Cyclin-dependent kinase inhibitor 1B (*CDKN1B*) gene variants in *AIP* mutation-negative familial isolated pituitary adenoma kindreds

Maria A Tichomirowa[1]*, Misu Lee[3]*, Anne Barlier[4,5]*, Adrian F Daly[1], Ilaria Marinoni[3], Marie-Lise Jaffrain-Rea[6,7], Luciana A Naves[8], Patrice Rodien[9], Vincent Rohmer[9], Fabio Rueda Faucz[10], Philippe Caron[11], Bruno Estour[12], Pierre Lecomte[13], Françoise Borson-Chazot[14], Alfred Penfornis[15], Maria Yaneva[16], Mirtha Guitelman[17], Emily Castermans[2], Catherine Verhaege[2], Jean-Louis Wémeau[18], Antoine Tabarin[19], Carmen Fajardo Montañana[20], Brigitte Delemer[21], Veronique Kerlan[22], Jean-Louis Sadoul[23], Christine Cortet Rudelli[18], Françoise Archambeaud[24], Sabine Zacharieva[16], Marily Theodoropoulou[25], Thierry Brue[26], Alain Enjalbert[4,5], Vincent Bours[2], Natalia S Pellegata[3] and Albert Beckers[1]

Departments of [1]Endocrinology and [2]Molecular Genetics, Centre Hospitalier Universitaire de Liège, Domaine Universitaire du Sart-Tilman, University of Liège, 4000 Liège, Belgium

[3]Institute of Pathology, Helmholtz Zentrum München, German Research Center for Environmental Health, Neuherberg, Germany

[4]Laboratory of Biochemistry and Molecular Biology, AP-HM Conception 11385 Marseille, France

[5]CRN2M, UMR 6231 CNRS, Aix Marseille University, 13344 Marseille, France

[6]Department of Experimental Medicine, University of L'Aquila, L'Aquila, Italy

[7]Neuromed, IRCCS, 86077 Pozzili, Italy

[8]Division of Endocrinology, University of Brasilia, Brasilia, Brazil

[9]Department of Endocrinology, Centre Hospitalier Universitaire de Angers, 49033 Angers, France

[10]Group for Advanced Molecular Investigation, Graduate Program in Health Science, Laboratory of Molecular Genetics, Pontifícia Universidade Católica do Paraná, Paraná, Brazil

[11]Service d'Endocrinologie, Maladies Métaboliques, Nutrition, CHU de Toulouse, Hôpital Larrey, Pôle Cardio-Vasculaire et Métabolique, 24 Chemin de Pouvourville, TSA 30030, FR-31059 Toulouse Cedex 9, France

[12]Department of Endocrinology, Centre Hospitalier Universitaire de Saint Etienne, 42055 Saint Etienne, France

[13]Unit of Endocrinology, Centre Hospitalier Régional Universitaire Tours, 37044 Tours Cedex 9, France

[14]Department of Endocrinology, Centre Hospitalier Universitaire de Lyon, 69495 Lyon, France

[15]Department of Endocrinology, Centre Hospitalier Universitaire de Besançon, 25030 Besançon, France

[16]Clinical Center of Endocrinology and Gerontology, 6 bd. "Damian Gruev", 1303 Sofia, Bulgaria

[17]Faculty of Medicine, Neuroscience Institute, University of Buenos Aires, Buenos Aires, Argentina

[18]Endocrinology, Diabetology and Metabolism and Hôpital Claude-Huriez, Centre Hospitalier Régional Universitaire de Lille, 59037 Lille, France

[19]Department of Endocrinology, Hôpital Haut Lévêque, CHU de Bordeaux, Avenue de Magellan, 33600 Pessac, France

[20]Department of Endocrinology, Hospital Universitario de la Ribera, 46600 Alzira, Valencia, Spain

[21]Department of Endocrinology, University Hospital of Reims, Reims, France

[22]Department of Endocrinology, Diabetes and Metabolic Diseases, CHU Brest, Hôpital de la Cavale Blanche, Université de Bretagne Occidentale, 29609 Brest Cedex, France

[23]Department of Endocrinology, Hopital de l'Archet, 06202 Nice, France

[24]Department of Internal Medicine and Endocrinology, Hopital du Cluzeau, 87042 Limoges, France

[25]Department of Endocrinology, Max Planck Institute of Psychiatry, 80804 Munich, Germany

[26]Service d'Endocrinologie, Diabète et Maladies Métaboliques, et Centre de Reference des Maladies Rares d'Origine Hypophysaires DEFHY, Hôpital de la Timone, 13385 Marseille, France

(Correspondence should be addressed to A Beckers; Email: albert.beckers@chu.ulg.ac.be)

*M A Tichomirowa, M Lee and A Barlier contributed equally to this work.

