

Rare Germline Mutations in Cyclin-Dependent Kinase Inhibitor Genes in Multiple Endocrine Neoplasia Type 1 and Related States

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Context: Germline mutation in the *MEN1* gene is the usual cause of multiple endocrine neoplasia type 1 (MEN1). However, the prevalence of identifiable germline *MEN1* mutations in familial MEN1 cases is only 70%. Some cases may have a germline mutation in another gene such as the *p27* cyclin-dependent kinase inhibitor (CDKI).

Objective: The aim of the study was to investigate cases of MEN1 or related states for germline mutations in all CDKI genes.

Methods: A total of 196 consecutive index cases were selected with clear or suspected MEN1 and no identifiable germline *MEN1* mutation. Every case was analyzed for germline mutation in each of the seven CDKI genes.

Results: We identified benign polymorphisms of the CDKI genes and also 15 other initially unclassified sequence variants. After detailed gene/protein analysis, seven of these 15 variants were classified as probably pathological mutations. Three of these seven were probable mutations of *p27*. The remaining four were probable pathological mutations in three of the other CDKI genes, thereby implicating these three genes in the germline of human tumors. The identification rates for probably pathological mutations among the 196 index cases were similarly low for each of four CDKI genes: *p15* (1%), *p18* (0.5%), *p21* (0.5%), and *p27* (1.5%). No characteristic clinical subtype related to MEN1 was identified among the seven index cases and their families.

Conclusion: Rare germline mutation in any among four (*p15*, *p18*, *p21*, and *p27*) of the seven CDKIs is a probable cause of MEN1 or of some related states. (*J Clin Endocrinol Metab* 94: 1826–1834, 2009)

Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant syndrome with tumors of many characteristic endocrine and nonendocrine tissues. The standard clinical criteria for a case of MEN1 are tumors in two of the three main associated endocrine tissues (parathyroid, duodenopancreas, and anterior pituitary) (1). By extension, familial MEN1 is defined clinically as an index case with MEN1 and at least one first-degree relative with tumor in one or more of these three tissues. The prevalence of identifiable heterozygous germline mutations in the *MEN1* gene is only 70% in index cases with familial MEN1 and considerably lower in sporadic MEN1 (2). Similarly, some other related states have only rare germline *MEN1* mutations. For instance, *MEN1* mutations have been

identified in only 10% of index cases with familial primary hyperparathyroidism (1°HPT) and in less than 1% of index cases with familial pituitary tumor (3–5). Some MEN1 and MEN1-like patients without an identifiable germline *MEN1* mutation may have an *MEN1* mutation that is undetectable by the methods used. However, among the possible mechanisms for non-detection, promoter mutations of the *MEN1* gene have not been reported, and large deletions of *MEN1* seem rare (6, 7). An important possibility is germline mutation of a different gene.

In mouse models, interactions of some cell cycle regulators can cause endocrine tumors. In particular, double knockouts combining germline losses of *p27* or *p21* with loss of *p18* resulted in endocrine tumors characteristic of both MEN1 and MEN2 (8)

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Abbreviations: CDK, Cyclin-dependent kinase; CDKI, CDK inhibitor; GRB2, growth factor receptor-bound protein 2; GST, glutathione S-transferase; 1°HPT, primary hyperparathyroidism; LOH, loss of heterozygosity; MEN1, multiple endocrine neoplasia type 1.

