EXPERIMENTAL STUDY

Exclusive underexpression of vitamin D receptor exon 1f transcripts in tumors of primary hyperparathyroidism

Pamela Correa, Göran Åkerström and Gunnar Westin
Department of Surgical Sciences, Endocrine Unit, Uppsala University Hospital, SE-751 85 Uppsala, Sweden
(Correspondence should be addressed to G Westin, Department of Surgical Sciences, Endocrine Unit, Uppsala University Hospital, Klinik forskningsavdelning 2, ingang 70, plan 3, lab 9, SE-751 85 Uppsala, Sweden; Email: gunnar.westin@surgsci.uu.se)

Abstract

Objective: Primary hyperparathyroidism (pHPT) is characterized by excessive production of parathyroid hormone (PTH) due to parathyroid adenomas while uremic secondary HPT (sHPT) is caused by parathyroid hyperplasia in response to renal failure. Active vitamin D, 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃), with the vitamin D receptor (VDR) is involved in regulation of the calcium homeostasis together with PTH. In a feedback loop, 1,25-(OH)₂D₃ has a direct action on the parathyroid gland to regulate PTH transcription, PTH secretion and cell proliferation. We have previously demonstrated reduced VDR mRNA expression in parathyroid adenomas and hyperplasia of sHPT using a probe detecting all 14 variant VDR transcripts expressed in parathyroid cells. Here we have assessed which of the 5’-terminal exon 1a, 1d and 1f variant VDR transcripts are reduced in pathological parathyroid glands.

Methods: The relative VDR/glyceraldehyde-3-phosphate dehydrogenase mRNA levels for each VDR exon were determined by real-time quantitative RT-PCR in five normal parathyroid glands, seventeen parathyroid adenomas and ten hyperplastic glands of sHPT.

Results: The results demonstrated exclusive underexpression of VDR exon 1f transcripts in parathyroid adenoma, while all measured VDR transcripts were reduced in secondary hyperplasia.

Conclusions: We suggest that exclusive underexpression of VDR exon 1f transcripts in adenomas of pHPT, which derive from a distal promoter active in tissues involved in calcium regulation by 1,25-(OH)₂D₃, may either reflect a defective cell type-specific transcription factor or other physiologically important pathway(s) for tissue-specific VDR gene expression.

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Introduction

Hyperparathyroidism (HPT) is characterized by hypercalcemia due to hypersecretion of parathyroid hormone (PTH). Monoclonality among adenomas of primary HPT (pHPT) is common and has also been associated with some hyperplastic glands of secondary HPT (sHPT) (1, 2). pHPT has a prevalence of 2–3% in postmenopausal women (3), whereas sHPT has become less frequent due to medical prevention in association with dialysis. The active form of vitamin D, 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃), functions as the ligand for the vitamin D receptor (VDR) allowing the modulation of target gene transcription, through vitamin D response element(s). 1,25-(OH)₂D₃ is crucial in the maintenance of serum calcium and regulates parathyroid gland activity by inhibiting PTH transcription, PTH secretion and cell proliferation (4–7). Reduced VDR mRNA or protein expression has been shown to occur both in parathyroid adenomas and hyperplastic glands of sHPT (8–10). Furthermore, VDR polymorphisms are associated with development of parathyroid adenomas (11, 12) and VDR knockout mice acquire low bone mass, hypocalcemia, HPT and a tenfold elevation of 1,25-(OH)₂D₃ (13).

The human VDR gene spans more than 60 kb, contains 14 exons and is transcribed from at least three promoters (14–17). Recently, four novel upstream exons were identified and denoted 1f, 1e, 1d and 1b (16). These give rise to several VDR transcripts which vary in the 5’-part of the mRNA (15, 16). The 5’-terminal exons 1a, 1d and 1f are found in five, five and four different transcripts respectively, of which only two exon 1d transcripts potentially encode proteins with N-terminal extensions of 50 or 23 amino acids. Recently, a novel N-terminal-extended VDR protein was detected in several cell types and was characterized by reduced transactivation activity (18). Most of the fourteen VDR transcripts have appeared to be expressed in one analyzed parathyroid adenoma (16). However,
expression of all four transcripts that originate from
exon 1f seems to be restricted to tissues with calcio-
trophic effects of vitamin D, such as kidney, parathyroid
and an intestinal carcinoma cell line. This suggests that
the distal promoter is cell type-specifically regulated
(16). Previously conducted VDR mRNA expression ana-
lysis in parathyroid tumors used RNase protection with
a probe located in VDR exon 9, subsequently detecting
all fourteen transcripts (9). Reduced VDR mRNA levels
could, however, be due to a reduction of any of the four-
ten transcripts.

In order to investigate which of the different exon 1a,
1d and 1f VDR transcripts could cause the reduced
VDR expression level in parathyroid tumors, we have
performed quantitative real-time RT-PCR analysis on
five normal parathyroid glands, seventeen parathyroid
adenomas and ten secondary hyperplastic glands.