Abstract

Familial isolated pituitary adenoma (FIPA) occurs in families and is unrelated to multiple endocrine neoplasia type 1 and Carney complex. Mutations in *AIP* account only for 15–25% of FIPA families. *CDKN1B* mutations cause MEN4 in which affected patients can suffer from pituitary adenomas. With this study, we wanted to assess whether mutations in *CDKN1B* occur among a large cohort of

AIP mutation-negative FIPA kindreds. Eighty-eight *AIP* mutation-negative FIPA families were studied and 124 affected subjects underwent sequencing of *CDKN1B*. Functional analysis of putative *CDKN1B* mutations was performed using *in silico* and *in vitro* approaches. Germline *CDKN1B* analysis revealed two nucleotide changes: c.286A>C (p.K96Q) and c.356T>C (p.I119T). *In vitro*, the K96Q change decreased p27 affinity for Grb2 but did not segregate with pituitary adenoma in the FIPA kindred. The I119T substitution occurred in a female patient with acromegaly. p27[I119T] shows an abnormal migration pattern by SDS–PAGE. Three variants (p.S56T, p.T142T, and c.605+36C>T) are likely nonpathogenic because *In vitro* effects were not seen. In conclusion, two patients had germline sequence changes in *CDKN1B*, which led to functional alterations in the encoded p27 proteins *in vitro*. Such rare *CDKN1B* variants may contribute to the development of pituitary adenomas, but their low incidence and lack of clear segregation with affected patients make *CDKN1B* sequencing unlikely to be of use in routine genetic investigation of FIPA kindreds. However, further characterization of the role of *CDKN1B* in pituitary tumorigenesis in these and other cases could help clarify the clinicopathological profile of MEN4.

Introduction

Among primary central nervous system tumors, pituitary tumors are the second most frequent by site (14.3%) and the third most frequent (13.1%) general group by histology (CBTRUS 2011). Cross-sectional studies reveal that clinically relevant pituitary adenomas are quite prevalent, occurring in approximately one in 1064–1288 of the general population (Daly *et al*. 2006*b*, Fernandez *et al*. 2010). Although usually histologically benign, these tumors have a significant burden in terms of disease effects (hormonal excess/deficiency and mass effects) and treatment (neurosurgery, biological medical therapy, and radiotherapy). In the case of genetic syndromes with a known pituitary adenoma predisposition, such as multiple endocrine neoplasia type 1 (MEN1) and Carney complex (CNC), mutation screening and clinical surveillance can aid early diagnosis. Familial isolated pituitary adenoma (FIPA) is a clinical syndrome unrelated to MEN1 and CNC (Daly *et al*. 2006*a*). Aryl hydrocarbon receptor interacting protein (*AIP*) gene mutations were shown by Vierimaa *et al*. (2006) to be associated with a low-penetrance familial form of pituitary tumors. However, *AIP* mutations explain only 15–25% of FIPA cases (Daly *et al*. 2007) and 12% of macroadenomas in young adults (Tichomirowa *et al*. 2011), the remaining cases have no currently identified genetic cause.

Among other syndromic conditions associated with pituitary adenomas is MEN4, which was originally described in a rat model that spontaneously developed a MEN1-like condition of neuroendocrine tumors (Fritz *et al*. 2002, Pellegata *et al*. 2006). In humans, as in rats, this is caused by mutation in the cyclin-dependent kinase inhibitor 1B (*CDKN1B*) gene that encodes p27 (IFI27), a cyclin-dependent kinase (CDK) inhibitor. Mutations in this and other CDKs can be associated with very rare cases of multiple endocrine tumorigenesis (Georgitsi *et al*. 2007*a*, Agarwal *et al*. 2009, Molatore *et al*. 2010). Interest in the role of *CDKN1B* mutations in other endocrine-related cancer has risen, with a recent study showing that 2/86 sporadic parathyroid adenoma patients had germline *CDKN1B* mutations, which, in turn, affected p27 protein levels or stability (Costa-Guda *et al*. 2011). Apart from endocrine neoplasia, *CDKN1B* mutations may also play a role in primary ovarian failure (Ojeda *et al*. 2011).

To date, large studies have not examined whether *CDKN1B* genetic variants play a role in the pathogenesis of FIPA kindreds that are negative for *AIP* mutations. We therefore performed a genetic sequencing and *in vitro* characterization study of *CDKN1B* gene variants in a large group of 88 well-characterized FIPA families with normal *AIP* sequences.

