Genetic polymorphisms of vitamin D receptor and calcium sensing receptor gene in uremic secondary hyperparathyroidism: a multicentric italian study

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Summary

The pathophysiology of hyperparathyroidism (HPT) relates to the loss of normal feedback control of parathyroid hormone secretion by extracellular calcium. Why the parathyroid cell loses its normal sensivity to calcium is unknown. Consistent with the essential role of vitamin D in parathyroid cell regulation, restriction fragment length polymorphisms (RFLPs) at the vitamin D receptor (VDR) gene locus have been recently postulated to be responsible for differential VDR transcription and/or mRNA stability, contributing to the parathyroid tumorigenesis. Particularly, common VDR allelic variants have been related to differences in the incidence of both primary and secondary HPT, and in serum PTH levels as well as in calcium regulation of PTH release. However, agreement on these relationships is not universal among different populations. In this study we investigated the role of VDR and calcium sensing receptor (CaSR) gene RFLPs in 100 uremic secondary HPT patients (mean age 58.2±6.7 yrs; mean dialytic age 7.1±2.0 yrs) compared to 200 age and sex matched controls. Apa I, Bsm I, and Taq I RFLPs at the 3'-end of VDR gene locus as well as Fok I RFLP at the translation initiation codon of the VDR gene were determined after PCR amplification and indicated respectively as A-a, B-b, T-t, and F-f, uppercase letter signifying the absence and lowercase letter the presence of the restriction site. The 3 polymorphisms in exon 7 of the CaSR gene (T/G at codon 986, A/G at codon 990, and G/C at codon 1011) were evaluated by PCR amplification and direct sequencing. Chi squared analysis revealed no significant difference in the distribution of any of the VDR or CaSR genotypes in subjects with secondary HPT compared to controls. Secondary HPT patients were divided into two major groups according to serum PTH levels: group 1 with higher PTH levels, requiring parathyroidectomy, and group 2 with serum PTH levels below 120 pg/ml. Interestingly, a trend for a higher prevalence of VDR genotype aabbTT in group 1, with respect to group 2 was observed (P=0.07; Chi squared test). Taken together, these results suggest that VDR and CaSR gene RFLPs are not directly involved in parathyroid tumorigenesis and in determining the incidence of secondary HPT. However, VDR gene RFLPs might be useful in predicting the progression of secondary HPT as well as the severity of the disease.

KEY WORDS: uremic hyperparathyroidism, VDR gene polymorphism, calcium sensing receptor gene, parathyroid tumorigenesis.

Introduction

The pathophysiology of hyperparathyroidism (HPT) relates to the loss of normal feedback control of parathyroid hormone secretion by extracellular calcium. However, why the parathyroid cell loses its normal sensivity to calcium is unknown. Parathyroid adenomas are of monoclonal origin, but it does not exclude influence by both stimulatory and inhibitory factors regulating neoplastic growth. Additionally, the monoclonal phase could be preceded by a period of polyclonal hyperplasia, which is possibly affected by several factors. The clonal origin of most parathyroid adenomas suggests a defect at the level of the gene(s) controlling the regulation and/or expression of PTH or also the proliferation of parathyroid cells (1-5). Vitamin D exerts an important role in parathyroid regulation and specific vitamin D receptors (VDR) have been identified in parathyroid glands (6). It has been demonstrated that calcitriol, via its receptor, constitutes an important regulator of parathyroid cell growth (7, 8) and directly interacts with parathyroid hormone (PTH) secretion by inhibiting PTH synthesis at the level of gene transcription (9, 10). Derangements in vitamin D receptor function and/or expression have recently been suggested in the pathogenesis of both primary and secondary HPT (11-13). Particularly, in the nodules of severe uremic parathyroid hyperplasia, an increased set-point and decreased expression of both the calcium sensing receptor (CaSR) and VDR have been observed. The VDR gene is located in the long arm of chromosome 12 and possesses several polymorphic sites (14). In the last years, several studies showed a relationship between VDR allelic variants and calcium metabolism, indicating the VDR gene as a major candidate gene for osteoporosis (15-16). Consistent with the essential role of vitamin D in parathyroid cell regulation, restriction fragment length polymorphisms (RFLPs) at the vitamin D receptor (VDR) gene locus