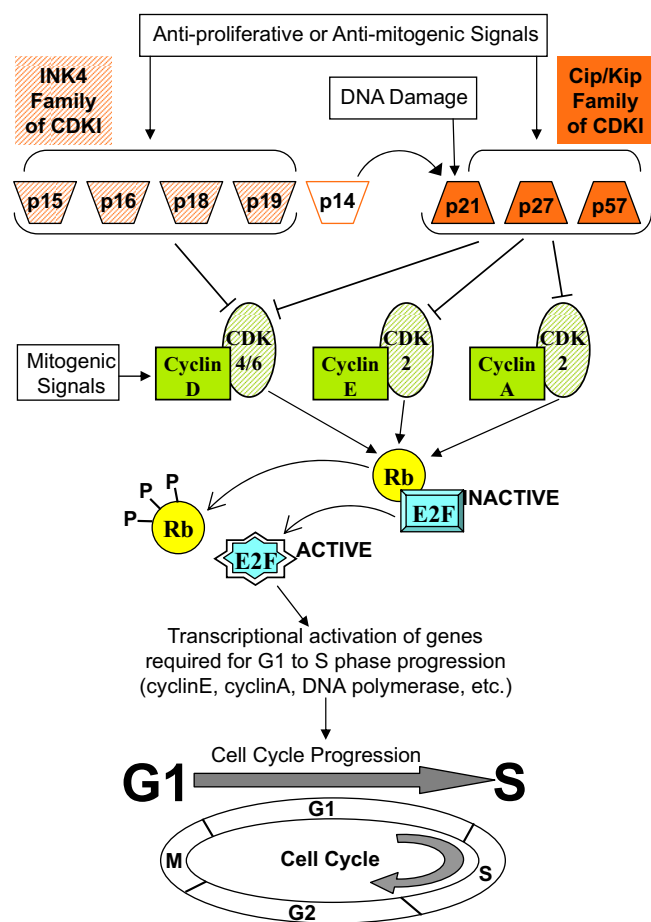


FIG. 1. Molecular pathways distal to CDKIs. Mitogenic signals promote entry into the G₁ phase through assembly and activation of cyclinD-CDK4 or cyclinD-CDK6 complexes. Subsequent progression through G₁/S transition and S phase is dependent on active cyclinE-CDK2 complexes and active cyclinA-CDK2 complexes. The complexes of cyclin (green solid) and CDK (green striped) phosphorylate members (only one shown) of the Rb (the retinoblastoma gene product) family. This releases from them the E2F family of transcription factors (only one factor shown). The E2F factors in turn transactivate genes for G₁ to S phase progression. Some other substrates (data not shown) of active cyclin-CDK complexes during G₁ and S phase are: for cyclinE-CDK2, NPAT (nuclear protein mapped to ATM locus), p27, and histone-H1; for cyclinA-CDK2, DNA polymerase α primase. Members of two families of CDKIs, INK4 family (orange striped) and Cip/Kip family (orange solid), negatively regulate the cell cycle. p14 is from an alternative reading frame of the p16 gene (see Supplemental Fig. 4); it does not bind to any cyclin-CDK complex, and it is not a CDKI. p14 inhibits the cell cycle through activating the inhibitory functions of p21 (9). Arrows, Activation; -, inhibition.

(Fig. 1). Despite their central roles in the cell cycle and in tumors of genetically engineered mice, most of these CDKIs have rarely been shown to cause tumors by germline mutation in man (see *Discussion*) [Online Mendelian Inheritance in Man (OMIM) numbers 116899, 600778, 600856, 600431, 603369, and 600927].

Recently, a syndrome that combined MEN1 and MEN2 (named MENX) was reported in a strain of rat (10); a germline homozygous frameshift mutation in *p27* caused this phenotype (11). A heterozygous germline nonsense mutation in *p27* was in

parallel identified as the cause of MEN1 in one human index case and in two of his other affected family members (11). A heterozygous germline frameshift mutation in *p27* was later reported in another MEN1 case (12). Among these four affected human cases, two had parathyroid tumor and three had pituitary tumor, secreting GH or ACTH. *p27* germline mutation was not found in three subsequent studies analyzing 34, 69, and 21 MEN1 or MEN1-like index cases without identified *MEN1* mutations, emphasizing the rarity of *p27* mutation in MEN1 (2, 13, 14). MEN1-like state caused by *p27* mutation in man was termed MEN4 (OMIM no. 610755). We have now tested for germline mutation in all of the CDKI genes¹, and we have examined these in a larger series of index cases with MEN1 and related states.

Patients and Methods

Patients

The study was approved by the Institutional Review Board of the National Institute of Diabetes and Digestive and Kidney Diseases. Each patient gave written informed consent.

DNA from each case had been referred for *MEN1* sequencing. Each of the 196 index cases [including the 34 cases analyzed previously for *p27* mutation (2)] had been tested through the National Institutes of Health (NIH) between 1997 and 2007, and each had no identified germline *MEN1* mutation. The criteria for *MEN1* testing were broad. In general, a sporadic case or a familial index case was tested if there was likely multigland parathyroid hyperfunction or parathyroid hyperfunction plus another likely MEN1-related tumor. Each case was triaged by one of the same six senior staff members. Most cases were characterized from available clinical information. The amount of additional testing among cases depended partly on whether they were ever evaluated as in-patients or out-patients at NIH or elsewhere. No more than one member of a family was accepted as an index case. Extra effort was made in cases and relatives of cases with probable mutation of a CDKI gene. During this time, a total of 347 index cases suspected of MEN1 had sequencing of *MEN1* plus or minus other genes. *CASR* or *HRPT2* tests were used selectively. The prevalences of identified mutations were: *MEN1*, 128 of 347 (37%), *CASR*, 13 of 347 (4%), and *HRPT2*, 10 of 347 (3%). Index cases with identified mutation of *MEN1*, *CASR*, or *HRPT2* were excluded from the analysis of the CDKI genes; analyses of CDKI germline mutation in this separate group will be of interest in the future but seem unlikely to uncover pathological CDKI mutations.

The distribution of nonoverlapping pretest diagnoses among the 196 index cases was: MEN1, 35%; familial MEN1, 7%; 1°HPT, 11%; multigland 1°HPT, 8%; familial 1°HPT, 17%; HPT-jaw tumor syndrome or parathyroid cancer, 7%; pituitary tumor, 1%; familial pituitary tumor, 4%; and other, 10%.

Isolation of genomic DNA and total RNA

Genomic DNA was isolated from peripheral blood, using the blood and cell culture genomic DNA maxi kit (QIAGEN, Valencia, CA). Total RNA from blood was isolated using the PaxGene blood RNA isolation kit (QIAGEN).

Parathyroid tumors (n = 5) were slowly frozen, stored under liquid nitrogen, and sectioned (8–10 μ m thickness) (15). Portions were scraped from slides for DNA and RNA isolation using the Picopure system for DNA or RNA (Arcturus, MountainView, CA).

¹ The INK4 CDKI family members specifically bind and inhibit cyclinD-CDK4 and cyclinD-CDK6; the genes in this family include *CDKN2A*, *CDKN2B*, *CDKN2C*, and *CDKN2D* encoding p16^{INK4a}, p15^{INK4b}, p18^{INK4c}, and p19^{INK4d}, respectively. The INK4 members are referred to here as p16, p15, p18, and p19. An alternate reading frame (ARF) of *CDKN2A* encodes p14^{ARF} (p19^{ARF} in mouse); this is not a CDKI and is referred to here as p14.

The Cip/Kip family members of CDKIs bind and inhibit a broad spectrum of cyclins-CDKs (cyclinD-CDK4, cyclinD-CDK6, cyclinE-CDK2, and cyclinA-CDK2); the genes in this family include *CDKN1A*, *CDKN1B*, and *CDKN1C* encoding p21^{Cip1}, p27^{Kip1}, and p57^{Kip2}, respectively. The Cip/Kip members are referred to here as p21, p27, and p57.

