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ORIGINAL ARTICLE

Evidence of a stabilizing mutation of β-catenin encoded by *CTNNB1* exon 3 in a large series of sporadic parathyroid adenomas

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Abstract Aberrant accumulation of β-catenin plays an important role in a variety of human neoplasms. This can be caused by stabilizing mutation of β-catenin (*CTNNB1*, exon 3) or by mutation or deregulated expression of other components of the WNT/β-catenin signaling pathway. Accumulation of non-phosphorylated active β-catenin has been reported to commonly occur in parathyroid adenomas from patients with primary hyperparathyroidism (pHPT), either due to the aberrantly spliced internally truncated WNT receptor LRP5 (LRP5Δ) or to a stabilizing mutation of β-catenin. The S37A mutation was reported to occur in 7.3 % in a single study of parathyroid adenomas, while in other studies no stabilizing mutations of β-catenin exon 3 were identified. The aim of this study was to determine the mutational frequency of the *CTNNB1* gene, specifically exon 3 in a large series of parathyroid adenomas. One hundred and eighty sporadic parathyroid adenomas were examined for mutations in exon 3 of *CTNNB1* by direct DNA sequencing, utilizing previously published primer sequences. The mutation S33C (TCT>TGT) was detected by direct-DNA sequencing of PCR fragments in 1 out of 180 sporadic parathyroid adenomas (0.68 %). Like serine 37, mutations of serine 33 have been reported in many neoplasms with resulting β-catenin stabilization, enhanced transcription, and oncogenic activities. Immunohistochemical analysis revealed an overexpression of the β-catenin protein in the lone mutant tumor. Taking also previous studies into account we conclude that activating mutations of the regulatory GSK-3β phosphorylation sites serine 33 and 37, encoded by *CTNNB1* exon 3, rarely occur in parathyroid adenomas from patients with pHPT.

Keyword Parathyroid · Primary hyperparathyroidism · Beta-catenin

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Introduction

Primary hyperparathyroidism (pHPT) is a relatively common disease, occurring in 2.1 % of the population [1]. The peak age of diagnosis is during the fifth to sixth decade of life, with a gender predominance toward females [2]. The disease is characterized by elevated serum calcium levels in the face of a physiologically inappropriately elevated PTH level. Patients can present with the typical signs and symptoms of bone loss, osteoporosis and fractures associated with decreased bone density, nephrolithiasis, pancreatitis, gastro-esophageal reflux disease, and vague symptoms of fatigue and difficulty with concentration and even significant cardiac abnormalities [2, 3].

Sporadic pHPT occurs predominantly due to a single hyperfunctioning gland (85 %), but can be due to multiglandular (which includes four gland hyperplasia) disease in 10–15 % [4] and is rarely from carcinoma (<1 %) [5]. Some cases of sporadic pHPT have been linked to direct exposure of radiation to the head and neck area, usually if the exposure occurred during childhood [6].

During the past decade and a half, studies of genetic predisposition, parathyroid tumorigenesis, and molecular genetics of familial hyperparathyroid disorders have started to unveil the molecular basis of pHPT [7]. Allelic loss (loss of heterozygosity; LOH) of chromosomal loci may identify tumor suppressor genes in neoplasia. LOH at the *MEN1* gene locus on chromosome 11q13 has been demonstrated in approximately 25–40 % of sporadic parathyroid adenomas, and somatic homozygous mutations of the recently identified *MEN1* gene are found in 12–17 % of adenomas, or about 50 % of those tumors with LOH at 11q13 [8, 9]. These findings clearly indicate that mutational aberrations in the *MEN1* gene contribute to parathyroid tumorigenesis. The *cyclin D1* oncogene, now recognized to have a central role in many forms of human neoplasia, was initially identified at the breakpoint of a parathyroid adenoma DNA rearrangement [10]. Cyclin D1 is a target of the Wnt/β-catenin signaling pathway [11]. Aberrations in the Wnt/β-catenin signaling pathway have been shown in parathyroid tumors, [12–14] including 7.3 % of examined parathyroid adenomas from a single Swedish cohort, which demonstrated activating mutations in exon 3 of *CTNNB1*. However, other studies have failed to show any such mutations [15–17]. An aberrantly spliced, internally truncated variant of LRP5 (LRP5Δ), a co-receptor for Wnt ligands, resulting in stabilization and accumulation of β-catenin seems to be present in a majority of parathyroid tumors of pHPT [18].

Subjects and methods

One hundred and eighty patients with surgically verified, non-familial pHPT due to a single adenoma were included in the study. Tumors were carefully evaluated by an experienced endocrine pathologist prior to use in the study. Tumors were dissected prior to processing to minimize contamination by normal cells. The tissue was snap frozen in liquid nitrogen and stored at −80 °C. None of the patients demonstrated evidence of familial disease or had a history of previous neck irradiation. Blood was collected after an overnight fast and serum (s-) calcium (reference range 2.20–2.60 mmol/L) and intact s-PTH (reference range 10–65 ng/L) were determined preoperatively. All patients were normocalcemic during routine follow-up, which lasted for at least 6 months (Table 1). Informed consent was obtained and the study was approved by the institutional review board.