have been recently postulated to be responsible for differential VDR transcription and/or mRNA stability, contributing to the parathyroid tumorigenesis. Common VDR allelic variants have been related to differences in the incidence of both primary and secondary HPT, and in serum PTH levels as well as in calcium regulation of PTH release (17-24). Moreover, association studies investigating intron 8 (*B/b* and *A/a* alleles) and exon 9 (*T/t* alleles) of *VDR* gene polymorphisms, detected by BsmI, ApaI and TaqI endonucleases respectively, showed a higher prevalence of *baT* haplotype in Swedish patients affected by primary hyperparathyroidism (PHPT) (25, 26). These results were not confirmed in a Japanese population study (27), even if an association study on a Spanish postmenopausal women population with PHPT failed to demonstrate a role of BsmI VDR polymorphism in the pathogenesis of parathyroid adenomas (28). However, agreement on these relationships is not universal among different populations and genetic differences among ethnical groups could account for such discrepancies. In the present study we investigated the role of VDR and calcium CaSR gene RFLPs in 100 uremic secondary HPT patients compared to 200 age and sex matched controls.

Materials and methods

Subjects

The study population comprised 100 patients affected with uremic secondary HPT and 200 age and sex matched controls. Patients had been recruited as part of a multicentric Italian study, including the Nephrology and Dyalisis Units of Bari, Cinisello Balsamo, Desio, Florence, Milan and Reggio Emilia. The inclusion criteria for the study were the following: age> 65 years, dyalitic age >2 years, mean Al levels <40 µg/l, serum intact PTH levels <120 pg/ml (Group 1) or >600 pg/ml (Group 2). The cause of renal failure was chronic glomerulonephritis in 43, nephroangiosclerosis in 14, tubulo-interstitial nephritis in 10, polycystic kidney disease in 10, diabetic glomerulosclerosis in 7, nephrotuberculosis in 1, and other causes in 15.
Controls were selected from a cohort of 500 women and 200 men visited in Florence for osteoporotic risk evaluation which had previously participated to genetic association studies on VDR polymorphisms (16, 29). Clinical characteristics of patients and controls are given in Table I.

Table I - General characteristics of uremic secondary HPT patients and controls.

Characteristic	Secondary HPT	Controls
Number	100	200
Gender (male/female)	57/43	108/92
Age (yrs)	58.2±6.7	59.5±8.4
Hemodyalisis (age)	7.1±2.0	–
PTH (pg/ml)	548±102	42±5

Genetic analysis

Genomic DNA was isolated from EDTA blood samples by a standard phenol-chloroform extraction procedure. Apa I and Taq I RFLPs at the 3'-end of VDR gene locus as well as Fok I RFLP at the translation initiation codon of the VDR gene were determined after PCR amplification and indicated respectively as *A-a*, *T-t*, and *F-f* uppercase letter signifying the absence and lowercase letter the presence of the restriction site (Fig. 1).

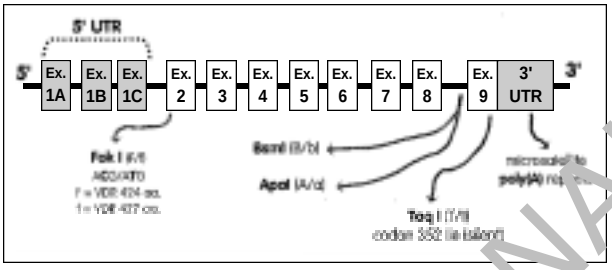

Figure 1 - Polymorphisms at the human VDR gene.