DNA sequence analysis

Primers were designed to amplify and sequence 16 coding exons, including the exon-intron boundaries, of CDKIs *p15*, *p16/p14*, *p18*, *p19*, *p21*, *p27*, and *p57* (Supplemental Table 1, which is published as supplemental data on The Endocrine Society's Journals Online web site at <http://jcem.endojournals.org>). The 3' part of the first coding exon of *p57* was not analyzed due to its highly variable PAPA (proline and alanine) repeats. PCR products were amplified using standard conditions with AmpliTaq Gold (ABI, Foster City, CA), purified, and sequenced (MWG Biotech, High Point, NC). Sequences were analyzed for variants by comparison with sequences obtained from normal control individuals (and with CDKI sequences deposited in GenBank), using MacVector (Oxford Molecular, Ltd., Cambridge, UK). Each sequence trace was visually analyzed.

Each DNA sample with a sequence variant was reamplified and resequenced. Sequence variants were also confirmed by independent PCR amplification using restriction enzyme digest (Supplemental Table 2).

Evaluation of CDKI sequence variants in controls

Two human random control DNA panels (HRC1 and HRC2), each consisting of genomic DNA from 96 healthy UK Caucasian blood donors were used (ECACC/Sigma-Aldrich, St. Louis, MO). These 192 control DNA samples were analyzed by PCR and sequencing for each of the 15 uncategorized sequence variants identified in the CDKI genes (Polymorphic DNA Technologies, Inc., Alameda, CA). For each of the 15 sequence variants, the same single nucleotide polymorphism was also searched in the HapMap database (hapmap.org), in the JSNPs database (snps.ims.u-tokyo.ac.jp), and in the GeneSNPs database (www.genome.utah.edu/genesnps).

Function analysis of CDKI variants

Quantitative analysis of protein expression (Western blot) and glutathione S-transferase (GST) pull-down assay was carried out using Alpha Innotech quantitation software. Further analyses of CDKI variants were with other methods (see *Supplemental Methods*, included in supplemental data on The Endocrine Society's Journals Online web site at <http://jcem.endojournals.org>).

We only scored a mutation as probably pathological if at least one relevant test showed a substantial defect in a function of the encoded CDKI protein. The criterion for such an effect to be judged probably pathological was a decrease to less than 11% (median = 0.1%) of control in a relevant bioassay and similar results at least three separate times.

Results

CDKI sequence variants and control sequences

Among the 196 index cases tested for the seven CDKI genes, we identified 15 germline heterozygous sequence variants (initially and temporarily scored as unclassified) (Table 1) and 15 common benign polymorphisms (10 previously known) (Supplemental Table 3).

Twelve of the 15 unclassified variants were not found in any among 192 control DNA samples, or in the HapMap database of 418 DNA samples, or in the JSNPs database of 934 DNA samples, or in the GeneSNPs database of 95 DNA samples.

However, the *p21* missense variant R84Q was seen in one of 192 controls, and two of the *p21* missense variants P4L and F63L were each found once in the HapMap database. Each of the three CDKI variants found in one among many apparently normal persons (an in-house control or a case in the HapMap database) was thus scored as a rare benign polymorphism.

Sequence homologies among CDKI variants

The sequence variants in the INK4 CDKI subfamily were compared with known germline or somatic disease-causing mu-

tations of *p16* (Table 1, and Supplemental Table 4) (18). Also, see supplemental Results (supplemental Table 5). No comparable analysis was possible in the Cip/Kip subfamily because analogous mutations were not reported previously for these. Both of the *p15* variants ($n = 2$) and the *p19* variant ($n = 1$) corresponded to previously identified and probably pathological somatic mutations in homologous amino acids of *p16* (18). Similar to our data about the two *p15* variants, the *p16* variants had also been shown previously to impair the binding of *p16* to CDK4/CDK6 (18). This abnormality was confirmed by us for both *p15* variants in the GST pull-down assays here, thereby extending evidence (see *CKDI binding by CDKI variants*) that both *p15* variants were probably pathological. The *p16* mutation that was similar to the *p19* variant has not been shown to affect *p16* activity (19). Therefore, this *p19* variant was of uncertain significance and was classified conservatively as benign.

Defects in functions of *p27* due to sequence variants

The nucleotide at the -7 position in the Kozak sequence affected by one *p27* DNA variant (ATG-7G>C) is conserved among vertebrate species (Supplemental Fig. 1). RT-PCR revealed similar expression of *p27* RNA from cells transfected with wild-type or variant-containing plasmid (Fig. 2A). However, the concentration of *p27* protein was reduced ($6 \pm 2\%$) in lysates of cells transfected with the variant-containing plasmid, suggesting deficient translation of *p27* protein (Fig. 2B). Thus this variant was scored as a probably pathological mutation.

p27 inhibits Ras activation by *p27* binding to growth factor receptor-bound protein 2 (GRB2), an adaptor protein involved in activation of Ras (21). *p27* contains a proline-rich region at amino acids 91 to 95 similar to that used by son of sevenless to bind the SH3 domain of GRB2. *p27* competes with SOS for binding to GRB2 at this region. In GST pull-down assays, the *p27* missense variant P95S had reduced ($0.1 \pm 0.05\%$) binding to GRB2 (Fig. 2C). Thus this variant was scored as a probably pathological mutation.

Expression analysis of the *p27* stop>Q variant protein showed a higher molecular weight than wild-type *p27*, as predicted from the extension of the open reading frame by 60 amino acids to the next in-frame stop codon; however, the protein was expressed at a lower level ($6 \pm 2\%$) (Fig. 2D). RT-PCR revealed similar expression of *p27* RNA from plasmids expressing wild-type or stop>Q variant (Fig. 2E). MG132, which blocks proteasomal degradation, brought expression of stop>Q protein to wild-type levels (Fig. 2D). Therefore, the *p27* stop>Q variation results in an unstable protein *in vitro*, prone to degradation by the proteasomal pathway. Thus, it was scored as a probably pathological mutation.