Materials and methods

Subjects

The study was performed in 88 FIPA families from France, Belgium, Italy, Brazil, Spain, Argentina, Germany, and Bulgaria. FIPA kindreds were defined as families with two or more related persons having pituitary adenomas without clinical or genetic evidence of MEN1 or CNC. *AIP* mutations were excluded from all FIPA kindreds by sequencing and multiplex ligation-dependent probe amplification.

The FIPA cohort consisted of 1 four-member, 3 three-member, 39 two-member homogeneous, and 45 two-member heterogeneous FIPA families. The four-member family presented with one corticotropinoma, one prolactinoma, and two somatotropinomas and the three-member family presented with two somatotropinomas and one nonfunctioning pituitary

adenoma. The 39 two-member homogeneous families had prolactinomas ($n = 23$), somatotropinomas ($n = 12$), corticotropinoma ($n = 2$), gonadotropinoma ($n = 1$), and nonfunctioning pituitary adenoma ($n = 1$) in the affected members. From the total of 181 FIPA patients, 124 were available for genetic testing.

The study was conducted in accordance with the guidelines of the Declaration of Helsinki, approved by Ethics Committee of the University of Liège, and all subjects provided informed written consent in their own language for the genetic screening.

CDKN1B genetic analysis and genotyping

Genomic DNA was isolated from blood samples from at least one affected member of each FIPA family. The structure of *CDKN1B* was based on Ensembl sequences ENSG00000111276. The primers used for the analysis (two sets of primers were used to amplify exon 1) were Ex1.1F, GTCTGTGTCTTTTGGCTC-CG; Ex1.1R, GGTCTGTAGTAGAACTCGGG; Ex1.2F, GACTTGGAGAAGCACTGCAG; Ex1.2R, CAAAGCTAAATCAGAATACGC; Ex2F, GGATC-CAGGATTGTGGGTG; and Ex2R, CCCAGCCTTC-CCCATTGC. Each 25 µl PCR reaction contained 140 ng genomic DNA, 1.25 µl of each primer, 1.5 mM MgCl$_2$, 10 mM Tris–HCL buffer (pH 8.3), 200 µM dNTPs, and 1.25 U FastStart Taq polymerase (Roche). PCR conditions were 95 °C for 10 min, followed by 35 cycles of 30 s at 95 °C, 45 s at 65 °C, 30 s at 72 °C, finishing with 7 min at 72 °C. PCR products were sequenced using ABI3130XL and BigDye Terminator v3.1 technology (Applied Biosystems, Foster City, CA, USA).

A group of control samples from normal individuals ($n = 476$) were studied to assess *CDKN1B* allelic frequencies compared with FIPA patients. These samples were derived from 326 Italian, 100 Belgian, and 50 French subjects. To explore the status of a variant discovered in a Brazilian family, further genotyping for this specific change was performed in 100 healthy subjects from Brazil.

Reagents

Cell culture materials were purchased from Life Technologies (Karlsruhe, Germany), Nunc (Wiesbaden, Germany), and Sigma. Inhibitors for protein kinases A/G/C, staurosporine, H8, and H89 were purchased from Biaffin (Kassel, Germany). The protein synthesis inhibitor cycloheximide (CHX) and the proteasome inhibitor MG132 were purchased from Sigma.

Expression vectors, cell lines, and transfections

The p27^{K96Q} and p27^{I119T} mutations were introduced by site-directed mutagenesis (Quikchange II Site-Directed Mutagenesis Kit; Stratagene, Waldbronn, Germany) in the wild-type human *CDKN1B* cDNA cloned in a pYFP and pHA backbone as described previously (Pellegata *et al.* 2006). MCF7 and HeLa cells (LGC Standards, Wesel, Germany) were maintained in RPMI 1640 and DMEM medium, respectively, supplemented with 10% FCS, 20 mM L-glutamine, 100 units/ml penicillin G sodium, and 100 µg/ml streptomycin sulfate. GH3 cells (ATCC) were grown in F12 medium supplemented with 15% horse serum, 2.5% FCS, 20 mM L-glutamine, 100 units/ml penicillin G sodium, and 100 µg/ml streptomycin. Transient transfection was performed as described previously (Pellegata *et al.* 2006).

