Mutations in \textit{CDKN2C} (\textit{p18}) and \textit{CDKN2D} (\textit{p19}) May Cause Sporadic Parathyroid Adenoma

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Dear Editor:

Hyperparathyroidism can arise from germline mutation of MEN1, CASR, or HRPT (Marx 2011). Recent studies also suggest that germline mutation of several cyclin-dependent kinase inhibitors is an uncommon cause of hyperparathyroidism (Pellegata et al, 2006; Agarwal, et al. 2009). A gene that predisposes to tumor via germline mutation also may predispose to similar tumor by somatic mutation; in fact, the MEN1 gene is mutated in about 30% of sporadic parathyroid tumors (Marx 2011). Screening a small number of sporadic parathyroid tumors by others, using whole exome analysis, did not show any CDKI gene mutation (Supplementary references: Cromer, et al. 2012; Newey, et al. 2012). In a recent study, all 7 CDKI genes were sequenced in 81 sporadic parathyroid adenomas. There were 5 novel DNA missense changes in p15, p18, and p21. 3-4 of these were also in the germline, and none of the five was unequivocally a pathological mutation, such as a truncation change (Costa-Guda 2013). We evaluated further whether some cases of sporadic parathyroid adenoma are caused by somatic mutation in CDKI genes.

We evaluated tumor from 42 patients, who underwent parathyroid surgery at NIH. Each patient had a postoperative diagnosis of sporadic parathyroid adenoma. Each gave written
informed consent to a protocol that had been approved by the NIDDK Institutional Review Board. See Supplement for additional Materials and Methods.

In 42 sporadic parathyroid adenomas, we found among the 7 CDKI genes 15 SNPS plus one deletion and one insertion. With the exception of two SNPs and the indels, the remaining SNPs were previously reported (Supplementary Table 1). Four unreported variations were found in CDKN2C and CDKN2D. CDKN2C encodes p18, a known tumor suppressor (Solomon, et al. 2008; van Veelen 2009). CDKN2D encodes p19, which is closely related to p18 but not previously considered as a tumor suppressor (Lam, et al. 2000; Zindy et al. 2000).

One parathyroid tumor showed a deletion in the p18 gene and one an insertion in p18; neither change was found in the patient’s germline. In the first (p18F71Ter), a 25 nucleotide deletion at c.1427_1454del causes a stop codon at Phe 71. In the second (p18V118fs*6), a 2 nucleotide insertion c.1567_1568insGG causes a frameshift, followed by new sequence GWSSWWstop, also predicting a shortened protein. The chromatograms in Figure 1A and Figure 1B show overlapping wild-type and mutant sequences, indicating heterozygosity or normal admixture. Western blots show stable expression of transfected wild type p18 (Figure 2A, lane 2) and no detectable expression of either changed p18 (Figure 2A: lanes 3 and 5). Transfection efficiency was checked with NPT-II, and did not vary significantly between lanes (not shown).
The p19 A164T change in two parathyroids, is absent in the germline of one patient but present in germline of the other; the p19 V123A change is absent in the patient’s germline (Supplementary Figure 1A and 1B). Transient transfection experiments demonstrate that both p19 changes are stably expressed at levels comparable to wild type (Figure 2B).

Each of the 7 CDKIs is conserved and consists of 4 or 5 ankyrin repeat motifs (Li et al, 2006). Missense mutations, thought to contribute to tumorigenesis by INK4 CDKI proteins, occur throughout the first four ankyrin repeats; they disrupt interactions with CDKs or with other proteins (Baumgartner et al. 1998; Li et al, 2006).

Even if expressed in vivo, neither of the observed p18 truncation mutants is likely to be active. The 70 amino acid p18 F71Ter lacks two of the four ankyrin repeats required for known interactions with CDK4 (Guo et al 2009). The p118V118 fs* (Guo 2009) omits the conserved V118 and truncates the protein with GWSSW prior to the fifth ankyrin repeat. The mutated frame-shifted residues do not align with the ankyrin repeat consensus sequence. Furthermore, the truncated p18 protein containing residues 1-137 and containing the first four N-terminal ankyrin repeats would be one tenth or less as active as the wild type (Guo, et al, 2009). In sum, both of the p18 indels are probably loss of function drivers of parathyroid tumorigenesis. Each predicts a severely truncated protein, modeling of the ankyrin repeats predicts impaired interaction with CDKs, and neither mutant protein was expressed in vitro. Thus the evidence for loss of function of p18 is particularly strong. This also supports a role of p18 in parathyroid
tumorigenesis, since p18 and most other members of the CDKI family participate in
tumorigenesis via loss of function (Yan 2008; Li 2011).

The C-terminal five amino acids of p19 are not identical among mammals, and are poorly
conserved in animals (Supplementary Figure 1A); these amino acids are predicted not to
make contact with the cyclic dependent kinases, and thus the p19A164T mutation is
unlikely to effect p19 function in the cell cycle. The p19V123A is in the fourth ankyrin
repeat (Supplementary Figure 1B) and is a conservative amino acid change found in the
homologous p16 and p15. Structural alignment shows that p19 and p16 closely overlap
in this region and in the remainder of the first 4 ankyrin repeats (Supplementary Figure
1C). In other species, alanine is easily accommodated in the region otherwise occupied
by wild type valine (Supplementary Figure 1B). Lastly, other amino acid changes in the
homologous position in p16 are benign (Pavletich 1999). Lacking strong evidence for
loss of function, we judged both p19A164T and p19V123A as polymorphisms that were
unlikely to drive parathyroid tumorigenesis. Still, the lack of either of these p19 missense
changes in normal controls indicates that either might be tumorigenic. Later studies might
uncover deleterious functional consequences of either of these missense changes in p19.

We have provided strong evidence for somatic inactivation of p18. The truncating
mutations observed in p18 also give conceptual support for the pathogenicity of some of
the previously reported missense mutations.
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**LEGENDS TO FIGURES**

Figure 1. Sequencing of *CDKN2C* exon 2.

A. Upper chromatogram shows normal germline *CDKN2C* exon 2 nucleotide sequence and the bottom shows DNA sequence from parathyroid tumor with the c.1427-1454 deletion. The perpendicular line pointing to the bottom trace indicates the location of this deletion. The upper trace shows the germline sequence with the location of deleted nucleotides bounded by the black vertical lines. Overlapping sequence is also present at upstream sites (not shown).

B. Upper chromatogram shows normal germline sequence and the bottom shows the tumor DNA sequence harboring the insertion at c.1567. The GG indicates the location of the insertion of two guanosines. Heterozygosity is suggested by overlapping base calls.

The text below compares the nucleotide sequence of wild type allele with the mutant allele containing the insertion.

Figure 2: Western blot of the expression of transfected *p18* and *p19* (wild-type and 2 variants of each). Transfection efficiency was similar for all lanes, judged by levels of NPT-II from the vector (not shown).

A. Western Blot of p18, p18F71Ter, and p18V118fs*6 isolated from transiently transfected HEK 293 cells. Lane 1: No vector; Lane 2: p18; Lane 3: p18V118fs*6 (p18c.1567_1568insGG) ; Lane 4: vector; Lane 5: p18F71Ter (p18 c.147_1454del); lane 6: molecular weight markers. Tubulin is the loading control.
B. Western blot of p19 and missense mutants. Lane 1: p19; Lane 2: p19V123A; Lane 3: p19A164T; Lane 4: vector; Lane 5: no vector. Tubulin is the loading control.
Figure 1

Wild type allele: GGTTGGTGA
Mutant allele: GGggGTGGTG
Figure 2