contrast, the FF genotype may increase the ability of intestinal VDR to absorb calcium, ultimately leading to hypercalciuria without bone loss. In a recent study, however, Vezzoli and co-workers did not observe any association between idiopathic hypercalciuria and kidney stones with the Fok I VDR genotype; however, they did find that the FF genotype was associated with low plasma phosphate concentrations in hypercalciuric stone forming patients [15].

The diversity in allele frequencies of VDR again underlies the extreme racial and ethnic genotype variability in the pattern of prevalence of the VDR alleles. Even though the mechanism of hypercalciuria does not seem linked to an unique VDR genotype, the association of urinary calcium with different VDR alleles observed in the present study, suggests a functional variation in VDR gene expression linked to VDR genotypes, as proposed for other conditions [23].

A relationship between VDR gene polymorphism and calcium metabolism has been shown by Gennari et al. [24], who found that intestinal calcium absorption was significantly lower in the BB genotype than the bb genotype in the presence of similar circulating levels of vitamin D in various genotypes. Ruggiero et al. [11] reported that patients with the bb genotype exhibited a higher calcium excretion than those with the BB genotype. However, Vezzoli et al. [15] did not observe any association between the Fok I genotype with idiopathic hypercalciuria and kidney stones. Our study suggests that there might be an association between the F allele and higher calcium excretion in nephrolithiatic subjects. These findings are in accordance with those reported earlier, confirming a possible association of hypercalciuria with the homozygous bb genotype. We found that both nephrolithiatic and hypercalciuric nephrolithiatic subjects with the bb genotype showed higher 24 h urinary calcium excretion than the homozygous BB patients. Our results, thus, support the hypothesis that intestinal calcium absorption and urinary calcium

excretion may be associated with an altered function of the VDR gene for the Bsm I and Fok I genotypes.

Besides the Fok I and Bsm I polymorphisms, other allelic variations in the VDR gene, such as Taq I and Apa I, are also associated with renal stone disease [14, 25]. It has been proposed that the VDR genotype may provide a tool to screen individuals who are at a risk of calcium nephrolithiasis [17]. Thus, studies such as ours provide useful information for understanding the pathogenesis of urinary calculi. However, further studies, with a larger sample size and different VDR gene variants are needed in different ethnic populations to confirm the influence of the VDR polymorphism in renal stone disease.