Protein expression from 11 other CDKI variants

Protein expression of missense variants of other CDKIs was analyzed as for two variants of *p27* (ATG-7G>C and stop>Q) in transiently transfected HEK293 cells. No abnormality in protein expression was detected for missense variants of *p16* (and its alternative reading frame *p14*), *p19*, two for *p21*, and one for *p27* (Fig. 3B, and Supplemental Fig. 2, A–D). The protein expression of *p18* V31L and *p21* R67L was decreased ($25 \pm 2\%$ and $11 \pm 2\%$) (Fig. 3, A and B) indicating likely underexpression or instability. Thus,

TABLE 1. Summary of CDKI sequence variants and data to distinguish probably pathological from benign variants

CDKI DNA variant ^A	CDKI DNA variant in 192 controls	CDKI DNA variant in 418 of HapMap, etc. ^E	CDKI DNA variant in family members	CDKI protein expression	CDKI protein interaction	Other information	Interpretation of variant ^L
<i>p15</i> N41D (AAC/GAC)	0	...	NA	NA	CDK6/Reduced (0.1% ± 0.05%)	Homologous <i>p16</i> mutation CDK4/6-binding abolished ^G	Pathological
<i>p15</i> L64R ^B (CTG/CGG)	0	...	Unaff daughter neg ^{F1}	NA	CDK6/Reduced (0.1% ± 0.05%)	Homologous <i>p16</i> mutation CDK4/6-binding abolished ^G	Pathological
<i>p16</i> A100S ^C (GCC/TCC)	0	...	NA	wt	CDK6/wt	Reported in other sporadic tumor, but functions like wt ^H	Benign
<i>p16</i> A127S ^D (GCA/TCA)	0	...	NA	wt	CDK6/wt	Reported in other sporadic tumor, but functions like wt ^H	Benign
<i>p18</i> V31L (GTC/CTC)	0	...	NA	Reduced (25% ± 2%)	CDK6/Reduced (0.1% ± 0.05%)	...	Pathological
<i>p18</i> IVS1-6 C>A	0	...	NA	ND	ND	Predicted splicing defect also seen in normal controls ^I	Benign
<i>p18</i> IVS2 + 19 G>A ^B	0	...	Unaff daughter pos ^{F2}	ND	ND	Predicted splicing defect also seen in normal controls ^I	Benign
<i>p19</i> V102M (GTG/ATG)	0	...	Aff mother pos ^{F3} . Unaff brother neg	wt	NA	Homologous tumor associated <i>p16</i> mutation ^F but functions like wt ^I	Benign
<i>p21</i> P4L (CCG/CTG)	0	1 (CEU)	NA	wt	CDK2/wt	...	Benign
<i>p21</i> F63L (TTC/TTA)	0	1 (YRI)	NA	wt	CDK2/wt	Also in Burkitt's lymphoma cell line DH978 ^K	Benign
<i>p21</i> R67L (CGT/CTT)	0	...	Aff sister pos ^{F4}	Reduced (11% ± 2%)	CDK2/wt	...	Pathological
<i>p21</i> R84Q (CGA/CAA)	1	...	NA	Increased	CDK2/wt	...	Benign
<i>p27</i> ATG-7 G>C	0	...	2 Asymp daughters pos ^{F5}	Reduced (6% ± 2%)	ND	No LOH in one parathyroid tumor	Pathological
<i>p27</i> P95S (CCC/TCC)	0	...	NA	wt	GRB2/Reduced (0.1% ± 0.05%)	...	Pathological
<i>p27</i> stop>Q (TAA/CAA)	0	...	Aff twin sister pos ^{F6}	Reduced (6% ± 2%)	CDK2/wt	ORF extends 60 amino acids	Pathological

^A Unequivocal common benign polymorphisms are omitted (Supplemental Table 3, published as supplemental data on The Endocrine Society's Journals Online web site at <http://jcem.endojournals.org>).

^B Two variants found in the same index case.

^C This *p16* variant also affects *p14* (*p14* G114V) because it lies in a shared coding exon.

^D Variant found in two index cases.

^E From Hapmap.org (total 418 HapMap samples include 120 CEU, European; 90 CHB, Chinese; 88 JPT, Japanese; 120 YRI, African), JSNPs (934 Japanese), and GeneSNPs (95 samples).

^{F1-F6} Number of family members examined for the variant found in the index case: F1 = 1, F2 = 1, F3 = 2, F4 = 1, F5 = 2, and F6 = 1.

^G Refs. 16 and 17.

^H Ref. 18.

^I Supplemental Fig. 3.

^J Ref. 19.

^K Ref. 20. NA or ..., Information not available; ND, not determined; Unaff, unaffected; Aff, affected; Asymp, asymptomatic; neg, negative; pos, positive; wt, expression or interaction similar to wild-type protein; reduced or increased, protein level altered with % in parentheses showing mean level and SD compared to wild type, or benign variant; CDK6/wt or CDK2/wt, CDKI protein binding to CDK6 or CDK2 similar to wild type, or benign variant; CDK6/Reduced or GRB2/Reduced, reduced CDKI protein binding to CDK6 or GRB2 with % in parentheses showing mean binding and SD compared to wild type, or benign variant. ORF, Open reading frame.

^L Yellow highlights: variants scored as 'probably pathological' plus the features pointing to this score; gray highlights: variants scored as benign plus the features pointing to this score.

protein expression analysis for two among these 11 missense variants contributed to their scores as probably pathological mutations.

