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

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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. p27119T 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.

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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. 2006b, 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. 2006a). 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, Pellegrata 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. 2007a, 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, GTCTGTGTCTTTTGCTC CG; Ex1.1R, GGTCGTGATAAGCTCAGG; Ex1.2F, GAGTGGAGGACTGCAG; Ex1.2R, CAAGGTAACAGTAATCC; Ex1.2R, CCCAGGATTGTGGGTG; and Ex2R, CCCAGGATTGTGGGTG. Each 25 μl PCR reaction contained 140 ng genomic DNA, 1.25 μl of each primer, 1.5 mM MgCl₂, 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 60°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 p27K96Q and p27I119T 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 (LG 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, p27K96Q, or p27I119T 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.ith.cnr.it/oriel/spliceview.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 non-functioning 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 non-pathological 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.
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, p27K96Q, and p27I119T. 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 p27K96Q and p27I119T 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, p27K96Q, and p27I119T. We observed a time-dependent reduction of p27-wt and p27K96Q following CHX treatment, while p27I119T levels did not decrease throughout the experiment (Fig. 2). These results show that the p27I119T 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 p27K96Q or p27I119T 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, p27K96Q 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, p27I119T 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 p27I119T 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 p27I119T 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, p27I119T 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 2 Stability of wild-type and mutant p27. The rate of the turnover of p27-wt, p27K96Q, and p27I119T proteins was measured in exponentially growing, transiently transfected GH3 cells using cycloheximide (CHX) with and without the proteasome inhibitor MG-132. p27-wt and p27K96Q have a half-life of ~10 h in GH3 cells. MG-132 has no effect on p27K96Q degradation. In contrast, p27I119T is more stable than p27-wt.

Figure 3 GST-Grb2 pull-down assay with p27K96Q. HeLa cells were transfected with p27-wt-YFP and p27K96Q-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 p27K96Q-YFP was pulled down compared with p27-wt. p27K96Q has a reduced affinity for Grb2 binding.
Theoretically, increased phosphorylation could explain the migration shift of p27<sup>I119T</sup>. To test this possibility, we transfected HeLa cells with p27-wt or p27<sup>I119T</sup> and then treated them with inhibitors of protein kinases A, C, and G (staurosporine, H89, and H8) or left untreated. 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<sup>I119T</sup> in SDS–PAGE gels is linked specifically to the isoleucine 119 residue, we substituted I<sub>119</sub> 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<sup>I119A</sup> showed the same migration pattern as p27<sup>I119</sup> (Fig. 4E). We then substituted I<sub>119</sub> with two phosphomimetic amino acids (aspartic acid, D; glutamic acid, E) to generate two mutant proteins, p27<sup>I119D</sup> and p27<sup>I119E</sup>. Surprisingly, these two proteins showed an even slower migration by SDS–PAGE compared with p27<sup>I119T</sup> (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. 2006a). 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. 2007b, 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, Pellegrata 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 & Pellegrata 2011). The interaction between p27 and Grb2 promotes p27 degradation in the cytoplasm (Pagano et al. 1995, Vervoorts & Lüscher 2008). Indeed, p27K96Q 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 p27I119T, 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 I19T residue could be caused by glycosylation of the protein (Dennis et al. 1999), thereby conferring greater stability. In agreement with this finding, p27I119T 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, may offer 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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