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# Molecular analysis of cyclin D1 modulators *PRKN* and *FBX4* as candidate tumor suppressors in sporadic parathyroid adenomas

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## Abstract

*Objective:* Primary hyperparathyroidism is most often caused by a sporadic single-gland parathyroid adenoma (PTA), a tumor type for which cyclin D1 is the only known and experimentally validated oncoprotein. However, the molecular origins of its frequent overexpression have remained mostly elusive. In this study, we explored a potential tumorigenic mechanism that could increase cyclin D1 stability through a defect in molecules responsible for its degradation.
*Methods:* We examined two tumor suppressor genes known to modulate cyclin D1 ubiquitination, *PRKN* and *FBXO4* (*FBX4*), for evidence of classic two-hit tumor suppressor inactivation within a cohort of 82 PTA cases. We examined the cohort for intragenic inactivating and splice site mutations by Sanger sequencing and for locus-associated loss of heterozygosity (LOH) by microsatellite analysis.
*Results:* We identified no evidence of bi-allelic tumor suppressor inactivation of *PRKN* or *FBXO4* via inactivating mutation or splice site perturbation, neither in combination with nor independent of LOH. Among the 82 cases, we encountered previously documented benign single nucleotide polymorphisms (SNPs) in 35 tumors at frequencies similar to those reported in the germlines of the general population. Eight cases exhibited intragenic LOH at the *PRKN* locus, in some cases extending to cover at least an additional 1.7 Mb of chromosome 6q25-26. *FBXO4* was not affected by LOH.
*Conclusion:* The absence of evidence for specific bi-allelic inactivation in *PRKN* and *FBXO4* in this sizeable cohort suggests that these genes only rarely, if ever, serve as classic driver tumor suppressors responsible for the growth of PTAs.

**Key Words**
- parathyroid adenoma
- cyclin D1
- PRKN
- PARK2
- FBXO4
- tumor suppressor

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## Introduction

Primary hyperparathyroidism (PHPT) is a common endocrine disorder that affects up to 36 people per 1000 population, disproportionately impacting women such that about 2% of post-menopausal women will eventually develop PHPT (1). Approximately 85% of all PHPT cases are caused by sporadic single-gland parathyroid adenomas (PTAs), benign tumors which typically release inappropriately high levels of parathyroid hormone (PTH) and cause hypercalcemia, in turn often leading to osteoporosis, kidney stones, and myriad neurocognitive symptoms. Knowledge of oncogenic pathways active in PTA remains incomplete, and increased understanding

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of its molecular pathogenesis could lead to advances in disease prevention, diagnosis, and development of nonsurgical treatment options.

Cyclin D1, encoded by *CCND1*, is the only known and experimentally validated PTA oncoprotein – yet the molecular origins of its frequent overexpression have remained mostly elusive. *CCND1* was initially identified and implicated as a driver oncogene via the discovery of a chromosome 11 rearrangement in PTA (2, 3, 4), in which the promoter for the parathyroid hormone gene (*PTH*) is juxtaposed to the coding region of *CCND1* and thus drives its overexpression in a parathyroid-specific manner (5). Subsequent studies in a mouse model confirmed the ability of overexpressed cyclin D1 to drive hyperparathyroidism (6). Interestingly, cyclin D1 overexpression at the protein level has been reported in 20-40% of PTAs (7, 8, 9, 10, 11, 12). This observation can be explained by DNA rearrangement involving the *CCND1* locus in about 8% of cases (13), suggesting that one or more additional causes of pathogenic cyclin D1 overexpression remain to be discovered. Some potential causes, such as stabilizing intragenic mutation (14, 15, 16) have not been substantiated by available evidence. Other possibilities, such as epigenetic upregulation via *CCND1* promoter hypomethylation (17, 18), have not yet been addressed in studies of parathyroid neoplasia, although methylation abnormalities have been interestingly reported in certain cyclin-dependent inhibitor genes in association with *CCND1* overexpression (19).