Genomic DNA was isolated from surgically resected and then snap-frozen fresh tumor samples and matched blood using standard protocols included in the commercially-available Qiagen DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA). All specimens were quality control checked for purity utilizing the nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Each sample (40 ng of DNA) was PCR amplified with the primers 5′-TGATGGAGTTGGACATGGCC (forward) and 5′-CTCATACAGGACTTGGGAGG (reverse), which yielded an amplicon measuring 182 bp in size. The fragments underwent both forward and reverse Sanger sequencing at the Keck DNA Sequencing Facility at Yale University. Data were analyzed with the use of Sequencing Analysis and Auto Assembler software (Applied Biosystems), and publically available web based resources (NCBI-Blast, GenBank). All novel DNA variants were verified by re-sequencing using genomic DNA from a separate extraction.

Immunohistochemical staining was preformed on the lone pathologic specimen and compared to normal parathyroid tissue which had been incidentally removed during thyroid surgery. Paraffin-embedded specimens were stained using an anti-β-catenin goat polyclonal antibody with an epitope mapping at the C-terminus (Santa Cruz Biotechnology, INC., Santa Cruz, CA; catalog no. sc-1496) (Fig. 2), as previously described [13].

Results

PCR amplicons from patients with sporadic primary hyperparathyroidism were analyzed for the presence of point mutations, insertions, and microdeletions of exon 3 of the *CTNNB1* gene. The S37A mutation found identically in nine adenomas by Björklund et al. [12] was not identified in any of the 180 samples studied. However, a single

Table 1 Clinical data for the cohort ($n = 180$)

	Main cohort ($n = 180$)	Patient with *CTNNB1* (S33A) mutation
Age (years)	62.57 (±1.61)	52
Gender M:F	15:98	F
Pre-op Ca^{2+} (mg/dl)	11.1 (±0.09)	11.1
Post-op Ca^{2+} (mg/dl)	9.50 (±0.05)	8.8
Pre-op PTH (pg/dl)	134.18 (±7.33)	95
Post-op PTH (pg/dl)	34.4 (±2.61)	22
Gland weight (mg)	891.5 (±107.13)	185

Standard deviation indicated in *parenthesis*

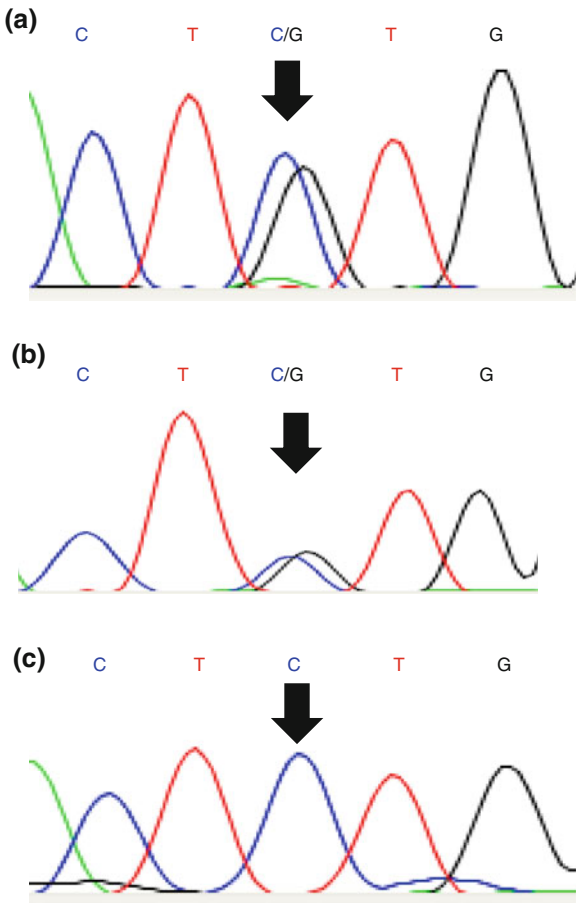

Fig. 1 Chromatogram of CTNNB1 (S33C) exon 3 mutation **a** 3′–5′ direction, **b** 5′–3′ direction when compared to germline non-mutant specimens **c** illustrating the S33C aberration

heterozygous somatic mutation was identified at codon 33 of the *CTNNB1* gene. The stabilizing mutation S33C (TCT>TGT) was detected by direct DNA sequencing of PCR fragments in 1 out of 180 sporadic parathyroid adenomas (0.68 %) (Fig. 1) [18]. To determine the *β*-catenin protein expression level in normal the pathological parathyroid gland, we performed immunohistochemical analysis on paraffin fixed tissue sections with a specific goat polyclonal peptide antiserum. Parathyroid cell staining in the parathyroid adenoma displaying a S33C mutation displayed an increased protein expression versus that in a control specimen (with wild-type *β*-catenin protein) (Fig. 2).