Drug treatments and pull-down assays

HeLa cells transfected with HA-p27-wt or HA-p27I119T were treated with 2 nM staurosporine, 2 µM H8, and 2 µM H89 for 24 h. To determine p27 half-life, GH3 cells that transfected the YFP-p27-wt, YFP-p27K96Q, or YFP-p27I119T were treated with 25 µg/ml CHX for the indicated times or with 20 µM of the proteasome inhibitor MG132 for 5 h. Cell lysates were prepared, separated, and blotted using standard procedures as described previously (Pellegata *et al.* 2006). Primary antibodies used were anti-p27 monoclonal antibody (BD Biosciences, Heidelberg, Germany), antiphospho p27 (Thr187; Santa Cruz Biotech, Santa Cruz, CA, USA), and α-tubulin (Sigma).

HeLa cells transfected with YFP-p27-wt or YFP-p27K96Q for 24 h were lysed in ice-cold buffer (5 mM EDTA and 1% Triton-X100). Total protein (500 µg) was pulled down with 5 µg Grb2-GST recombinant protein already bound to 2.5 µl glutathione agarose beads (Upstate, Charlottesville, VA, USA). After extensive washing, immunoprecipitates were resuspended in 25 µl Laemmli buffer. Immunoblotting was performed using the anti-p27 and subsequently the anti-Grb2 antibodies (Santa Cruz Biotech).

Immunofluorescence

Immunofluorescence was performed on MCF7 cells transfected with p27-wt, p27^{K96Q}, or p27^{I119T} on a coverslip; 24 h later, transfected cells were fixed in 2% paraformaldehyde in PBS for 30 min at room temperature. Cell nuclei were stained with 1 µg/ml Hoechst for 5 min at room temperature and mounted on glass slides. Images were generated using a Zeiss

Axiovert 200 epifluorescence microscope including an Apotome unit (Zeiss, Jena, Germany) using the YFP and the DAPI channel and processing was carried out using Zeiss computer software (AIM 3.2).

In silico analysis

To predict splice signals, the following programs were used: SpliceView (http://bioinfo.itb.cnr.it/oriel/splice-view.html and http://www.fruitfly.org/seq_tools/splice.html). The web-based ESEfinder 3.0 program (available at: http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi) searches for sequences that act as binding sites for four members of the serine/arginine-rich family of splicing enhancer proteins. Input sequences were screened for consensus-binding sequences for the SR proteins SF2/ASF (SRSF1), SC35 (SRSF2), SRP40 (SRSF5), and SRP55 (SRSF6), developed using the SELEX (systematic evolution of ligands by exponential enrichment) procedure. Increased threshold values of 2.5 for SF2/ASF (from 1.956) and 3.0 for SC35 (from 2.383), SRP40 (from 2.670), and SRP55 (from 2.676) were used in order to minimize false-positive results.

Results

CDKN1B sequencing

Genetic sequencing in the *CDKN1B* gene revealed two heterozygous allelic variants, one that did not occur in the control population, i.e. c.286A>C (p.K96Q), and one that occurred at a very low frequency, c.356C>T (p.I119T; 1/476, 0.2% of healthy controls). Two other variants were detected in the matching control populations: p.S56T (c.167G>C) and p.T142T (c.426G>A). The c.167G>C (S56T) base substitution was found in

both brothers of a Brazilian two-member heterogeneous FIPA family with a somatotropinoma and a nonfunctioning pituitary adenoma and appeared among 100 Brazilian controls: 198 chromosomes were G and two were C (genotype: 196 G/G and two G/C). An intronic change, c.605+36C>T, was seen in one FIPA family member (male with a giant prolactinoma) and did not occur in the control subjects; however, *in silico* modeling indicated that this variant had no strong effect on splicing and was deemed to probably represent a nonpathological change. The previously reported T142T (c.426G>A) variant was found in three unrelated prolactinoma patients (one male and two females) across three different FIPA families. The findings from the genotyping of the control cohort ($n=476$ healthy individuals) were as follows: c.286A>C (p.K96Q), all 952 chromosomes were A; c.605+36C>T, all 952 chromosomes were C; c.356T>C (p.I119T), 950 chromosomes were T and one chromosome was C (genotype: 950 T/T and one T/C); c.426G>A (p.T142T), 945 chromosomes were G and seven chromosomes were A (genotype: 945 G/G and seven G/A).