The expression of *p21* R84Q was increased (Fig. 3B), indicating that the variant was more stable or was produced more efficiently than the wild-type protein. More importantly, this was not scored as a probably pathological mutation because this sequence change was also detected in one of 192 in-house control samples (Table 1).

Cyclin-dependent kinase (CDK) binding by CDKI variants

To examine the possible consequence of CDKI sequence change on binding to CDKs, GST pull-down assays were performed. The variants of *p21* and *p27* were analyzed for binding to GST-CDK2, and the variants of *p15*, *p16*, *p18*, and *p19* were analyzed for binding to GST-CDK6. No abnormality in binding was observed for the missense variants of *p16*, *p21*, or *p27* (Supplemental Fig. 2, E–I). However, missense variants of *p15* (N41D

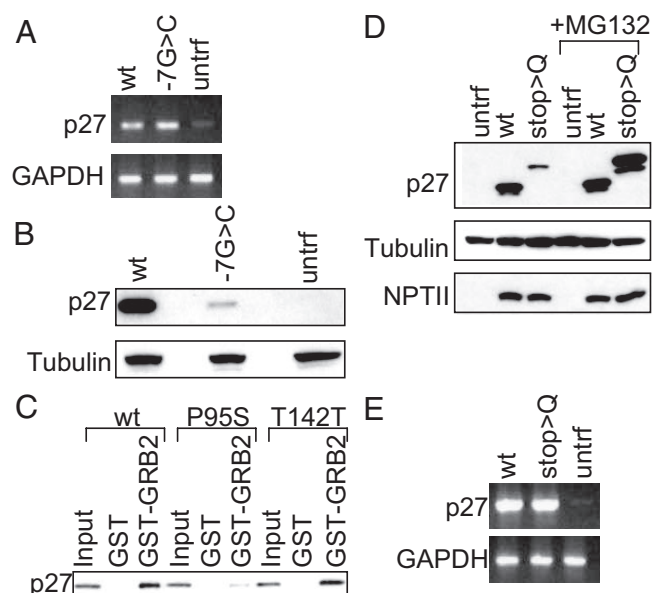


FIG. 2. Function assays of *p27* variants among index cases. **A**, mRNA. RT-PCR of HEK293 cells untransfected or transfected with wild-type or *p27* variant (ATG-7G>C) genomic constructs. *Top panel* shows RT-PCR products (at 30 cycles) with *p27* primers, and *lower panel* shows products with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers. **B**, Protein. Western blot of whole cell lysates from HEK293 cells untransfected or transfected with wild-type or *p27* variant (ATG-7G>C) genomic constructs. *Top panel* was probed with anti-*p27* antibody and *lower panel* with antitubulin antibody. **C**, Binding to GRB2. GST pull-down assay for binding with GST-GRB2 of ³⁵S labeled *in vitro* translated (IVT) wild-type *p27* or missense variant P95S or benign polymorphism T142T. The bound IVT protein was detected by SDS-PAGE and autoradiography. Input lane contained 10% of the IVT product incubated with GST or GST-GRB2. **D**, Protein. Western blot of whole cell lysates from HEK293 cells transfected with wild-type or *p27* (stop>Q) expression constructs, untreated or treated with 20 μ M MG132 (proteasome inhibitor). *Top panel* was probed with anti-*p27* antibody, *middle panel* with antitubulin antibody, and *lower panel* with anti-NPTII antibody (index of transfection efficiency). **E**, mRNA. RT-PCR of HEK293 cells from panel D. *Top panel* shows RT-PCR products (at 25 cycles) with *p27* primers, and *lower panel* shows RT-PCR products with GAPDH primers. untrf, Untransfected; wt, wild-type.

and L64R) and *p18* (V31L) were deficient in binding to GST-CDK6 ($0.1 \pm 0.05\%$) (Fig. 3, C and D). Thus these three were scored as probably pathological mutations. This confirmed and strengthened the protein expression data of *p18* V31L.

CDKI variants in family members

Germline DNA from five first-degree relatives of four index cases with probably pathological CDKI variants was sequenced. Two affected relatives aged 47 and 48 had the appropriate variant. No affected relative tested negative for the appropriate variant. Two unaffected relatives tested positive for a variant, and one tested negative for a variant. Small size of each family precluded testing for genetic linkage to any of the probably pathological CDKI variants (Fig. 4). Thus family patterns were not used as a main criterion for assigning probably pathological roles of the CDKI variants.

Clinical characteristics

Based on biochemical analyses of CDKI variants, seven index cases and their families had probably pathological mutations

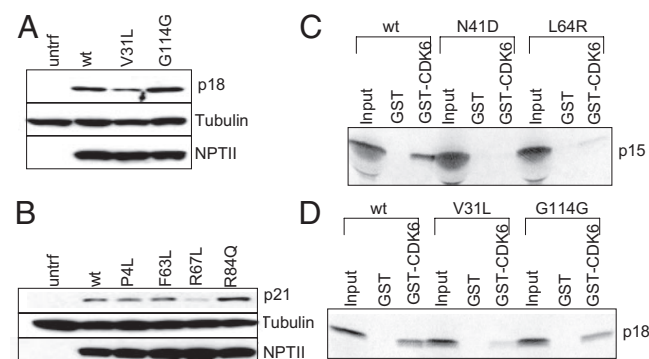


FIG. 3. Expression and interaction assays of several CDKI missense variants. **A** and **B**, Protein. Western blot of whole cell lysates from HEK293 cells transfected with wild-type or mutagenized cDNA expression constructs. *Top panels* were probed with the indicated specific anti-CDKI antibody, *middle panels* with antitubulin antibody, and *lower panels* with anti-NPTII antibody. **C** and **D**, Protein. Binding of ³⁵S-labeled IVT wild-type or variants of *p15* or *p18* to CDK6 in GST pull-down assay. The bound IVT protein was detected by SDS-PAGE and autoradiography. Input lane contained 10% of the IVT product incubated with GST or GST-CDK6.