We hypothesized that in some PTAs, excess cyclin D1 may be attributable to enhanced stability of the protein (20, 21). In fact, several mechanisms by which cyclin D1 could resist proteolysis remain unexplored in PTA. Given the absence of stabilizing alterations in the *CCND1* coding sequence itself (14, 15, 16), other regulators of cyclin D1 stability may prove worthwhile investigatory targets – in particular, tumor suppressor genes that encode cyclin D1-targeting degradation machinery. For example, ubiquitin ligase complex components PRKN (PARK2 or Parkin), an E3 ubiquitin ligase, and FBXO4 (FBX4), a substrate recognition protein, have been implicated as important regulators of proteasomal cyclin D1 degradation and other tumor-suppressive functions (22, 23, 24, 25). Their eponymous genes, *PRKN* and *FBXO4*, exhibit evidence consistent with potential two-hit tumor suppressor inactivation (26, 27) across many types of cancer. Specifically, somatic copy number loss, microdeletions, missense mutations, and nonsense mutations are frequently reported in both *PRKN* and *FBXO4* (28, 29, 30, 31, 32). Therefore, we investigated a cohort of typically presenting, single-gland, sporadic PTAs for somatic inactivation via coding or splice site mutations or allelic loss in *PRKN* and *FBXO4*.

## Materials and methods

### Patients and samples

Patient samples were obtained with informed consent and utilized in accordance with UConn Health Institutional Review Board approved protocols and policies of the University of Connecticut. Eighty-two cases of sporadic parathyroid adenoma were selected for this study according to the following criteria: (i) referral for parathyroidectomy following a diagnosis of biochemical PHPT, that is, hypercalcemia with elevated or inappropriately normal parathyroid hormone levels; (ii) absence of personal or family history suggestive of a heritable or syndromic form of parathyroid disease; (iii) single-gland lesion resected at parathyroidectomy, and (iv) histological confirmation of a high-purity, adenomatous tumor free of any atypical or malignant features. Median patient age was 59 at the time of parathyroidectomy (range 19–90). The cohort included 59 females, 21 males, and 2 samples where gender was not noted in deidentified pathology reports. Apart from the need to have adequate quantity of tissue available for research, and quality of samples for study, cases were otherwise unselected in terms of clinical or demographic criteria.

Tumor DNA was isolated from fresh frozen patient tumor samples by proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation. Matched germline DNA was isolated from peripheral blood using PureGene Blood Kit (Qiagen) or from muscle tissue using the phenol-chloroform method.

### Sequencing

The coding regions of *PRKN* and *FBXO4* and intron-exon boundaries were amplified by PCR using self-designed primers (Supplementary Table 1, see section on supplementary materials given at the end of this article), AmpliTaq Gold DNA Polymerase with Buffer II and MgCl2 (Applied Biosystems). Each reaction contained 25 ng template DNA, 1 U polymerase, 0.5 μM each of forward and reverse primers, 200 μM dNTPs, and 1.5 mM of $MgCl_2$ in a 20 μL reaction. PCR was conducted as follows: denaturation for 10 min at 95°C; 35 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 55°C, and extension

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for 30 s at 72°C; and a final extension step for 10 min at 72°C. Gel electrophoresis of PCR product was run on a 1.5% (w/v) agarose, 0.6 µg/mL ethidium bromide gel at 120 volts for approximately 30 min. PCR product that yielded clear bands was enzymatically purified with ExoSAP-IT (Applied Biosystems) and Sanger sequenced in forward and reverse directions (GENEWIZ, Inc., South Plainfield, NJ, USA). Sequence data were aligned to NCBI reference sequences (*PRKN*, NM_004562.3; *FBXO4*, NM_033484.2) and analyzed with Sequencher software (Gene Codes Corporation, Ann Arbor, MI, USA). The entire coding sequence and intron-exon boundaries were examined for mutations. Variant databases dbSNP (33), COSMIC (34), and ClinVar (35) were queried for any identified variants. Variants were assessed by predictive modeling tools SIFT (36) and Poly-Phen (37), and meta prediction tools REVEL (38) and MetaLR/dbNSFP (39), all available through Ensembl (40).