The I119T change was found in one member of a two-person homogeneous FIPA family with somatotropinomas. The other affected member could not be genetically tested (Supplementary Figure 1, see section on supplementary data given at the end of this article). The K96Q variant was found in a homogeneous FIPA family presenting with prolactinomas, but the variation did not segregate with prolactinoma-affected patients. The patient with the K96Q change had hyperprolactinemia due to a suspected prolactinoma that was treated chronically with cabergoline when referred, who also developed breast cancer at the age of 41. The unaffected sister of this patient was also a carrier of this variant.
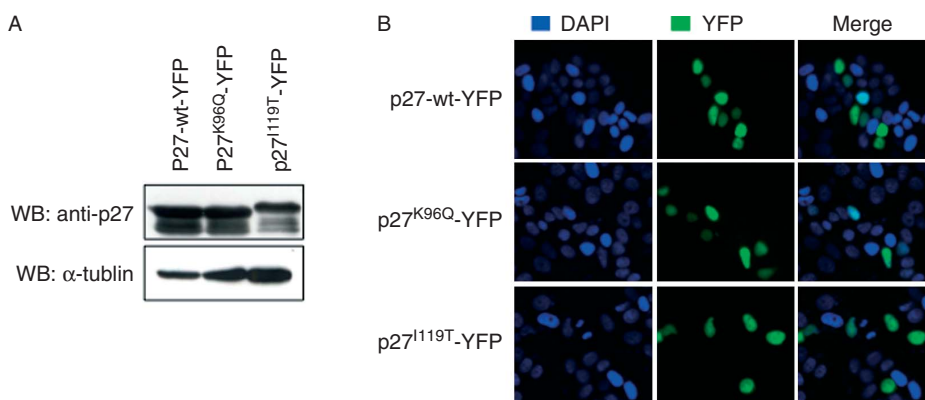
Figure 1 Subcellular localization of wild-type and mutant p27. (A) HeLa cells were transfected with YFP-p27 constructs containing p27-wt, p27^{K96Q}, or p27^{I119T} mutant proteins and examined by western blotting. Expression and size of p27 were compared in wt and mutant transfected cells. (B) MCF7 cells were transfected as in (A) and were determined using fluorescent microscopy. All fusion proteins (wild-type and mutants) were located primarily in the nucleus.
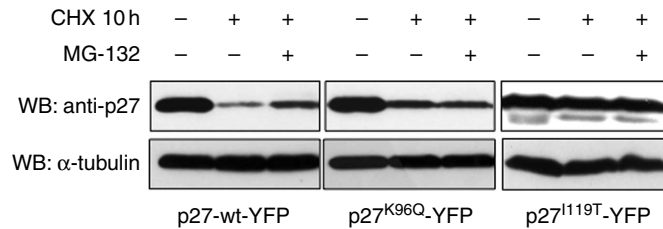
Figure 2 Stability of wild-type and mutant p27. The rate of the turnover of p27-wt, p27^{K96Q}, and p27^{I119T} proteins was measured in exponentially growing, transiently transfected GH3 cells using cycloheximide (CHX) with and without the proteasome inhibitor MG-132. p27-wt and p27^{K96Q} have a half-life of ∼10 h in GH3 cells. MG-132 has no effect on p27^{K96Q} degradation. In contrast, p27^{I119T} is more stable than p27-wt.

In vitro analysis of mutant p27 proteins

The subcellular localization, stability, and function of the K96Q and I119T mutant proteins were studied *in vitro*. To determine the effect of the p27 changes on protein localization and stability, the following YFP-tagged proteins were generated: p27-wt, p27^{K96Q}, and p27^{I119T}. Transient transfections performed in MCF7 cells revealed that the mutant proteins are expressed at similar levels (Fig. 1A). Both wild-type and mutant p27 proteins localize to the nucleus (Fig. 1B). We noted that the p27^{I119T} protein migrates slower than p27-wt by SDS–PAGE (Fig. 1A).

As previously reported MEN4-associated *CDKN1B* mutations often affect the stability of the encoded p27 protein, we analyzed the turnover of p27^{K96Q} and p27^{I119T} in exponentially growing, p27-negative GH3 cells. We blocked *de novo* protein synthesis with CHX, and at various time points thereafter, we analyzed the amount of p27-wt, p27^{K96Q}, and p27^{I119T}. We observed a time-dependent reduction of p27-wt and p27^{K96Q} following CHX treatment, while p27^{I119T} levels did not decrease throughout the experiment (Fig. 2). These results show that the p27^{I119T} protein is more stable *in vitro* than p27-wt. Although the intracellular amount of p27 is mainly regulated by ubiquitin-mediated proteasomal degradation, inhibiting the proteasome has been shown to stabilize p27 (Pagano *et al*. 1995). Proteasome inhibition of transfected GH3 cells by MG-132 partially recovered p27-wt but not p27^{K96Q} or p27^{I119T} proteins (Fig. 2), indicating that the mutant p27 proteins are resistant to proteasome degradation.

