Analysis of CYP27B1, Encoding 25-Hydroxyvitamin D-1α-Hydroxylase, as a Candidate Tumor Suppressor Gene in Primary and Severe Secondary/Tertiary Hyperparathyroidism

Kelly Lauter and Andrew Arnold

ABSTRACT: *CYP27B1*, encoding 25-hydroxyvitamin D-1α-hydroxylase, converts 25-hydroxyvitamin D to its active form, 1,25-dihydroxyvitamin D, and is expressed primarily in the kidney but also in nontraditional sites including the parathyroid glands. Whereas the role of locally produced 1,25-dihydroxyvitamin D is not yet clear, it is possible that it contributes importantly to vitamin D–mediated inhibition of parathyroid cell growth, so *CYP27B1* can be considered a candidate parathyroid tumor suppressor gene in that its acquired inactivation in a parathyroid cell could confer a tumorigenic growth advantage. Expression of *CYP27B1* has also been reported to be altered in parathyroid neoplasms. Because detection of inactivating mutations is the central criterion for validating a candidate tumor suppressor, we directly sequenced the coding region and all splice sites of *CYP27B1* in 31 sporadic parathyroid adenomas and 31 parathyroid tumors from patients with refractory secondary/tertiary hyperparathyroidism. No nonsense, frameshift, or other inactivating mutations were found, and there was no sign of homozygous deletion. Our findings indicate that *CYP27B1* does not commonly serve as a classical tumor suppressor gene in the development of sporadic parathyroid adenomas or of refractory secondary/tertiary hyperparathyroidism.
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Key words: parathyroid, vitamin D, PTH

INTRODUCTION

25-HYDROXYVITAMIN D-1α-HYDROXYLASE [1α-(OH)ase], a mitochondrial cytochrome P450 enzyme encoded by *CYP27B1*, is responsible for the conversion of 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D [$1,25(OH)_2D_3$], which is the active form of the vitamin. $1,25(OH)_2D_3$ has a multitude of actions, among them increasing calcium absorption in the intestine and, in the parathyroid glands, inhibiting PTH gene transcription, PTH secretion, and parathyroid cell growth.[1,2]

Mutations in *CYP27B1* are responsible for the autosomal recessive disease in humans termed vitamin D–dependent rickets type I (VDDR I) or pseudovitamin D deficiency rickets (PDDR). Affected individuals develop rickets and osteomalacia, as well as secondary hyperparathyroidism, growth retardation, hypotonia, and abnormal mineral homeostasis.[3,4] The phenotype is reflected by a mouse knockout model of *CYP27B1*.[5,6]

The primary site of *CYP27B1* expression is the proximal tubule of the kidney[7]; however, the enzyme is also expressed in various extrarenal sites, including the parathyroid glands,[8] colon, and pancreas.[9] Because 1α-(OH)ase is expressed in the parathyroids, it is possible that local 1α-hydroxylation is responsible for a significant proportion of the total effect of $1,25(OH)_2D_3$ on the parathyroid gland. This local production may be especially important in patients with chronic renal failure, because of their diminished ability to produce $1,25(OH)_2D_3$ in the kidneys and their low circulating $1,25(OH)_2D_3$ levels. That said, the role of locally active 1α-hydroxylation in the parathyroids remains uncertain, and the important possibility that its acquired loss could cause a clonal selective advantage is not directly spoken to by the hyperparathyroid phenotype in humans and mice with germline inactivation of the 1α-(OH)ase gene.

Monoclonality is a central feature of sporadic primary adenomas,[10] as well as the majority of parathyroid glands removed because of severe refractory secondary/tertiary hyperparathyroidism,[11] indicating that these tumors developed from a single cell with a selective growth advantage. Tumor suppressor genes are defined as those in which inactivating mutations contribute to tumorigenesis,[12] with *MEN1* and *HRPT2* being two such genes already implicated in parathyroid neoplasia.[13,14] Whereas many tumor suppressors have normal functions in processes like cell cycle control or signaling, a wide variety of tumor suppressor functions exists.[15] Given the antiproliferative properties of $1,25(OH)_2D_3$ in the parathyroid and reported abnormalities in its expression, a crucial question is whether acquired inactivation of *CYP27B1* is a primary driving contributor to clonal parathyroid growth, serving as a classic tumor suppressor gene. Segersten et al.[8] found the major-

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Center for Molecular Medicine and Division of Endocrinology and Metabolism, University of Connecticut School of Medicine, Farmington, Connecticut, USA.
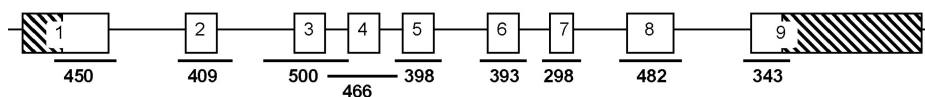
FIG. 1. Schematic diagram of *CYP27B1* with PCR amplification and sequencing strategy. All nine exons are shown as boxes: protein coding, open; 5′ and 3′ UTRs, hatched. PCR products are shown below, with their corresponding size in base pairs.

ity of parathyroid adenomas to have strong but somewhat variable and irregular immunohistochemical staining for the 1α-(OH)ase enzyme, whereas a subset of parathyroid adenomas had weak staining. These patterns are consistent with the possibility that *CYP27B1* acts as a tumor suppressor gene in adenomas, because one would generally expect that a subset of tumors would result from inactivation of a specific tumor suppressor, with some of these showing decreased protein levels. Glands from patients with renal failure had irregular strong staining, but the intensity was notably less in areas of nodular (often monoclonal) growth, potentially consistent with an even higher prevalence of *CYP27B1* inactivation. However, despite the intriguing nature of these expression patterns, they could reflect secondary changes or epiphenomena, and mutational analyses are therefore crucial in definitively addressing *CYP27B1*'s candidacy.[12] We analyzed *CYP27B1* for inactivating mutations by direct sequencing in sporadic parathyroid adenomas and also in parathyroid tumors from patients with severe refractory secondary/tertiary hyperparathyroidism.