### Loss of heterozygosity

For each tumor and germline sample, five highly polymorphic microsatellite loci in and around *PRKN* (D6S1599, D6S305, and D6S1581) and *FBXO4* (D5S418 and D5S2082) were amplified using fluorophore-tagged primers, whose sequences are available through the UCSC Genome Browser (41). Each reaction contained 25 ng template DNA, 1 U polymerase, 0.4 µM each of forward and reverse primers, 250 µM dNTPs, and 2.5 mM of $MgCl_2$ in a 15 µL reaction. PCR was conducted as follows: denaturation for 12 min at 95°C; 10 cycles of denaturation for 15 s at 95°C, annealing for 15 s at 55°C, and extension for 30 s at 72°C; 20 cycles of denaturation for 15 s at 89°C, annealing for 15 s at 55°C, and extension for 30 s at 72°C; and a final extension step for 10 min at 72°C. Gel electrophoresis of PCR product was run on a 2% (w/v) agarose, 0.6 µg/mL ethidium bromide gel at 120 volts for approximately 30 min. PCR product that yielded clear bands was submitted to fragment analysis (GENEWIZ, Inc., South Plainfield, NJ, USA). Allele peaks for each microsatellite locus were called with GeneMarker software (SoftGenetics, LLC, State College, PA, USA) (42). The allelic ratio (AR) of the fluorescent signals of an individual's discrete alleles were calculated as follows:

$$AR = \frac{(A_T / B_T)}{(A_G / B_G)}$$

The ratio of fluorescent signal from discreet alleles A and B was compared between tumor (T) and matched germline (G) samples. Consistent with prior studies (43, 44, 45, 46, 47), cases with allelic ratios above 2 or below 0.5 – indicative of a two-fold or greater change in allele signal ratio in the tumor sample compared to the germline sample – were scored as having undergone LOH.

## Results

### Sequencing

In order to detect inactivating genetic alterations such as frameshift indels, early stop codons, or damaging substitutions in this cohort of 82 typical PTAs, the coding regions and intron-exon boundaries of the 12 exons in *PRKN* and 5 exons in *FBXO4* were amplified by PCR and subjected to Sanger sequencing. Sequencing did not reveal any clearly inactivating mutations that might be expected to attenuate the tumor-suppressive function of these cyclin D1-modulating genes. In *PRKN*, we identified six heterozygous single nucleotide polymorphisms (SNPs) among 35 patients, all of which are previously documented SNPs found in the germline at the population level (33). Two of the SNPs (rs9456711 and rs144340740) were synonymous nucleotide substitutions and annotated as benign variants in ClinVar (35). The remaining four SNPs (rs1801474, rs9456735, rs1801582, and rs1801334) correspond with missense substitutions (S167N, M192L, V380L, and D394N, respectively) that are annotated as benign by ClinVar (35). Predictive modeling tools SIFT (36) and Poly-Phen (37), and meta prediction tools REVEL (38) and MetaLR/dbNSFP (39), largely predicted that these substitutions would be benign. Exceptions include the REVEL prediction of M192L as likely disease causing, and the SIFT/MetaLR prediction of D394N as deleterious or damaging. However, neither of these variants are documented as somatic changes in cancer (34), and both occur at frequencies similar to those in the overall population (33) and are not otherwise associated with a tumor phenotype or aberrant or defunct proteins. In *FBXO4*, we observed a single heterozygous synonymous SNP (rs144096644) in one case. This polymorphism is reported at a low population level frequency (33) and is not associated with tumors (34) or other disease.

### Loss of heterozygosity

To detect LOH in *PRKN* and *FBXO4*, we probed gene-flanking and intragenic microsatellite markers by PCR and fragment analysis for the loss of an allele in all 82 tumors

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and their matched germline controls. We examined the status of two markers that lie within *PRKN* intronic regions and a third 1.5 MB upstream of the gene (Fig. 1A) in addition to two markers that flank *FBXO4* (Fig. 1B). A total of 8/82 cases (9.8%) exhibited LOH at one or more *PRKN* microsatellite markers (Figs 1A and 2). However, only one tumor exhibited LOH at both of the intragenic microsatellites (Fig. 1A, case 4). LOH at the upstream marker, in this case, allows for the possibility that *PRKN* LOH may be nonspecific, that is, part of a much larger stretch of chromosomal loss and therefore not indicative of specific selective pressure attributable to deletion of the *PRKN* locus. Similarly, the upstream marker exhibited LOH in five other samples where results indicated LOH at one intragenic marker, but were uninformative at the other (Fig. 1A; cases 13, 29, 53, 57, and 66). Thus, in the absence of evidence for concurrent intragenic inactivating second hits on the other allele, these observations do not implicate *PRKN* as a classic driving tumor suppressor gene. Finally, no tumors in the cohort exhibited LOH at either of the *FBXO4*-flanking markers assayed.