The 740 base pair fragment of the VDR gene, including the Apa I and Taq I restriction sites in intron 8 and exon 9, was amplified according to Morrison et al. (15), by using the specific primers 5'- C A G A G C A T G G A C A G G A G C A A -3' and 5'- G C A A C T C C T C A T G G C T G A G G T C T C -3'. PCR products were then digested with *Apa I* and *Taq I* restriction endonucleases (Roche Diagnostics GmbH, Mannheim, Milan, Italy), respectively at 37° C and 65° C, for 4 hours.
The 265-bp fragment of genomic DNA containing the Fok I polymorphic portion of exon 2 was amplified by PCR, as described by Gross et al. (26), with the following primers: 5'-A G C T G G C C C T C G C A C T G A C T C T G C T C T-3' and 5'-A T G G A A C A C C T T G C T T C T T C T C C C T C-3'. PCR products were digested with Fok I restriction endonuclease (New England Biolabs, Beverly, MA, USA) at 37° C for 4 h and then electrophoresed through a 3% low melting point agarose gel, containing ethidium bromide. The presence of the restriction site, that generates two fragments of 196 bp and 69 bp, was indicated with *f*, while its absence, resulting in a single uncut 265 bp fragment, with *F*.
Subjects were scored as *aa*, *tt*, *ff* homozygotes, Aa, Tt, Ff heterozygotes and AA, TT, FF homozygotes according to the digestion pattern.
Exon 7 polymorphisms at the CaSR gene locus (Fig. 2) were determined by direct dideoxy sequencing (ABI Prism 310, Perkin Elmer, Monza, Italy) using fluorescently labelled primers as previously described (30-32).

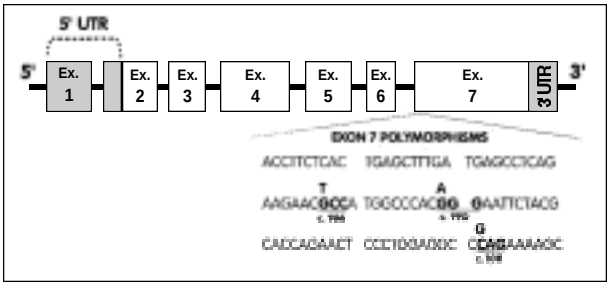

Figure 2 - Polymorphisms at the human CaSR gene.

Results

The frequency distribution of VDR and CaSR genotypes were in Hardy-Weinberg equilibrium. The distribution of *Apa I* and *Taq I* genotypes was very similar to what previously reported in Caucasian populations of European ancestry, while significantly differed from what observed in populations of Asiatic ancestry (14). The distribution of CaSR genotypes did not vary from that previously observed in Canadian population (31).
Chi squared analysis revealed no significant difference in the

distribution of any of the VDR or CaSR genotypes in subjects with secondary HPT compared to controls. When controls were subgrouped in osteoporotics and non osteoporotics, according to W.H.O. criteria (33), we observed a statistically significant increased prevalence of "*AA*", "*tt*" and "*ff*" VDR genotypes in osteoporotics than in non osteoporotics (Tab. II), and of "*ff*" VDR genotype in secondary HPT patients than in osteoporotics (Tab. III). Conversely, CaSR polymorphism distributions did not vary among secondary HPT, osteoporotic and non osteoporotic groups (Tab. IV). Secondary HPT patients were divided into two major groups according to serum PTH levels: group 1 including patients with higher PTH levels, unresponsive to medical treatment and thus requiring parathyroidectomy, and group 2 including subjects with serum PTH levels below 120 pg/ml. Interestingly, as shown in Table V, a trend for a two-fold increased prevalence of VDR genotype "*aaTT*" in group 1, with respect to group 2 was observed (P=0.07; Chi-squared test).

Table II - Distribution of VDR 3'-end (Apa I and Taq I) genotypes in uremic secondary HPT patients and controls (osteoporotics and non osteoporotics).

Genotype	SHPT	Controls	
		OP	Non OP
AAtt	14	24*	8
AATt	15	14	10
AATT	2	6	6
AaTt	33	38	39
AaTT	16	11	19
aaTT	20	7*	18

* P<0.05 OP vs non OP,Chi-squared test.

