CASE REPORT

Functional characterization of a rare germline mutation in the gene encoding the cyclin-dependent kinase inhibitor p27Kip1 (*CDKN1B*) in a Spanish patient with multiple endocrine neoplasia-like phenotype

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Abstract

Objective: The aim of this study was to investigate the presence of germline mutations in the *CDKN1B* gene that encodes the cyclin-dependent kinase (Cdk) inhibitor p27 in multiple endocrine neoplasia 1 (MEN1)-like Spanish index patients. The *CDKN1B* gene has recently been identified as a tumor susceptibility gene for MEN4, with six germline mutations reported so far in patients with a MEN-like phenotype but negative for MEN1 mutations.

Design and methods: Fifteen Spanish index cases with MEN-like symptoms were screened for mutations in the *CDKN1B* gene and the mutant variant was studied functionally by transcription/translation assays *in vitro* and in transiently transfected HeLa cells.

Results: We report the identification of a heterozygous GAGA deletion in the 5'-UTR of *CDKN1B*, NM_004064.3:c.-32_-29del, in a patient affected by gastric carcinoid tumor and hyperparathyroidism. This deletion falls inside the region that is responsible for *CDKN1B* transcription and is predicted to destroy a secondary stem and loop structure that includes the GAGAGA element responsible for ribosome recruitment. Accordingly, *in vitro* studies of coupled transcription/translation assays and transient transfection in HeLa cells showed that the GAGA deletion in the *CDKN1B* 5'-UTR significantly impairs the transcription of downstream reporter luciferase (of ~40–60%) and, possibly, the translation of the corresponding mRNA. This mutation was associated with a significant reduction in the amount of *CDKN1B* mRNA in peripheral blood leukocytes from the patient, as demonstrated by quantitative real-time PCR.

Conclusions: Our results confirm that germline *CDKN1B* mutations may predispose to a human MEN4 condition and add novel evidence that alteration in the transcription/translation rate of *CDKN1B* mRNA might be the mechanism implicated in tumor susceptibility.

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Introduction

Multiple endocrine neoplasia (MEN) syndromes represent autosomal dominant disorders characterized by the occurrence of tumors in two or more endocrine glands. So far, two MEN syndromes have been well recognized: MEN type 1 (MEN1), caused by germline mutations in the *MEN1* tumor suppressor gene, and MEN2, caused by germline mutations in the *RET* proto-oncogene respectively. Recently, Pellegata *et al.* (1) identified a novel MEN-like syndrome in rat, denoted MENX. Animals affected by MENX spontaneously develop multiple endocrine tumors, with symptoms

that overlap both human MEN1 and MEN2. Genetic studies in the rats predisposed to MENX identified a loss-of-function germline mutation in the *Cdkn1b* gene that encodes the cyclin-dependent kinase (Cdk) inhibitor p27 as the causative mutation for the MENX syndrome (1). Subsequently, mutations in the human homolog *CDKN1B* gene were identified in patients with multiple endocrine tumors that were negative for *MEN1* or *RET* mutations (2, 3, 4). As a consequence of these observations, a novel human MEN-like syndrome, caused by germline mutations in *CDKN1B*, was recognized as a separate disease and denoted MEN4 (5).

P27 is an inhibitor of Cdks that negatively regulate cell cycle progression in mammalian cells. Loss of p27 protein expression has been reported as a frequent event in human cancers (6, 7). Importantly, reduced p27 expression in cancer cells is correlated with tumor aggressiveness, invasion, and poor differentiation, predicting shorter disease-free and/or overall survival (7, 8, 9, 10, 11). Yet, *CDKN1B* does not fit all classic features of a tumor suppressor gene, since it is rarely mutated in cancer (12, 13, 14). Point mutations in the coding region of *CDKN1B* are rare (12, 15, 16) and loss of heterozygosity has been observed only in some tumors (16, 17, 18). Therefore, alternative mechanisms that act at the post-transcriptional or post-translational level have been suggested. These alternative mechanisms include a lower translation rate of *CDKN1B* mRNA (19), an increase in protein degradation (20, 21), and the cytosolic mislocalization of p27 protein (22, 23, 24).

Importantly, experiments with *Cdkn1b*-deficient mice have established that *Cdkn1b* is a haploinsufficient tumor suppressor gene. Both *Cdkn1b*-null and heterozygous mice are predisposed to spontaneous pituitary adenomas (25, 26, 27), as well as to radiation- and ethylnitrosourea-induced tumors in multiple tissues (28).