Materials and methods

Seventeen tumors of pHPT and ten of sHPT were
obtained from patients undergoing parathyroidec-
omy in routine clinical treatment. The patients comprised
three males and fourteen females in the pHPT group
and seven males and three females in the sHPT group.
Biopsies of five normal parathyroid glands were also
obtained of which three were normal glands from patients with parathyroid adenomas and
two were inadvertently removed at operation of goitre
patients. Patients operated for hyperparathyroidism
secondary to uremia were all hypercalcemic and four
patients received dialysis and four had undergone
renal transplantation. Informed consent and approval
of the local ethical committee was obtained. None of
the patients included in this study had a history of
familial hypercalcemia, or showed signs of multiple
docrine neoplasia or had previously received
irradiation to the neck. All parathyroid tumors were
histopathologically analyzed to confirm the diagnosis.

Total serum calcium corrected for albumin (reference
range 2.20–2.60 mM), intact serum PTH (reference
range 1.3–5.8 pmol/l) and serum creatinine (refer-
ence range 0.60–1.30 mM) were analyzed preopera-
tively. Weights of pathological glands were measured
immediately after removal of the glands.

Total RNA was extracted with TriZol Reagent (Gibco-
BRL, Life Technologies, Gaithersburg, MD, USA) from
intra-operatively frozen tissue. According to standard
procedures the RNA was treated with RQI DNase
(Wizard: Promega, Madison, WI, USA) and proteinase
K. First strand cDNA synthesis kit (Amersham Phar-
cacia Biotech, Uppsala, Sweden) was used for reverse
transcription to cDNA. Real-time quantitative RT-PCR
was carried out as previously described (19). Briefly,
cDNA from the above-described subjects and reference
cDNA (purified PCR fragments covering the sites for
probes and primers) were amplified by PCR together
with the presence of a probe tagged with a 5′ fluores-
center reporter dye and a 3′ quencher dye (5′FAM-sequence-
3′TAMRA. Applied Biosystems, Foster City, CA, USA).
During amplification, the exonuclease activity of Taq
DNA polymerase cleaves the reporter dye from the
probe and the fluorescence released is measured by a
laser detector in the ABI Prism 7700 Sequence Detec-
tion System (Applied Biosystems). Quantification of the
relative amount of target in samples was accomplished
by using standard curves for each VDR transcript and
glyceraldehyde-3-phosphate dehydrogenase (GAPDH).
The standard fragment for each transcript was estab-
lished by amplifying a purified PCR fragment covering
the sites for probe and primers. All samples were ana-
yzed in triplicate. The PCR mixture contained 5 μl
DNA template, 1× TaqMan buffer A, 5.5 mM MgCl2,
200 μM dATP, dCTP and dGTP, 400 μM dUTP,
100 nM probe, 200 nM of each primer, 0.01 U
AmplErase UNG and 0.05 U AmpliTaqTM Gold. All
reagents were supplied in the TaqMan PCR core
Reagent Kit (Applied Biosystems). The sequence of
primers and dual fluorogenic probes for VDR exon 9,
1f, 1a, 1d and GAPDH were (5′-3′): exon 9 forward,
cct tca cca tgg aca acg atg; exon 9 reverse, tca cgt cag
tga gct cag ac; exon 9 probe, gca gct gta gcc acc
agg act acg; exon 1f forward, acg cag ggc gcc caa
gca; exon 1f reverse, gag tgt ctc aat gat gag gaa t;
exon 1f probe, gca gaa agg aag agg gct tgt t;
exon 1a forward, tgt gcc cgg gga gcc cgg c; exon 1a
forward, agg tgt ctc acc cgc cgg cgc gac; exon 1d forward,
aga aag gga ggc att gcc tg; exon 1d probe, gca gat
cac tgc tgg aat gag gac; exon 1f forward; gag gat
gag gac; exon 1d forward, gag gat gat; GAPDH
forward, gag gat gat gat; GAPDH

gac; exon 1a forward, gag gat gat gat atc and GAPDH
probe, cca gtc ctc tgt cag cc.

Values are presented as means±S.E.M. Differences in
mean values were calculated with ANOVA followed by
Fischer’s PLSD using P < 0.05 as significant. All calcu-
lations were made with StatView.