References

1. Resnick M, Pridgen D, Goodman H (1968) Genetic predisposition to formation of calcium oxalate renal calculi. N Engl J Med 278: 1313
2. Robertson W, Peacock M, Baker M, Marshall D, Pearlman B, speed R, Sergeant V, Smith A (1983) Studies on the prevalence and epidemiology of urinary stone disease in men in Leeds. Br J Urol 55: 595
3. Coe FL, Parks JH, Moore ES (1979) Familial idiopathic hypercalciuria.300: 337
4. Zerwekh JE, Yu XP, Breslau NA, Manolagas S, Pak CYC (1993) Vitamin D receptor quantitation in human blood mononuclear cells in health and disease. Mol Cell Endocrinol 96: 1
5. Li XQ, Tembe V, Horwitz GM, Bushinsky DA, Favus MJ (1993) Increased intestinal vitamin D receptor in genetic hypercalciuric rats: a cause of intestinal calcium hyperabsorption. J Clin Invest 91: 661
6. Scott P, Ouimet D, Valiquette L, Guay G, Proulx Y, Trouve ML, Gagnon B, Bonnardeaux A (1999) Suggestive evidence for a susceptibility gene near the vitamin D receptor locus in idiopathic calcium stone formation. J Am Soc Nephrol 10: 1007
7. Dawson-Hughes B, Harris SS, Finneran S (1995) Calcium absorption on high and low calcium intakes in relation to vitamin D receptor genotype. J Clin Endocrinol Metab 80: 3657
8. Wishart JM, Horowitz M, Need AG, Scopacasa F, Morris HA (1997) Relations between calcium intake, calcitriol, polymorphisms of vitamin D receptor gene, and calcium absorption in premenopausal women. Am J Clin Nutr 65: 798
9. Sutton RAL, Walker VR (1995) Mild hyperoxaluria in idiopathic calcium oxalate stone formers. Urolithiasis Consensus and Controversies. Proceedings of Fifth European Urolithiasis Symposium. p 354s
10. Sambrook J, Russell DW (2002) Preparation and analysis of eukaryotic genomic DNA. In: Sambrook J, Russell DW (eds) Molecular cloning: a laboratory manual, 3rd edn, vol 1. Cold Spring Harbor Laboratory, Cold Spring Harbor p 6.4
11. Ruggiero M, Pacini S, Amato M, Aterini S, Chiarngi V 1999) Association between vitamin D receptor gene polymorphism and nephrolithiasis. Miner Electrolyte Metab 25: 185
12. Chen WC, Chen HY, Lu HF, Hsu CD. Tsai FS (2001) Association of vitamin D receptor gene start codon *Fok*I polymorphism with calcium oxalate stone disease. Br J Urol:87: 167
13. Heilberg IP, Teixeira SH, Martini LA, Boim MA (2002) Vitamin D receptor gene polymorphism and bone mineral density in hypercalciuric calcium-stone-forming patients. Nephron 90: 51
14. Nishijima S, Sugaya K, Naito A, Morozumi M, Hatano T, Ogawa Y (2002) Association of vitamin D receptor gene polymorphism with urolithiasis. J Urol 167: 2188
15. Vezzoli G, Soldati L, Proverbio MC, Adamo D, Rubinacci A, Bianchi G, Mora S (2002) Polymorphism of vitamin D receptor gene start codon in patients with calcium kidney stones. J Nephrol 15: 158
16. Chen WC, Chen HY, Hsu CD, Wu JY, Tsai FJ (2001) No association of vitamin D receptor gene *Bsm*I polymorphisms with calcium oxalate stone formation. Mol Urol 5: 7
17. Jackman SV, Kibel AS, Ovuworie CA, Moore RG, Kavoussi LR, Jarrett TW (1999) Familial calcium stone disease: Taq I polymorphism and the vitamin D receptor. J Endourol 13: 313
18. Tsuruoka S, Bushinsky DA, Schwartz GJ (1997) Defective renal calcium reabsorption in genetic hypercalciuric rats. Kidney Int 51: 1540
19. Mossetti G, Vuotto P, Rendina D, Numis FG, Viceconti R, Giordano F, Cioffi M, Scopacasa F, Nunziata V.J (2003) Association between vitamin D receptor gene polymorphisms and tubular citrate handling in calcium nephrolithiasis. Intern Med 253: 194
20. Zerwekh JE, Hughes MR, Reed BY (1995) Evidence for normal vitamin D receptor messenger ribonucleic acid and genotype in absorptive hypercalciuria. J Clin Endocrinol Metab 80: 2960
21. Gross C, Eccleshall TR, Malloy PJ et al. (1996)The presence of translation initiation site of vitamin D receptor gene is associated with low mineral density in post menopausal-Americans. J Bone Miner Res11: 1850
22. Arai H, Miyamoto KI, Taketani et al. (1997) A vitamin D receptor gene polymorphism in the translation initiation codon: effect on protein activity and relation to bone mineral density in Japanese women. J Bone Miner Res 12: 915
23. RuggeiroM, Pacini S, Aterini S, Fallai C, Rugiero C, Pacini P (1998) Vitamin D receptor gene polymorphism is associated with metastatic breast cancer. Oncol Res 10: 43
24. Gennari L, Becherini L, Masi L, Gonnelli S, Cepollaro C, Martini S, Mansani R, Brandi ML (1997) Vitamin D receptor genotypes and intestinal calcium absorption in postmenopausal women. Calcif Tissue Int 61: 460
25. Ozkaya O, Soylemezoglu O, Misirlioglu M, Gonen S, Buyan N, Hasanoglu E (2003) Polymorphisms in the vitamin D receptor gene and the risk of calcium nephrolithiasis in children. Eur Urol 44: 150