Mutations in *CDKN2C* (p18) and *CDKN2D* (p19) may cause sporadic parathyroid adenoma

Dear Editor

Hyperparathyroidism (HPT) can arise from germline mutation of multiple endocrine neoplasia type 1 (*MEN1*), *CASR*, or *HRPT1* (*HRPT1*; Marx 2011). Recent studies also suggested that germline mutation of several cyclin-dependent kinase inhibitors (CDKIs) is an uncommon cause of HPT (Pellegata et al. 2006, Agarwal et al. 2009). A gene that predisposes to tumor via germline mutation may also 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 sequencing analysis, did not show any CDKI gene mutation (Cromer et al. 2012, Newey et al. 2012). In a recent study, all seven CDKI genes were sequenced in 81 sporadic parathyroid adenomas. There were five novel DNA missense changes in *p15*, *p18*, and *p21*. Three to four of these were also in the germline, and none of the five were unequivocally a pathological mutation, such as a truncation change (Costa-Guda et al. 2013). We further evaluated whether some cases of sporadic parathyroid adenoma are caused by somatic mutation in CDKI genes.

We evaluated tumors from 42 patients, who underwent parathyroid surgery at NIH. Each patient had a post-operative diagnosis of sporadic parathyroid adenoma. Each patient gave written informed consent to a protocol that had been approved by the NIDDK Institutional Review Board. See [Supplementary Materials and methods](#), see section on [supplementary data](#) given at the end of this article.

In 42 sporadic parathyroid adenomas, we found 15 SNPs plus one deletion and one insertion among the seven CDKI genes. With the exception of two SNPs and the indels, the remaining SNPs were previously reported ([Supplementary Table 1](#), see section on [supplementary data](#) given at the end of this article). Four unreported variations were found in *CDKN2C* and *CDKN2D*. *CDKN2C* encodes p18, a known tumor suppressor (Solomon et al.

2008, van Veelen et al. 2009). *CDKN2D* encodes p19, which is closely related to p18 but not previously considered as a tumor suppressor.

One parathyroid tumor showed a deletion in the *p18* gene and one showed 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 two-nucleotide insertion c.1567_1568insGG causes a frameshift, followed by new sequence GWSSWWstop, also predicting a shortened protein. The chromatograms in [Fig. 1A](#) and [B](#) show overlapping WT and mutant sequences, indicating heterozygosity or normal admixture. Western blots show stable expression of transfected WT *p18* ([Fig. 2A](#), lane 2) and no detectable expression of either changed *p18* ([Fig. 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 the germline of the other; the p19 V123A change is absent in the patient's germline ([Supplementary Fig. 1A and B](#), see section on [supplementary data](#) given at the end of this article). Transient transfection experiments demonstrate that both *p19* changes are stably expressed at levels comparable to WT ([Fig. 2B](#)).

Each of the seven CDKIs is conserved and consists of four or five 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

Figure 1

Sequencing of *CDKN2C* exon 2. (A) Upper chromatogram shows normal germline *CDKN2C* exon 2 nucleotide sequence and the lower chromatogram 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 lower chromatogram shows the tumor DNA sequence harboring the insertion at c.1567. The GG indicates the location of the insertion of two guanines. Heterozygosity is suggested by overlapping base calls. The text below compares the nucleotide sequence of WT allele with the mutant allele containing the insertion.

(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 frameshift 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 WT (Guo *et al.* 2009). In sum, both the p18 indels are probably loss-of-function drivers of parathyroid tumorigenesis. Each predicts a severely truncated protein, modeling of the ankyrin repeats predict 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, as p18 and most other members of the CDKI family participate in tumorigenesis via loss of function (Li *et al.* 2011).

The C-terminal five amino acids of p19 are not identical among mammals and are poorly conserved in animals (Supplementary Fig. 1A); these amino acids are predicted not to make contact with the CDKs, and thus the p19A164T mutation is unlikely to affect p19 function in the cell cycle. The p19V123A is in the fourth ankyrin repeat (Supplementary Fig. 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 four ankyrin repeats (Supplementary Fig. 1C). In other species, alanine is easily accommodated in the region otherwise occupied by WT valine (Supplementary Fig. 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.

Figure 2

Western blot of the expression of transfected p18 and p19 (WT and two 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); and 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; and lane 5, no vector. Tubulin is the loading control.