that supported the reported tumorigenic implication of *p27*, or that newly implicated three other CDKI genes in endocrine tumors (Table 1). The seven index cases and five of their relatives (two affected, one unaffected, and two asymptomatic carriers) were tabulated separately by CDKI gene and were also examined clinically as one large group. We did not tabulate data about skin tumors of MEN1 because these data were too incomplete. Cases were classified as four index cases with sporadic or familial MEN1, two index cases with familial isolated 1°HPT, and one index case with the MEN1-associated combination of sporadic multigland 1°HPT and meningioma (22) (Fig. 4 and Table 2). Endocrine tumor onset ages seemed similar to those for MEN1. We did not encounter pituitary tumor from *p27* germline mutation although it had been reported in three of four affected cases with proven or likely *p27* mutation previously (11, 12). There were two prolactinomas in one family (with *p21* R67L). Two cases (*p15* L64R and *p27* P95S) had Zollinger-Ellison syndrome that had not been previously reported with *p27* mutation. Tumors of uncertain importance in affected cases or in their possibly affected first-degree relatives included esophageal carcinoid, prostate cancer, and cutaneous schwannoma.

Discussion

CDKI mutations

Among 196 index cases of MEN1 or related states, without identifiable germline *MEN1* mutations, seven showed probably pathological germline mutations in four CDKI genes (Supplemental Fig. 1); identification rates² of probably pathological mutation among these 196 index cases were similarly low for each of these four CDKIs: *p15* (1%), *p18* (0.5%), *p21* (0.5%), and *p27* (1.5%). Eight CDKI variants of the initially unclassified 15 were scored here as benign variants (Table 1); it remains possible

² The overall identification rates of probably pathological mutations in the CDKI genes among all of our 347 MEN1 or MEN1-like index cases with or without identified MEN1 mutation were the following: *p15* (0.58%), *p18* (0.29%), *p21* (0.29%), and *p27* (0.86%).

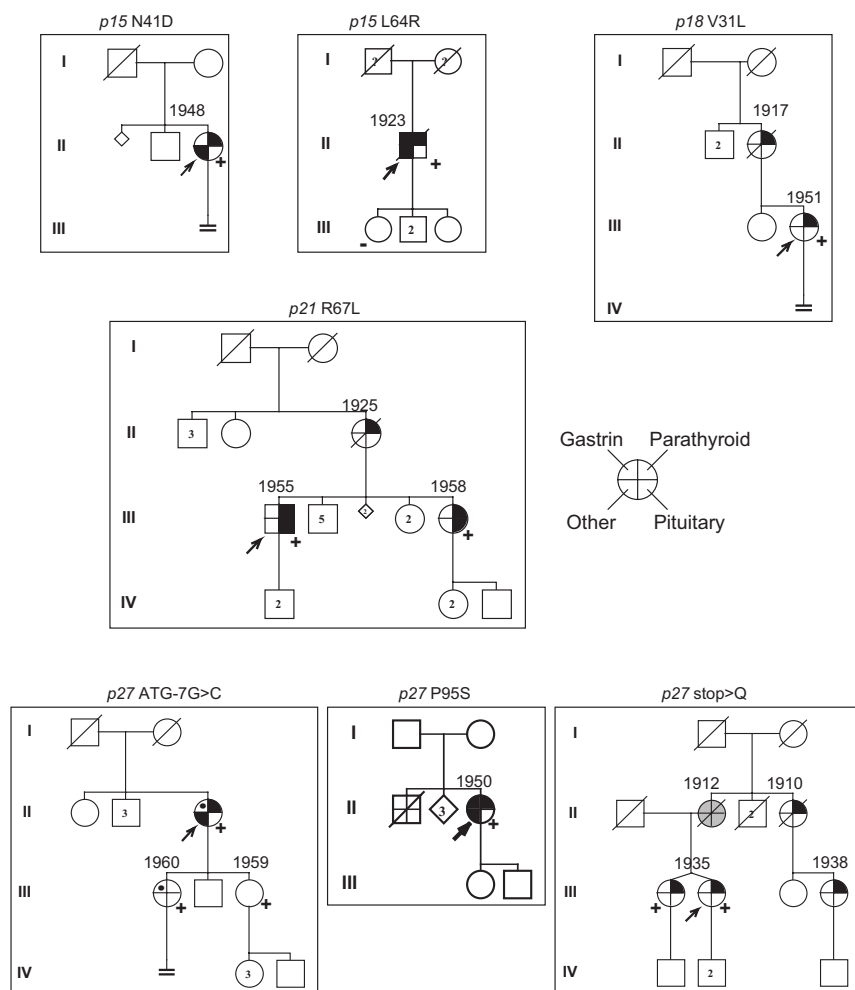


FIG. 4. Pedigrees and tumors of index cases with probably pathological CDKI mutations, their affected relatives, and all of their other first-degree relatives (see Table 2 for details). Germline heterozygous CDKI mutation is shown above each pedigree. Roman numerals indicate generation number. Arrows indicate index case. Circle, Female; square, male; diamond, sex not known; small diamond, miscarriage of unknown gender; double line below descent line, no offspring; number in symbol, number of unaffected offspring of that gender; empty symbol, unaffected family member; slashed symbol, deceased; stippled circle, obligate carrier by family position; question mark in symbol, offspring is adopted; question mark near symbol, offspring information not known; filled quadrant in symbol, tumor; dot in quadrant, likely tumor; upper right quadrant, parathyroid tumor; upper left quadrant, gastrinoma; lower right quadrant, pituitary tumor; lower left quadrant, other MEN1-associated tumor [e.g. adrenal cortex ($n = 2$), meningioma ($n = 1$), uterine fibroids ($n = 1$)] (see also Table 2); (+), CDKI mutation positive; (–), CDKI mutation negative; numbers on top of symbols, year of birth for all carriers. First-degree relatives without CDKI mutation information were shown as affected syndrome carrier if they expressed at least one of the three main tumors of MEN1.

that one or more of these eight might eventually prove to be a pathological mutation.