## Discussion

The oncoprotein cyclin D1 is one of the few validated drivers of PTA tumorigenesis, yet in many instances the molecular mechanism causing cyclin D1 overexpression, found in up to 40% of these tumors, remains unknown (1). While a pathogenic DNA rearrangement activates the cyclin D1 gene in some cases, other potential activating mechanisms such as stabilizing mutation (14, 48, 49) and amplification (50) of the cyclin D1 gene *CCND1* occur rarely, if at all, in PTA. Although mounting evidence suggests that regulators of cyclin D1 stability via proteolytic degradation may play a prominent role as tumor suppressors, such cyclin D1 pathway components like PRKN and FBXO4 have thus far been underexplored in PTA. The genetic aberrations in the *PRKN* and *FBXO4* genes reported in many types of cancer are consistent with those characteristics of classical two-hit tumor suppressor inactivation, including allelic loss and/or intragenic mutations that would be expected to impair both alleles.

In this study, we report results of mutational and allelic loss analysis of *PRKN* and *FBXO4* in 82 typically presenting, sporadic, single-gland PTAs. We uncovered no evidence of bi-allelic inactivation; indeed, our observations identified only previously documented SNPs with no known pathogenicity and only occasional loss of intragenic *PRKN* marker(s). Because *PRKN* lies within the large common fragile site FRA6E, which is prone to instability (51), allelic loss of *PRKN* may often be nonspecific and would need to, at minimum, occasionally be accompanied by co-occurrence of specific inactivating mutations on the other allele in order to constitute strong evidence invoking *PRKN* as a tumor suppressor whose inactivation yields a selective advantage (27, 52). Our investigation of *FBXO4* did not reveal any non-synonymous intragenic alterations or allelic loss.

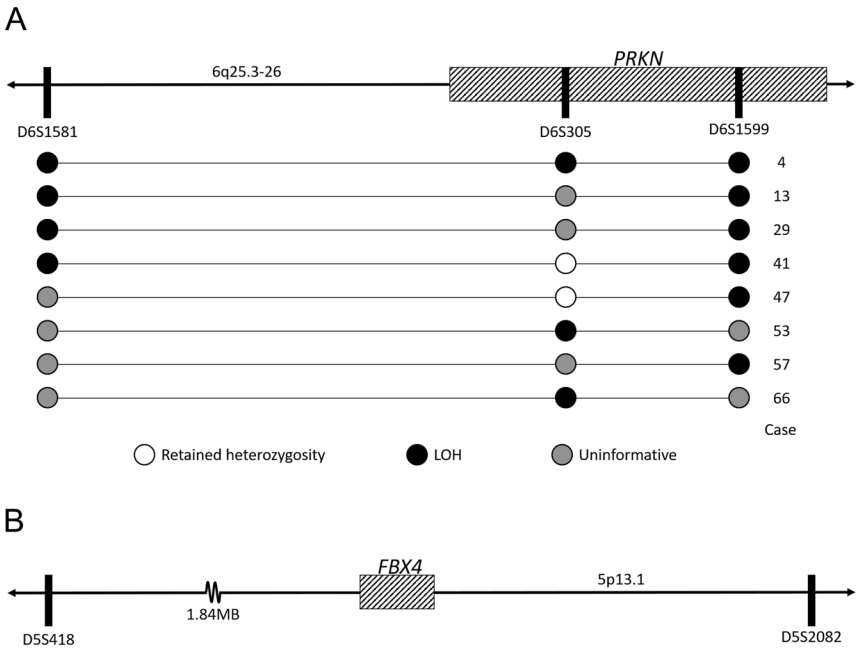


**Figure 1**
Schematic of LOH markers and allelic loss. (A) Microsatellite loci D6S1581, D6S305, and D6S1599 were used to assay heterozygosity at the *PRKN* locus. D6S1581 is located approximately 1.5 MB upstream, while D6S305 and D6S1599 lie within intronic regions of *PRKN*. LOH occurred at one or more *PRKN* loci in eight cases, each represented by a line with shaded circles indicating heterozygosity status at each microsatellite locus. (B) The two microsatellite loci nearest to either end of *FBXO4* were used to infer LOH status at that locus. D5S418 lies approximately 1.9 MB upstream of *FBXO4*, while D5S2082 lies approximately 84 kB downstream. LOH was not detected at either locus in any of the samples.