In this study, we report on the identification of a germline mutation in the 5'-UTR of *CDKN1B*, corresponding to the deletion of four nucleotides GAGA centered at position -27, NM_004064.3:c.-32_-29del, in a patient with multiple endocrine tumors showing MEN1-like phenotype. *In vitro* functional assays demonstrated that the c.-32_-29del in the 5'-UTR of *CDKN1B* gene can account for the reduced expression of p27 mRNA in patients affected by MEN-like syndrome.

Materials and methods

Patients

Each index case (proband) had been previously referred for MEN1 genetic screening. Only one of them presented familial antecedents related to the disease at the moment this study was performed (Table 1). Written informed consent to collect phenotypic and genotypic data was obtained from all participants in accordance with Institution Review Board-approved protocols for each center. To examine the presence of the variant in the control population, a group composed of 400 individuals was used. Controls were Spanish individuals free cancer recruited between 1999 and 2005 through the following sources: 287 (72%) from the Menopause Research Centre at the Instituto Palacios, Madrid, and 113 (28%) from the National Blood Transfusion Centre, Madrid. Informed consent was obtained from all participants.

DNA extraction and sequencing

DNA extraction was carried out by using QIAamp DNA Blood Kit (Qiagen). Germline DNA sequences of *CDKN1B*, representing the coding regions and intron-exon boundaries, were amplified by PCR. PCR was performed with AmpliTaq Gold with GeneAmp (Applied Biosystems, Foster City, CA, USA). The conditions were: 10 min at 94 °C for Taq Gold activation, 35 cycles at 94 °C for 30 s, annealing at 60 °C (exon 1) or 55 °C (exon 2) for 30 s, extension at 72 °C for 60 s, and then for 5 min at 72 °C.

Table 1 Clinical characteristics of index cases. The age of onset indicates the age at diagnosis of the first tumor. The clinical presentation refers to the clinical signs developed by the patients; the age at diagnosis of each tumor is indicated in brackets.

Patient ID	Gender	Onset (years)	Clinical presentation	MEN1 gross deletion ^a	Family
1	Female	55	HPT (adenoma) (67), pituitary adenoma (GH) (55)	NA	No
2	Female	56	HPT (56), pituitary adenoma (GH) (56)	NA	No
3	Female	68	HPT (68) ^b , pituitary adenoma (non-secretory) (68)	Negative	No
4	Male	62	HPT (62), pituitary macroadenoma (ACTH) (65)	Negative	No
5	Female	44	HPT (53), pituitary adenoma (GH) (44)	Negative	No
6	Female	56	HPT (63), pituitary adenoma (non-secretory) (56)	NA	No
7	Female	69	Gastric carcinoid tumor (69), HPT (74)	Negative	No
8	Female	65	Insulinoma (65), adrenal adenoma (65)	Negative	No
9	Female	62	Insulinoma (62), adrenal adenoma (67)	Negative	No
10	Female	62	Insulinoma (62), adrenal adenoma (67)	Negative	No
11	Male	73	HPT (73), macroprolactinoma (73)	Negative	No
12	Female	78	HPT insulinoma carcinoid tumor (lung) goiter	Negative	No
13	Female	59	HPT (63), pituitary adenoma (GH) (59)	NA	No
14	Female	—	HPT pituitary adenoma (GH), renal carcinoma	Negative	Yes ^c
15	Female	54	Carcinoid tumor (bronchial) (54), collagenoma (55), adrenal adenoma (55)	Negative	No

PRL, prolactin; NA, not assessed.

^aMEN1 gross deletion was assessed by multiplex ligation-dependent probe amplification (MLPA), according to the manufacturer's instructions (MRC-Holland, Amsterdam, Holland).

^bMultiglandular.

^cPatient with familial antecedents (son diagnosed with HPT 1° at the age of 32 years).

Part of exon 1 (605 bp) was amplified with primers 5'-GGCGCTTTGTTTTGTTCCG-3' and 5'-CCCTCCAGC-AGGCAAAGCG-3'. Exon 2 (333 bp) was amplified with primers 5'-CCTTAAAGCCACTGGGGATG-3' and 5'-CGTGAGGTCTGAAGGCC-3'. PCR products were purified using QIAquick PCR Purification Kit (Qiagen) and sequenced in one or both directions. Sequencing results were both machine-read and manually read by two of the authors (D Malanga and S De Gisi) independently.