Results

In order to determine the relative contribution of 5′-
terminal exon variant VDR gene transcripts (16) to
the previously observed underexpression of VDR
mRNA (exon 9), in lesions of pHPT and sHPT (9), we
designed primers and probes specific for the VDR
exons 1f, 1a, 1d and 9 (Fig. 1). The relative
VDR/GAPDH mRNA levels for each exon were deter-
mined by real-time quantitative RT-PCR in five
normal parathyroid glands, seventeen parathyroid ade-
nomas and ten hyperplastic glands of sHPT. Clinical
characteristics of the patients are shown in Table 1.
Compared with normal parathyroid glands, a sig-
nificantly reduced level of VDR exon 9 transcripts was
seen both in hyperplasias of sHPT and parathyroid

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adenomas (Fig. 2), in agreement with published results where an RNase protection assay was used for quantification (9). In the hyperplastic glands of sHPT, levels of VDR transcripts containing exons 1f, 1a and 1d were all significantly reduced compared with normal glands (P < 0.04; P < 0.001), but in parathyroid adenomas there was no significant difference (P > 0.05) for exon 1a and exon 1d (Fig. 2). Only the level of VDR transcripts with the most distal exon 1f was significantly reduced (P < 0.001) in parathyroid adenomas compared with normal glands (Fig. 2). Thus, the reduced VDR mRNA level in adenomas reflects a specific underexpression of exon 1f-containing transcripts. We note, however, that a few individual adenomas displayed low expression of VDR exon 1d transcripts. No correlations between VDR expression level for any of the different transcripts and clinical characteristics such as gland weight, serum PTH, serum calcium or serum creatinine were found.

Discussion

In this study we have shown that VDR transcripts with the most distal exon 1f are exclusively underexpressed in parathyroid adenomas in contrast to hyperplastic tumors which demonstrated an overall underexpression of all three analyzed 5’-terminal variant VDR gene transcripts. Expression of the VDR gene, which consists of 14 exons, seems to be regulated by at least three promoters located upstream of the non-coding exons 1f, 1a and 1c (14–17). Interestingly, exon 1f transcripts have so far only been detected in tissues involved in calcium regulation by 1,25-(OH)2D3, such as a parathyroid adenoma, an intestinal carcinoma cell line and kidney. The distal 1f promoter, located approximately 9 kbp upstream of exon 1a, was thus suggested to regulate tissue-specific VDR gene expression (16). The observed underexpression of exon 1f transcripts in parathyroid adenomas can be explained by several default mechanisms. The VDR 1f promoter may be directly involved through mutations in promoter elements or, more likely, indirectly through inactivating mutations or aberrant expression of a parathyroid transcription factor gene. Other possible mechanisms include changed mRNA stability or other physiologically important pathways for tissue-specific VDR gene expression. The VDR exon 1d transcript expression was not significantly reduced in parathyroid adenomas, although a few individual adenomas displayed low levels. This could represent reduced expression of the two exon 1d/exon 1c-containing VDR transcripts described (16), or of only the one encoding an N-terminal-extended VDR protein (18). The non exon-specific down-regulation of VDR transcripts in hyperplastic glands of sHPT may be due to synergistic effects of hypocalcemia and hyperphosphatemia.

1,25-(OH)2D3 is crucial in the maintenance of serum calcium and regulates parathyroid gland activity by inhibiting PTH transcription, PTH secretion and cell proliferation (4–7). Many of these regulatory pathways involve, or are expected to involve, the VDR. Furthermore, suboptimal vitamin D nutrition stimulates growth of parathyroid adenomas (20), and over-representation of certain VDR alleles in Caucasian patients are predictive factors for the development of pHPT (11, 12). Reduction of the cell type-specific VDR exon 1f transcripts in pHPT and of exon 1f, 1a and 1d transcripts in sHPT is expected to interfere with the regulatory effects of 1,25-(OH)2D3 in the pathological glands and to contribute to parathyroid tumorigenesis. Elucidating the precise regulatory mechanisms for VDR gene expression and identification of 1,25-(OH)2D3 target genes in parathyroid glands, whose expression is sensitive to a reduced VDR level, demands further studies.

Table 1 Clinical characteristics of the patients. Values are means±S.E.M.

<table>
<thead>
<tr>
<th></th>
<th>Age (years)</th>
<th>Calcium (mmol/l)</th>
<th>PTH (pmol/l)</th>
<th>Gland weight (g)</th>
<th>Creatinine (µmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (n = 5)</td>
<td>59.3±6.8</td>
<td>2.62±0.29</td>
<td>ND</td>
<td>ND</td>
<td>88±12</td>
</tr>
<tr>
<td>pHPT (n = 15)</td>
<td>67.4±3.5</td>
<td>2.92±0.05</td>
<td>12.7±2.8</td>
<td>1.13±0.21</td>
<td>90±5.7</td>
</tr>
<tr>
<td>sHPT (n = 10)</td>
<td>51.1±2.6</td>
<td>2.2±0.06</td>
<td>83.3±22.6</td>
<td>3.42±1.2</td>
<td>663±87</td>
</tr>
</tbody>
</table>

The group of five normal gland biopsies consisted of three normal glands from patients with parathyroid adenomas and two inadvertently removed at operation of goitre patients. Two additional pHPT patients with ordinary clinical characteristics were included in the study. ND, not determined.

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References


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