The K96Q missense change is situated in the proline-rich domain (amino acids 90–96) of p27. This domain mediates the binding of p27 to the adaptor protein GRB2, an interaction that eventually leads to p27 degradation. In a pull-down assay using anti-Grb2 antibody, p27^{K96Q} displayed reduced Grb2 binding compared to p27-wt (Fig. 3), and this may be

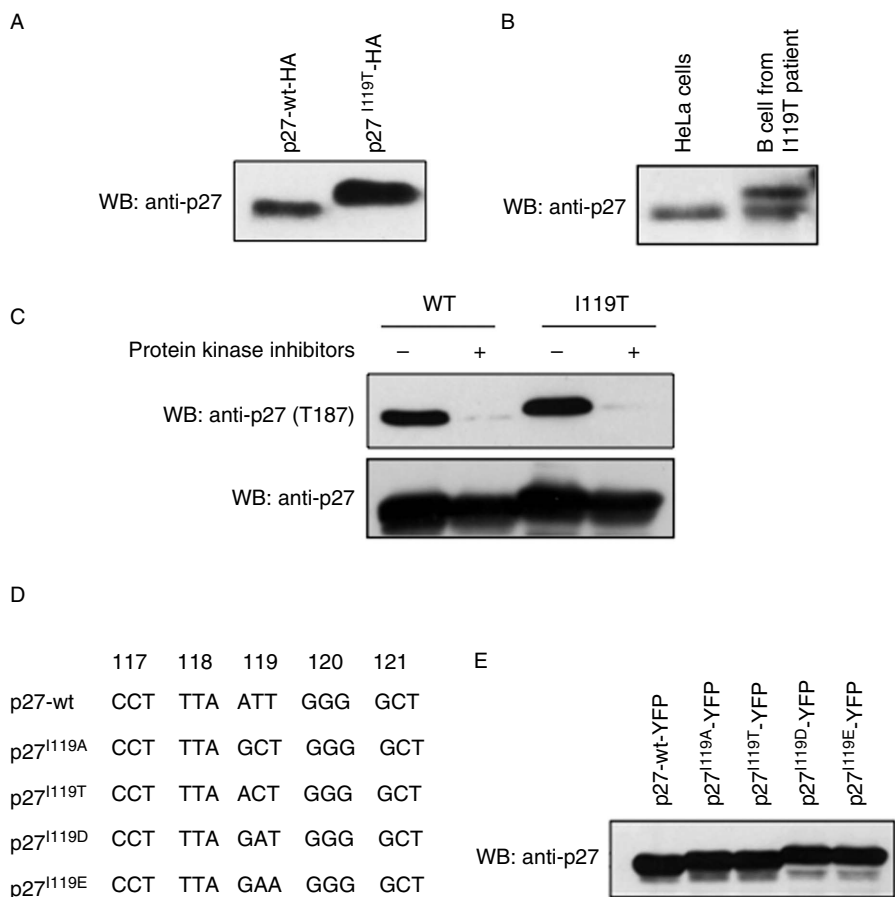responsible for the observed proteasome resistance of this mutant p27 protein.

As already mentioned, p27^{I119T} migrates more slowly than p27-wt by SDS–PAGE (Fig. 1A). To exclude that this abnormal migration pattern could be an artifact of the cloning, we subcloned both p27-wt and p27^{I119T} cDNA into a different vector (having an HA tag) and checked the expression of the proteins upon transfection in GH3 and HeLa cells. Western blotting analysis showed that HA-tagged p27^{I119T} also migrated more slowly than HA-tagged p27-wt (Fig. 4A).

B lymphocytes from the variant-positive patient grown in culture were available and were analyzed for *p27* expression. We observed the presence of two bands by western blotting, one corresponding to the wild-type allele and one to the I119T mutant protein migrating gradually in the gel (Fig. 4B). Thus, p27^{I119T} is expressed *in vivo* in the patient's blood.

p27 is a target of phosphorylation at various residues, and these posttranslational modifications regulate its function, stability, and intracellular localization (reviewed in Vervoorts & Lüscher (2008)).
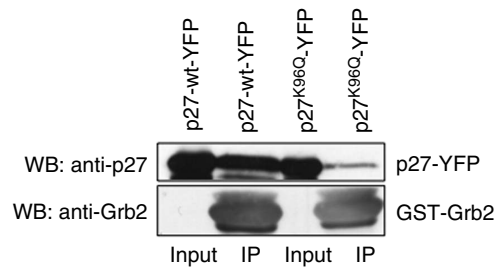
Figure 3 GST-Grb2 pull-down assay with p27^{K96Q}. HeLa cells were transfected with p27-wt-YFP and p27^{K96Q}-YFP. Cell lysates (INPUT) were incubated with recombinant GST-Grb2. After washing the Grb2-GST resin, all the bound proteins (IP) were eluted. The SDS–PAGE was loaded with 50 μg of input and the whole IP. Immunoblotting was performed as described previously with anti-p27 antimouse antibody and subsequently with anti-Grb2 antirabbit antibody. As shown, little p27^{K96Q}-YFP was pulled down compared with p27-wt. p27^{K96Q} has a reduced affinity for Grb2 binding.