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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Supplementary data

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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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References

- Agarwal SK, Mateo CM & Marx SJ 2009 Rare germline mutations in cyclin-dependent kinase inhibitor genes in multiple endocrine neoplasia type 1 and related states. *Journal of Clinical Endocrinology and Metabolism* **94** 1826–1834. (doi:10.1210/jc.2008-2083)
- Baumgartner R, Fernandez-Catalan C, Winoto A, Huber R, Engh RA & Holak TA 1998 Structure of human cyclin-dependent kinase inhibitor p19INK4d: comparison to known ankyrin-repeat-containing structures and implications for the dysfunction of tumor suppressor p16INK4a. *Structure* **6** 1279–1290. (doi:10.1016/S0969-2126(98)00128-2)
- Costa-Guda J, Soong C-P, Parekh VI, Agarwal SK & Arnold A 2013 Germline and somatic mutations in cyclin-dependent kinase inhibitor genes CDKN1A, CDKN2B, CDKN2C in sporadic parathyroid adenomas. *Hormones & Cancer* **4** 301–307. (doi:10.1007/s12672-013-0147-9)
- Cromer MK, Starker LF, Choi M, Udelsman R, Nelson-Williams C, Lifton RP & Carling T 2012 Identification of somatic mutations in parathyroid tumors using whole-exome sequencing. *Journal of Clinical Endocrinology and Metabolism* **97** E1774–E1781. (doi:10.1210/jc.2012-1743)
- Guo Y, Mahajan A, Yuan C, Joo SH, Weghorst CM, Tsai M-D & Li J 2009 Comparisons of the conformational stability of the cyclin-dependent kinase (CDK) 4-interacting ankyrin repeat (AR) proteins. *Biochemistry* **48** 4050–4062. (doi:10.1021/bi802247p)
- Li J, Mahajan A & Tsai MD 2006 Ankyrin repeat: a unique motif mediating protein–protein interactions. *Biochemistry* **45** 15168–15178. (doi:10.1021/bi062188q)
- Li J, Poi MJ & Tsai MD 2011 Regulatory mechanisms of tumor suppressor P16(INK4A) and their relevance to cancer. *Biochemistry* **50** 5566–5582. (doi:10.1021/bi200642e)
- Marx SJ 2011 Hyperparathyroid genes: sequences reveal answers and questions. *Endocrine Practice* **17** (Suppl 3) 18–27. (doi:10.4158/EP11067.RA)
- Newey PJ, Nesbit MA, Rimmer AJ, Attar M, Head RT, Christie PT, Gorvin CM, Stechman M, Gregory L, Mihai R *et al.* 2012 Whole-exome sequencing studies of nonhereditary (sporadic) parathyroid adenomas. *Journal of Clinical Endocrinology and Metabolism* **97** E1995–E2005. (doi:10.1210/jc.2012-2303)
- Pavletich NP 1999 Mechanisms of cyclin-dependent kinase regulation: structures of Cdk, their cyclin activators, and Cip and Ink4 inhibitors. *Journal of Molecular Biology* **287** 821–828. (doi:10.1006/jmbi.1999.2640)
- Pellegata NS, Quintanilla-Martinez L, Siggelkow H, Samson E, Bink K, Hoffer H, Fend F, Graw J & Atkinson MJ 2006 Germ-line mutations in p27Kip1 cause a multiple endocrine neoplasia syndrome in rats and humans. *PNAS* **103** 15558–15563. (doi:10.1073/pnas.0603877103)
- Solomon DA, Kim JS, Jenkins S, Ransom H, Huang M, Coppa N, Mabanta L, Bigner D, Yan H, Jean W *et al.* 2008 Identification of p18 INK4c as a tumor suppressor gene in glioblastoma multiforme. *Cancer Research* **68** 2564–2569. (doi:10.1158/0008-5472.CAN-07-6388)
- van Veelen W, Klompmaier R, Gloerich M, van Gasteren CJ, Kalkhoven E, Berger R, Lips CJ, Medema RH, Hoppener JW & Acton DS 2009 P18 is a tumor suppressor gene involved in human medullary thyroid carcinoma and pheochromocytoma development. *International Journal of Cancer* **124** 339–345. (doi:10.1002/ijc.23977)

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