Discussion

The molecular mechanisms underlying benign sporadic parathyroid tumorigenesis are incompletely understood. Clearly, germline and sporadic mutations of the *MEN1* tumor suppressor gene at 11q13 play an important role in parathyroid tumorigenesis. Activation of *cyclin D1* has also been implicated as a potential driver of parathyroid tumorigenesis. The Wnt/*β*-catenin signaling pathway, an upstream activator of *cyclin D1* expression, may contribute to parathyroid tumorigenesis [7]. Aberrant accumulation of *β*-catenin was dependent of the LRP5Δ receptor in most parathyroid tumors from patients with pHPT and sHPT [19]. In 7.3 % of analyzed parathyroid adenomas not expressing LRP5Δ, the *β*-catenin S37A stabilizing mutation was identified in a select cohort of patients [12, 14, 18], a finding that has not been substantiated by additional studies [15–17, 19]. This led

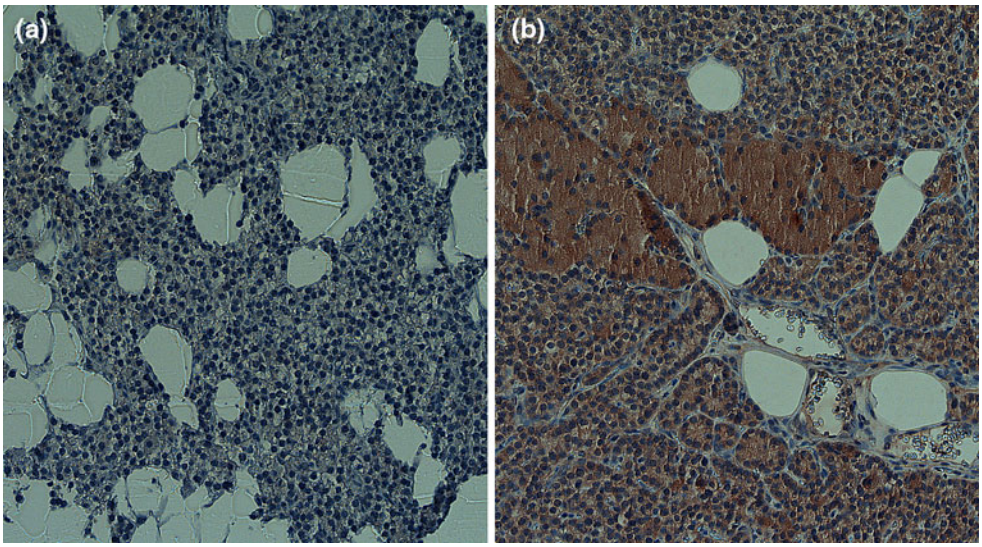

Fig. 2 Immunohistochemical staining of **a** normal parathyroid tissue. **b** Pathological tissue of the S33C mutant patient in our cohort stained for *β*-catenin utilizing goat polyclonal antibody (Santa Cruz Biotechnology, INC., Santa Cruz, CA; catalog no. sc-1496)

us to believe that there may be other mutations of the *CTNNB1* gene in exon 3, which required a large series of 180 parathyroid adenomas for validation.

Similar to the four studies previously mentioned we were unable to accurately identify any S37A mutation within exon 3 of the *CTNNB1* gene. We were, however, able to identify a single somatic mutation within one parathyroid adenoma at codon 33, where a serine was substituted for cysteine. Serine 33 is one of the GSK-3β phosphorylation sites regulating β-catenin stability. A mutation at this site leads to protein accumulation, enhanced transcriptional and oncogenic activities. This novel parathyroid mutation has been implicated in a multitude of other neoplastic processes with variable mutational frequency [20]. Recently, this mutation was concomitantly identified in an Italian cohort, with a frequency of 1.8 % [21].

To determine the β-catenin protein expression level in normal parathyroid tissue and the pathological parathyroid gland, we performed immunohistochemical analysis on paraffin fixed tissue sections with a specific goat polyclonal peptide antiserum. Accumulation of β-catenin through stabilizing mutations or other mechanisms may result in deregulated transcription of Wnt signaling target genes and promotion of oncogenic signals that lead to tumor formation [20].

Aberrant accumulation of β-catenin, caused by stabilizing mutations like S33C and S37A, may drive parathyroid tumorigenesis in a small number of cases. Further assessment of this gene and its functional influences on parathyroid diseases is warranted.

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