A

p27-wt-HA p27^{I119T}-HA

WB: anti-p27

B

HeLa cells B cell from I119T patient

WB: anti-p27

C

	WT		I119T	
Protein kinase inhibitors	−	+	−	+

WB: anti-p27 (T187)

WB: anti-p27

D

	117	118	119	120	121
p27-wt	CCT	TTA	ATT	GGG	GCT
p27^{I119A}	CCT	TTA	GCT	GGG	GCT
p27^{I119T}	CCT	TTA	ACT	GGG	GCT
p27^{I119D}	CCT	TTA	GAT	GGG	GCT
p27^{I119E}	CCT	TTA	GAA	GGG	GCT

E

p27-wt-YFP p27^{I119A}-YFP p27^{I119T}-YFP p27^{I119D}-YFP p27^{I119E}-YFP

WB: anti-p27

Figure 4 p27^{I119T} is associated with abnormal SDS–PAGE migration. Western blotting analysis of the whole lysates from p27-wt-HA- or p27^{I119T}-HA-transfected GH3 cells (A) and lymphoblastoid B cell of p27^{I119T} mutation carrier (B). (C) HeLa cells were transfected with p27-wt-HA and p27^{I119T}-HA constructs. After 24 h, cells were incubated for 6 h in a culture medium supplemented with a pool of protein kinase inhibitors (staurosporine, H89, and H8) or left untreated. (D) Scheme of the mutations introduced at the isoleucine 119 residue. (E) HeLa cells were transfected with YFP-p27 constructs containing p27-wt, p27^{I119A}, p27^{I119T}, p27^{I119D}, or p27^{I119E} mutant proteins and examined by western blotting. The altered mobility of all of the I119 variants is evident.

Theoretically, increased phosphorylation could explain the migration shift of p27^{I119T}. To test this possibility, we transfected HeLa cells with p27-wt or p27^{I119T} and then treated them with inhibitors of protein kinases A, C, and G (staurosporine, H8, and H89). We then checked for alterations in the SDS–PAGE migration behavior of both proteins, but we did not observe any differences in the presence or absence of the inhibitors (Fig. 4C).

To confirm whether the atypical migration of p27^{I119T} in SDS–PAGE gels is linked specifically to the isoleucine 119 residue, we substituted I119 with three different amino acids (Fig. 4D) and analyzed their migration behavior. We introduced the amino acid Ala (A) that cannot be phosphorylated at position 119 by mutagenesis. Upon transfection in HeLa cells, p27^{I119A} showed the same migration pattern as p27^{I119} (Fig. 4E). We then substituted I 119 with two

phosphomimetic amino acids (aspartic acid, D; glutamic acid, E) to generate two mutant proteins, p27^{I119D} and p27^{I119E}. Surprisingly, these two proteins showed an even slower migration by SDS–PAGE compared with p27^{I119T} (Fig. 4E). Thus, genetic changes that substitute the residue at position 119 of p27 affect the migration pattern of the protein.

Discussion

FIPA is a syndrome of pituitary adenomas occurring in a familial setting in the absence of MEN1 and CNC (Daly *et al*. 2006*a*). Since the discovery by Vierimaa *et al*. (2006) that *AIP* is an inherited cause of pituitary adenomas in 2006, extensive studies have demonstrated its involvement in the pathophysiology of 15–25% of FIPA kindreds (Georgitsi *et al*. 2007*b*, Daly *et al*. 2010, Igreja *et al*. 2010, Tichomirowa *et al*. 2011).

In an effort to study other potential genetic causes of FIPA, we examined *CDKN1B* sequences in 124 individuals from 88 FIPA *AIP* mutation-negative kindreds, as previous studies had concentrated largely on *MEN1*-negative MEN1 cohorts (Igreja *et al*. 2009). We found two new germline *CDKN1B* changes in patients with pituitary adenomas from *AIP* mutation-negative FIPA kindreds. Although these sequence changes were identified in a familial setting and they altered p27 function or structure *in vitro*, the K96Q variant did not segregate with pituitary adenomas in one kindred. In the case of I119T variant that affected *CDKN1B* molecular weight/migration, one of the two family members affected with a pituitary adenoma was not available for genetic testing, so it cannot be fully ruled in or out as a cause of the clinical phenotype. Based on these findings, *CDKN1B* changes alone are not a frequent or likely cause of the FIPA tumor phenotype but could represent a contributing factor. Nevertheless, the *CDKN1B* sequence variants described here add to growing evidence of a role for p27-related dysfunction in the development of a subset of many endocrine tumors within and outside of the setting of MEN4.