Table III - Distribution of VDR transcription initiation codon (Fok I) genotypes in uremic secondary HPT patients and controls (osteoporotics and non osteoporotics).

Genotype	SHPT	Controls	
		OP	Non OP
FF	58	37	44
Ff	39	44	47
FF	3*	19	9

* P<0.05 SHPT vs OP,Chi-squared test.

Table IV - Distribution of Exon 7 CaSR genotypes in uremic secondary HPT patients and controls (osteoporotics and non osteoporotics).

	SHPT	Controls	
		OP	Non OP
Codon 986			
G/G	75	63	65
T/G or T/T	25	37	35
Codon 990			
A/A	93	90	88
A/G	7	10	12
G/G	–	–	–
Codon 1011			
C/C	88	91	93
C/G	12	9	7
G/G	–	–	–

Table V - Distribution of VDR 3'-end (Apa I and Taq I) genotypes in uremic secondary HPT patients.

Genotype	PTH<120 pg/ml	PTH>600 pg/ml
AAtt	8 (16.6%)	4 (10.3%)
AATt	5 (10.4%)	12 (29.5%)
AATT	2 (4.2%)	–
AaTt	18 (37.5%)	11 (28.2%)
AaTT	9 (18.8%)	5 (12.8%)
aaTT	6 (12.5%)	11 (28.2%)

Discussion

Patients on long term hemodialysis caused by chronic renal failure commonly have accompanying renal bone disease, one of the major factors underlying this disease being secondary hyperparathyroidism. Parathyroid hyperplasia and high levels of immunoreactive PTH are present in the early stage of secondary hyperparathyroidism (34). Clonal analysis has shown that in renal hyperparathyroidism the parathyroid glands initially grow diffusely and polyclonally, with successive development of foci of nodular hyperplasia and possibly of monoclonal neoplasia. Somatic changes of specific genes have been suspected of being responsible for parathyroid tumorigenesis in uremic hyperparathyroidism. However, the genetic loci responsible for tumor development remain to be identified and heterogeneous genetic abnormalities may contribute to the progression of secondary parathyroid hyperplasia. The initial stimulus for PTH hypersecretion is thought to be predominantly a chronic reduction of ionized calcium in the extracellular fluid, caused by the reduced production of 1,25 $(OH)_2$ vitamin D and/or to phosphate retention. Although secondary hyperparathyroidism is sometimes managed by calcium supplementation, by oral calcitriol therapy and by restriction of phosphate intake, many patients remain unresponsive to these conservative treatments (35). Lack of response to treatment may be due to alterations in the calcium sensing set-point and/or to an increased functional parathyroid gland mass (36). To date, there are conflicting data regarding the relative contribution of gland size and set-point abnormalities to non-suppressible PTH in hemodialysis patients with secondary hyperparathyroidism (37-40). Many prior studies provided evidence for a calcium sensing abnormality in a subset of patients with severe hyperparathyroidism (37), but recent studies have failed to identify calcium set point abnormalities in normocalcemic patients with secondary hyperparathyroidism of different severity (38). Similarly, in spite of parathyroid gland enlargement, it has been difficult to conclusively show that gland size is responsible for impaired calcium-mediated PTH suppression in these patients. Whereas some studies have found a relationship between basal circulating PTH concentrations and parathyroid gland size (38), other investigations have not (40). Several studies have also suggested that resistance of parathyroid cells to 1,25 $(OH)_2$ vitamin D might underlie the secondary hyperparathyroidism in uremia