No mutation herein of a CDKI gene predicted truncation of a CDKI protein, so possible pathology from mutation needed careful scrutiny. Easton *et al.* (23) analyzed 1433 such *BRCA1* and *BRCA2* variants of unknown clinical significance. Typically, the DNA variations judged to be pathological predicted defective splicing, were located in highly conserved positions, were located in likely functional domains, segregated with disease in pedigrees, or affected the protein in assays of function. If the assay of function is relevant and the measured impairment is significant, then the defect caused by the missense mutation is probably important and pathological. Our expression studies were done in a heterologous nonendocrine cell line (HEK293 cells); human islet or

parathyroid cell lines could be even more informative, but such lines are not available.

Prior reports of the effects of CDKI mutation offered aspects for comparisons to our study. *In vitro* CDK binding studies using amino acid substitutions in CDKIs have identified amino acids critical for optimal CDKI-CDK binding and for kinase inhibitor activity (24, 25). Furthermore, several missense variants of *p16* associated with a tumor phenotype have been shown to impair its CDK binding activity or to affect its expression/stability *in vitro* (16–18). Results from similar assays helped us to identify several variants of homologous CDKIs (*p15* and *p18*) as probably pathological. We also analyzed variants by sequence homology to *p16* mutations. The many sequence homologies in the INK4 family and the extended data on mutations of *p16* combined to help categorize two variants (*p15* N41D and *p15* L64R) as probably pathological and to categorize another (*p19* V102M) as probably benign.

Our analyses of CDKI function cannot be exhaustive because some aspects of CDKI function are unknown. Furthermore, some known aspects were not studied, e.g. interaction with CDK-cyclin complex or effect on CDK activity (26, 27).

The seven probably pathological CDKI gene mutations predict functional impairment by combinations among protein expression, protein interaction, and absence of mutation in controls. This adds three new CDKI genes as a likely cause in MEN1 and related states. Six of the seven CDKI genes are now implicated by germline mutation in one tumor syndrome (see *Clinical features of tumors associated with CDKI mutation*). With likely pathological CDKI gene mutation identified in only 4% of our index cases, other unidentified genes/proteins in the cell

cycle pathway may also cause a similar syndrome among some of the remaining cases. Another interpretation of the 4% rate of total identified mutations is that additional mutations of these CDKI genes were not identified by our methods. In particular, large deletions are undetected by sequencing, and this is a major mechanism for mutation of *p16* (28).

The implication of germline mutations in four CDKIs in MEN1 and related syndromes (see *Clinical features of tumors associated with CDKI mutation*) raises the possibility that one or more CDKI could contribute by mutation or inactivation mechanisms in common sporadic endocrine tumors. It is thus relevant that some CDKIs (*p16*, *p18*, *p21*, and *p27*) have previously been analyzed for mutation and expression in sporadic endocrine tumors (Refs. 29–31 and references therein). Expression changes

TABLE 2. Clinical characteristics with probably pathological CDKI mutations in index cases and families, including two published

Gene	Mutation	Syndrome summary	Hormone excess, and other known or suspected tumors ^A of index case	Others affected in family	Additional possible tumor
1 <i>p15</i>	N41D	1°HPT	1°HPT (3 parathyroid tumors) ^{B1} , skin schwannoma, meningioma, liver hemangioma	0	
2 <i>p15</i>	L64R	MEN1	1°HPT (3 parathyroid tumors) ^{C1} , ZES ^{C2} , adrenal mass ^{C3} , prostate cancer	0	Son (age 56) has prostate cancer (not tested for <i>p15</i> variant). Unaffected daughter (age 52) is negative for the same <i>p15</i> variant.
3 <i>p18</i>	V31L	1°HPT (familial)	1°HPT (2 parathyroid tumors) ^{D1} , breast cancer	1	Mother had 1°HPT (1 parathyroid tumor) ^{D2} (not tested for <i>p18</i> variant).
4 <i>p21</i>	R67L	MEN1 (familial)	1°HPT ^{E1} , macroprolactinoma ^{E2}	2	Mother had 1°HPT ^{E3} (not tested for <i>p21</i> variant). Sister has 1°HPT ^{E4} , macroprolactinoma ^{E5} , and is positive for same <i>p21</i> variant.
5 <i>p27</i>	ATG-7G>C	MEN1	1°HPT (1 parathyroid tumor) ^{F1} , stomach problems, bilateral adrenal mass nonfunctioning ^{F2} , uterine fibroids	0	Two asymptomatic daughters (ages 47 and 48); one has stomach problems, and both are positive for same <i>p27</i> variant.
6 <i>p27</i>	P95S	MEN1	1°HPT (2 parathyroid tumors) ^{G1} , ZES ^{G2} , masses in duodenum and tail of pancreas ^{G3}	0	Family was outside United States and not reachable.
7 <i>p27</i>	Stop>Q	1°HPT (familial)	1°HPT (3 parathyroid tumors) ^{H1}	3	Identical twin sister 1°HPT (1 parathyroid tumor) ^{H2} , and is positive for same <i>p27</i> variant. Aunt ^{H3} and cousin ^{H4} have 1°HPT (not tested for <i>p27</i> variant).
8 <i>p27</i>	W76X	MEN1 (familial)	1°HPT, GH-pituitary tumor	2	3 family members tested positive for same <i>p27</i> variant (phenotype of two undetermined). Father had acromegaly (not tested for the <i>p27</i> variant). Sister had renal angiomyolipoma (positive for same <i>p27</i> variant); her son has testicular cancer. Published (Ref. 11).
9 <i>p27</i>	K25fs	MEN1	1°HPT, ACTH-pituitary tumor, carcinoid tumor of uterine cervix	0	Published (Ref. 12).