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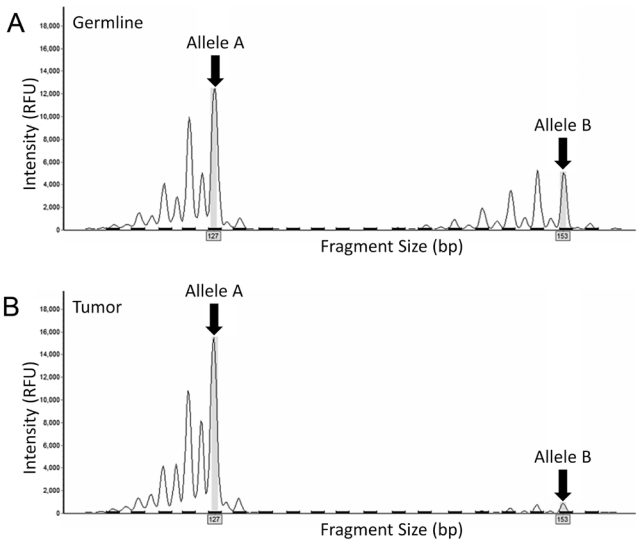


**Figure 2**
Representative example of LOH at a microsatellite locus. (A) Germline DNA from one case exhibits a strong fluorescent signal at the two discrete length alleles, highlighted by gray bars, of the *PRKN* intronic D6S1599 microsatellite locus. Allele A is 127 bp and Allele B is 153 bp. Allele peak intensity is measured in relative fluorescent units (RFU). Stutter peaks, a phenomenon frequently observed when amplifying microsatellite loci, trail each major allele peak and represent products of polymerase slippage during PCR. (B) In tumor DNA from the same case, fluorescent signal for Allele B has decreased significantly compared to the signal from Allele A. The allelic ratio is 0.15.

Tumor suppressor genes as defined by the classic two-hit model are often inactivated by intragenic mutations and/or allelic loss. Although tumor suppressor genes may also be inactivated by a variety of other means – such as noncoding mutation, chromosomal rearrangement, aberrant methylation, and transcriptional dysregulation, most of which necessarily lie outside the scope of this study – direct, bi-allelic inactivation remains an essential and most definitive means of identifying tumor suppressor genes that behave as a bona fide drivers of tumorigenesis, as opposed to downstream effectors of tumorigenic events. Our observations in a sizeable cohort of PTAs thus argue strongly against the hypothesis that *PRKN* and *FBXO4* commonly function as classical inactivated tumor suppressor genes in PTA.

Additional investigations are required to reveal the remaining undiscovered causes of cyclin D1 overexpression in PTA. Promising avenues for future research into PTA tumorigenic drivers will likely involve further investigation into genes relating to cyclin D1 overexpression as well as those in other parathyroid-relevant pathways. For example, *CRYAB* encodes an essential component of the PRKN-FBXO4 ubiquitination complex (29, 53) and provides an appealing subject for future study as a potential parathyroid tumor suppressor gene. *GSK3B* is likewise an appealing candidate for future exploration because it plays a multi-faceted role in cyclin D1 regulation: in addition to its function in inhibiting Wnt signaling, of which *CCND1* is a transcriptional target, GSK3B also mediates phosphorylation of cyclin D1 at T268, which is required for nuclear export (54) and may also be required for cyclin D1 ubiquitination by the PRKN-FBXO4 complex (53). The multiplicity of cyclin D1-relevant pathways in which *GSK3B* is involved, as well as its decreased expression reported in some parathyroid tumors (55), recommend it for study as a potential tumor suppressor in parathyroid neoplasia. Extending beyond cyclin D1-centric pathways, the molecular causes of epigenetic dysregulation of parathyroid-relevant genes such as *CASR* (56) may also reveal novel parathyroid tumorigenic drivers.

**Supplementary materials**
This is linked to the online version of the paper at https://doi.org/10.1530/EC-21-0055.

**Declaration of interest**
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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