MATERIALS AND METHODS

Patients and samples

We studied 31 sporadic parathyroid adenomas and 31 parathyroid tumors from patients with chronic kidney disease (CKD) and refractory secondary or tertiary hyperparathyroidism. All parathyroid adenomas were typical examples of single gland disease, both clinically and histologically. Criteria for parathyroidectomy in CKD patients were as previously described,[11,16] based on development of hypercalcemia and/or symptoms resistant to standard medical therapy, and are known to yield a high prevalence of monoclonal parathyroid neoplasia. All tissue specimens were obtained in accordance with institutional review board–approved protocols for human studies.

Parathyroid specimens were frozen in liquid nitrogen after surgical removal and stored at –80°C. High molecular weight genomic DNA was extracted from frozen tissue using standard procedures including proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation.

CYP27B1 sequencing

The entire nine exon coding region and splice sites of *CYP27B1* were amplified by PCR (Fig. 1), using published primer sequences.[3] Primers were optimized at an annealing temperature of 55°C, with the exception of primers for exon 6, which had an annealing temperature of 59°C. PCR reactions were performed in 20-μl reaction volumes containing 25 ng genomic DNA, 20 pmol of each primer, 200 μM of each dNTP, 2 mM magnesium chloride, and 1.25 U of Amplitaq Gold DNA Polymerase (Applied Biosystems,

Foster City, CA, USA). Each PCR reaction was performed in conjunction with a negative control that did not contain any template DNA; such control reactions were uniformly and appropriately negative. The thermocycling conditions included an initial denaturation step of 95°C for 10 min; 35 cycles of 95°C for 30 s, the optimized Ta for 30 s, and 72°C for 30 s; and a final extension step at 72°C for 10 min. PCR products were purified and sequenced using standard methods on ABI3730xl instruments (Agencourt Bioscience). Sequence data were analyzed with Sequencher software (GeneCodes, Ann Arbor, MI, USA) and compared with the reference sequence (RefSeq ID: NM_000785).

RESULTS

We analyzed 31 sporadic parathyroid adenomas and 31 parathyroid tumors from patients with severe secondary or tertiary hyperparathyroidism by direct sequencing for intragenic mutations of the *CYP27B1* gene. We sequenced all coding regions and splice sites in a search for clonal inactivating mutations. No point mutations, deletions, or insertions were uncovered in any of the 62 samples studied. All exons of each sample were able to be amplified by PCR, providing evidence against homozygous deletion. Furthermore, in a prior report from our group, loss of heterozygosity, a common mechanism for tumor suppressor activation, was present at chromosome 12q, the location of *CYP27B1*, in only 1 of the 29 examined adenomas and in none of the secondary hyperparathyroid tumors,[16,17] thus reinforcing our current observations.

DISCUSSION

The active form of vitamin D, $1,25(OH)_2D_3$, has a crucial function in the parathyroid glands, where it decreases PTH gene expression and secretion and parathyroid cell growth. Local expression of 1α-(OH)ase in the parathyroid glands may be required to achieve adequate levels of the hormone to suppress cell growth. Thus, *CYP27B1* is an important candidate tumor suppressor gene that could acquire loss of function mutations specifically in the parathyroid cell genome, leading to a selective advantage and tumor development. Our approach was designed to rigorously test the possibility that *CYP27B1* serves as a classic tumor suppressor in the parathyroids. Only recurrent inactivating mutations can provide this critical evidence for a primary driving role,[15] because gene expression changes can reflect secondary phenomena not necessarily involved in tumorigenesis. Therefore, we explored the mutational status of this gene and found no mutations in any of the 62 tumors studied. Whereas our approach does not exclude the possibility of epigenetic changes or mutations in the unsequenced re-

gions like introns, the promoter region, or the untranslated regions (UTRs), it would be unprecedented if inactivating alterations in an authentic tumor suppressor gene were to be found exclusively in these regions without ever appearing in the coding region.

These observations complement previous study of the vitamin D system in this context. The vitamin D receptor gene (VDR) was sequenced in sporadic parathyroid adenomas and in parathyroids from patients with severe hyperparathyroidism of uremia, and it was found that clonal mutations in VDR occur rarely, if at all, in these tumors.[18,19] However, it remains possible that influences on local production of $1,25(OH)_2D_3$ could still contribute to parathyroid tumorigenesis in a less direct fashion,[20,21] but our observations make a direct driving role very unlikely.

In summary, our observations strongly suggest that *CYP27B1* acts infrequently, if at all, as a classical tumor suppressor gene in the molecular pathogenesis of parathyroid adenomas or refractory secondary/tertiary hyperparathyroidism.

ACKNOWLEDGMENTS

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Address reprint requests to:
Andrew Arnold, MD
Center for Molecular Medicine
University of Connecticut Health Center
263 Farmington Avenue
Farmington, CT 06030-3101, USA
E-mail: molecularmedicine@uchc.edu