Number of parathyroid tumors was estimated for cases with one to three parathyroidectomies. ZES, Zollinger-Ellison syndrome, including high basal acid output, and high gastrin (more than 3-fold over upper limit of normal gastrin).

^A We did not tabulate data about skin tumors of MEN1 because these data were too incomplete.

^{B–H} Onset age of endocrine tumor (age in years in parentheses): B1(49), C1(59), C2(55), C3(55), D1(44), D2(69), E1(44), E2(43), E3(?), E4(?), E5(?), F1(61), F2(63), G1(50), G2(50), G3(50), H1(50), H2(66), H3(52), H4(?). Question mark indicates that the age information is not available. Onset age for an endocrine tumor was the age of first symptom or first sign.

of uncertain importance were identified, but mutations were not found.

Loss of wild-type CDKI allele in tumors

Although tumorigenesis herein seems likely to be caused by the loss of CDKI gene function (32), three of four CDKIs affected by probably pathological mutations could not be tested for the loss of the wild-type allele due to the unavailability of tumors. Unfortunately, tumor was available from only one case with a probably pathological germline variant *p27* (ATG-7G>C); there was no loss of the wild-type DNA copy in that tumor. For this variant, there was a reduction in *p27* protein amount (analyzed by transient transfection assay in HEK293 cells; Fig. 2B) that might initiate tumors by a haploinsufficiency expression (33).

Prior mutation analyses of *p27* in human and rodent tumors, in particular those from germline heterozygous knockout mice, have usually not shown biallelic *p27* inactivation in the tumor (33, 34). This is similar to much data on *p27* somatic mutations in several nonendocrine human tumors (35).

Inactivation of a single allele of *p18*, *p21*, or *p27* has been shown to be sufficient to sensitize mice to radiation-induced or carcinogen-induced tumors (33, 36, 37). Among the CDKIs, only *p16* was found previously to have consistent biallelic inactivation in a variety of human cancers (32, 38). Among other CDKIs, mutation with a parallel loss of heterozygosity (LOH) has seldom been seen in human tumors (28, 38). Considering prior reports, CDKIs may sometimes function as tumor suppressor genes via haploinsufficiency or via silencing of the wild-type allele without LOH, e.g. by promoter methylation.

Clinical features of tumors associated with CDKI mutation

The clinical phenotype of previously reported human tumors from proven or likely germline *p27* mutation has so far been based on only four affected cases (and two silent carriers). We have now extended this via six more cases (four affected and two asymptomatic carriers) with probable *p27* mutation. We also identified five cases affected with probable mutation in any one of three other CDKI genes (*p15*, *p18*, and *p21*). Because no spe-

cific phenotype for mutation of any one CDKI gene was evident, these were all pooled with *p27* for preliminary examinations of phenotype in the CDKI gene family. Further studies will be required to determine age-related penetrance of tumors for each CDKI gene. In particular, penetrance could be determined in the future in part from larger kindreds than we have encountered. The overall features here and in two previous reports with germline *p27* mutation (11, 12) were those of MEN1, including that of the closely related familial or multigland 1°HPT. Among the pretest diagnoses for index cases, probable CDKI mutations were most prevalent (10%; $P < 0.05$; χ^2) in familial 1°HPT. Such features may typify the full tumor spectrum of the mutant CDKIs; however, they might also reflect our own referral biases toward MEN1 or 1°HPT.

The current phenotype (MEN1 or related states) from mutation in one of four CDKI genes contrasts with two other phenotypes from other CDKI genes (Supplemental Fig. 4). First, familial melanomas with pancreatic ductal adenocarcinomas can arise from inactivating mutation of *p16*. Secondly, tumors (Wilms' tumor, adrenocortical carcinoma, and rhabdomyosarcoma) in the Beckwith-Wiedemann syndrome arise from inactivation by mutation or other changes of *p57* (39, 40). Tumor specificity in the latter two phenotypes is robust from the *p16* or *p57* gene, respectively, although with unknown details of the relationship to that gene. It may explain why a mutation in the *p16* or the *p57* CDKI gene was not found in any of our index cases that had been selected for a different or MEN1-related tumor spectrum. The predominance of MEN1-associated tumors in our series may also explain why CDKI mutations (except *p16*) were not previously found in many common nonendocrine tumors (32, 38). It is notable that we did not find C cell or chromaffin tumors of MEN2 in any family with probable CDKI mutation, although tumors of MEN2 and MEN1 were seen in both rat and mouse models of CDKI mutation (8, 11).

In conclusion, we confirm and extend reports that germline *p27* mutations account for a small but important fraction of cases with MEN1 and related states. Germline mutations in three of six other CDKI genes (*p15*, *p18*, and *p21*) are also shown for the first time as a probable cause of MEN1 or related states. The rarity of *p15*, *p18*, and *p21* mutations in these cases and the lack of unequivocal pathological features of the identified mutations indicate that further research will be needed before applying these findings to the clinic. The involvement of three previously unimplicated CDKIs may indicate a broad relevance of this gene family to endocrine tumors.

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