(41) and reduction of VDR density is believed to participate in the pathogenesis of this resistance. However, the results of biochemical studies addressing VDR numbers in the parathyroids are still controversial, some finding a decreased VDR content in the parathyroid glands of uremic patients (42) and animals (43, 44), while others finding no such decrease (45, 46). Moreover, some other studies have recently shown a reduced nuclear uptake of calcitriol-VDR complex by uremic ultrafiltrate, suggesting an inhibited interaction of calcitriol receptor with DNA during chronic renal failure (47, 48). Consistent with the essential role of active vitamin D in parathyroid regulation, a varied VDR expression due to allelic polymorphisms at the VDR gene locus could be relevant in chronic renal failure and may play a role in the increased proliferation of parathyroid cells as well as in the progression of HPT in uremic patient, accounting for the variation in the degree of secondary HPT observed in this patient population. To this regard, Howard et al. firstly associated the VDR "*BB*" genotype with a lower decrease in PTH levels after oral administration of calcitriol, suggesting that different 1,25 dihydroxyvitamin D concentrations are required to produce similar calciotropic responses in the different *Bsm I* homozygotes of the VDR gene (24). Possibly, the parathyroid gland in the low bone density "*BB*" group could result less sensitive to the negative feedback of 1,25-$(OH)_2$ vitamin D. On the contrary, in 1995 Carling et al. described an increased prevalence of the VDR "*bb*" genotype but not of the "*BB*" genotype in patients affected with primary HPT respect to controls (17), suggesting that the presence of the "*b*" allele may blunt the antiproliferative effect of vitamin D on parathyroid cells and the inhibitory action of vitamin D on PTH transcription, possibly by impaired VDR expression. However, the same authors did not observe any difference in the distribution of the "*bb*" VDR genotype in a subset of 34 uremic secondary HPT subjects (19). Subsequent association studies on the relationship between VDR polymorphisms and uremic secondary HPT yielded conflicting results, some describing an increased prevalence of "*bb*" genotype in secondary HPT, while others reporting no significant differences in the distribution of genotypes (18, 20-23). Data from the present study confirm the significant segregation of VDR genotypes with osteoporotic risk in Italian population, as previously observed in larger samples (16, 29), but failed to detect a major role of these polymorphisms in the determination of secondary HPT, in uremia. Interestingly, a trend for a two-fold increased prevalence of "*aaTT*" VDR genotype in group 1 (high PTH levels), with respect to group 2 (low PTH levels) was observed, suggesting a more severe degree of HPT in those patients with "*aa*" or "*TT*" (and thus "*bb*", being this allele in linkage disequilibrium with "*aa*" and "*TT*" alleles) VDR allelic variants. The latter finding is in keeping with those from previous studies showing increased intact PTH levels in secondary HPT subjects with "*aa*", "*bb*", and "*TT*" VDR genotypes (20-23). Moreover, a trend for a decreased prevalence of "*ff*' VDR allelic variant in secondary HPT patients with respect to controls was observed. The possibility of an association of secondary HPT with the Fok I polymorphism in the VDR gene deserves further investigation in larger populations as well as in longitudinal studies.

Finally, in the same population of uremic secondary HPT patients we analyzed, for the first time, the role of polymorphisms at an additional candidate gene in parathyroid diseases, the human CaSR gene. The analyzed polimorphisms were located in exon 7 of the CaSR gene, respectively in codon 986 (Ala/Ser), codon 990 (Arg/Gly), and codon 1011 (Gln/Glu). They all encode non-conservative aminoacid changes in the cytoplasmic tail of the CaSR, but their functional significance is not known. Indeed, the codon 986 T/C polymorphism, has been recently associated with variation in serum calcium levels in a Canadian female population (31), giving rise to the possibility of a role of this polymorphism in disorders of calcium regulation such as hyperparathyroidism. However, in the present preliminary study, we did not observe any significant correlation between the codon 986 CaSR polymorphism and uremic secondary HPT or osteoporosis. Similar negative results were observed for what concern codon 990 and codon 1011 CaSR polymorphisms.

Taken all together, these results suggest that both VDR and CaSR gene polymorphisms are not directly involved in parathyroid tumorigenesis and in determining the incidence of secondary HPT. Further studies are needed to evaluate the possible role of VDR gene allelic variant in predicting the progression of secondary HPT as well as the severity of the disease.

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