The involvement of p27 in pituitary tumorigenesis has been demonstrated in animal studies. Indeed, *p27*-null mice develop pituitary intermediate lobe adenomas (Fero *et al*. 1996, Kiyokawa *et al*. 1996, Nakayama *et al*. 1996), and heterozygous $p27^{+/-}$ mice display pituitary hyperplasia (Fero *et al*. 1998). While human pituitary adenomas only rarely showed somatic *CDKN1B* mutations, downregulation of p27 is observed frequently in these tumors, especially in corticotropinomas (Kawamata *et al*. 1995, Ikeda *et al*. 1997, Jin *et al*. 1997, Takeuchi *et al*. 1998). Interest was renewed by the discovery that germline *CDKN1B* mutations in both the rat MENX and the human MEN4 syndromes are associated with development of pituitary adenomas (Fritz *et al*. 2002, Pellegata *et al*. 2006). Among the eight MEN4 patients identified to date, three (37.5%) had pituitary adenomas (a somatotropinoma, Cushing disease, and a nonfunctioning adenoma), so it appears to be a distinctive disease feature among these patients, although not as pronounced as primary hyperparathyroidism (7/8 patients, 87.5%).

The K96Q mutation is situated in the proline-rich domain (amino acids 90–96) of p27, which mediates the binding to the adaptor molecule Grb2, which in turn recruits and leads to activation of Ras (Marinoni & Pellegata 2011). The interaction between p27 and Grb2 promotes p27 degradation in the cytoplasm (Pagano *et al*. 1995, Vervoorts & Lüscher 2008). Indeed, $p27^{K96Q}$ displayed less Grb2 binding during a pull-

down assay compared with p27-wt. These findings echo the altered Grb2 interaction reported by Agarwal *et al*. (2009) in a patient with a missense mutation at the previous amino acid residue (P95S) that led to parathyroid tumors and a metastatic gastrinoma.

The I119T variant affects a residue located in the so-called 'scatter domain' of p27 (amino acids 118–158), which is responsible for actin cytoskeletal rearrangement and cell migration, processes involved in metastatic spread of human tumors (McAllister *et al*. 2003). This change causes a shift in the migration of the p27 protein in SDS–PAGE gels. The unique migration pattern of $p27^{I119T}$, indicative of posttranslational modifications, was not affected by multiple kinase inhibitors, suggesting that it is not due to phosphorylation at this newly created threonine residue. As glycosylation occurs at serine, threonine, or aspartic acid residues, the migration shift associated with the 119T residue could be caused by glycosylation of the protein (Dennis *et al*. 1999), thereby conferring greater stability. In agreement with this finding, $p27^{I119T}$ is more stable than the p27-wt *in vitro*.

The I119T sequence change was previously described as a somatic genetic mutation in a patient with myeloproliferative disorder (presence of the change in the patient's germline was not studied; Pappa *et al*. 2005); also the W76X nonsense *CDKN1B* mutation found in a MEN4 patient had been previously identified as a somatic change in hematological malignancies (Morosétti *et al*. 1995). Moreover, the c.356T/C (I119T) change has been reported in a study of hereditary prostate cancer (Chang *et al*. 2004), but the association of the C variant allele with the predisposition to the disease could not be demonstrated. The observations that this variant allele is expressed and translated into protein in our mutation carrier, in addition to the association of the I119T change with other tumor types and its potential effect in the function of p27, make a plausible case that it may play a role in tumor predisposition.

In conclusion, this study is the first extensive study of *CDKN1B* germline variants in a set of 88 FIPA families that do not have *AIP* mutations. According to our data, mutations of *CDKN1B* are not a cause of FIPA. However, *CDKN1B* germline variants associated with *in vitro* molecular phenotypes were seen in nearly 2% of cases studied. Altered p27 function may infrequently play a role in general pituitary disease outside of MEN4, although screening for *CDKN1B* mutations systematically appears unjustified in the setting of the >75% of FIPA kindreds not caused by *AIP* mutations (Jaffrain-Rea *et al*. 2011).

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-11-0362.

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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