Constructs

We cloned the minimal regulatory region of the *CDKN1B* gene that has been shown to be able to efficiently drive transcription of a downstream reporter gene (29, 30, 31). DNA fragments from the 5' region of the *CDKN1B* gene between nucleotides -575 and -1 were generated through PCR using genomic DNA of a donor sample with forward 5'-GGTACCGGTACCCACCTTAAGGCCGCTCG-3' and reverse 5'-AAGCTTA AGCTTCTTTCTCCCGGGTCTGCACG-3' primers containing KpnI and HindIII sites respectively. PCR products were cloned into the pGL3-basic promoterless plasmid (Promega) to generate pGL3-5'-UTR-WT and to drive expression of the downstream luciferase gene. After cloning, the construct was subject to site mutagenesis using the site-directed mutagenesis system kit (Invitrogen) to generate the pGL3-5' -UTR-MUT, carrying the c.-32_-29del using the following primers: forward 5'-GTTTTTTTGAGAGTGCGAGGCGGTCGTG-CAG-3' and reverse 5'-CTGCACGACCGCTCGCAC-TCTCAAAAAAAC-3'. Plasmids generated for each genotype were confirmed by direct sequencing. To generate pGL3-promoter-5' -UTR-WT and pGL3-promoter-5'-UTR-MUT, we amplified from the previous corresponding constructs the *CDKN1B* 5'-UTR with forward 5'-AAGCTTAAGCTTCCCCACCTTAAGGCCGCGCTCG-3' and reverse 5'-CCATGGCCATGGTGGCTTACCAACAGTACCGGAAT-3' primers containing HindIII and NcoI sites respectively. The PCR products were inserted in the HindIII and NcoI restriction sites of pGL3 promoter (Promega). For *in vitro* coupled transcription/translation experiments, the 2 kb KpnI/XbaI restriction fragments from pGL3-5'-UTR-WT and pGL3-5'-UTR-MUT were cloned in pcDNA3 (Invitrogen) to obtain the constructs pcDNA3-5'-UTR-WTLuc and pcDNA3-5'-UTR-MUTLuc respectively.

Cell culture and transfection

HeLa cells (American Type Culture Collection) were incubated at 37 °C in a humidified 5% CO₂ atmosphere in DMEM – high glucose medium (Sigma–Aldrich) supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 10% fetal bovine serum. Transfections were performed with Trans IT-LT1 Transfection Reagent (Mirus Bio LLC, Madison, WI, USA) following the manufacturer's protocol.

In vitro transcription

In vitro transcription (IVT) reactions were performed by the Riboprobe *in vitro* transcription systems (Promega). The constructs were linearized with StuI and subjected to the reaction following manufacturer's recommendations. The IVT RNAs were separated using 1.5% denaturing agarose gel electrophoresis and analyzed by densitometry.

Luciferase reporter gene assays

For Luciferase activity assays, cells were transfected with 1 µg of the promoter/luciferase reporter plasmids, and with 100 ng pRL Renilla as control vector (Promega) to correct for differences in transfection efficiency. Cells were collected 24 and 48 h after transfection by centrifugation, washed twice with PBS, and lysed with 100 µl cell lysis reagent (Dual-Luciferase Reporter Assay System, Promega). Cell lysates were dispensed into luminometer plates, followed by sequential auto-injection of the Luciferase Assay Reagent for firefly luciferase activity measurement and Stop&Glo Reagent for Renilla luciferase activity measurement (Dual-Luciferase Reporter Assay System, Promega). Basal luciferase activity was examined in cells transfected with an empty vector (pGL3 Basic or Promoter Vector). Three independent transfections were performed for each construct in triplicate.

IVT/translation

Coupled transcription/translation reactions were performed by the TNT Coupled Reticulocyte Lysate (Promega) standard rabbit reticulocyte systems. The constructs were linearized with PvuI and subjected to 90 min reaction following manufacturer's recommendations. After completion of the reaction, 5 µl of the reaction mixture was added to 100 µl Luciferase Assay Reagent for firefly luciferase activity measurement.

cDNA synthesis and real-time quantitative RT-PCR

We obtained cDNA from RNA isolated from HeLa cells or from peripheral blood leukocytes of the patient genotyped in the study and from five healthy controls. One microgram of total RNA was reverse-transcribed using Superscript II Reverse Transcriptase (Invitrogen) and an oligo dT18 primer, according to the manufacturer's instructions. The amount of *CDKN1B* mRNA was quantified by real-time PCR with the Sequence Detection System 7900HT (Applied Biosystems), using primers p27QF (5'-CCCTAGAGGGCAAGTACGAGT-3') and p27QR (5'-AGTAGAACTCGGGCAAGCTG-3'). For the evaluation of luciferase and Renilla mRNAs,

we used the following primers respectively: luciferase forward (5'-ATCCATCTTGCTCCAACACC-3') and reverse (5'-TTTTCCGTCATCGTCTTTCC-3') and Renilla forward (5'-ACAAGTACCTCACCGCTTGG-3') and reverse (5'-GACACTCTCAGCATGGACGA-3'). The amplification conditions were: an initial step at 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Normalization was carried out with the internal standard GAPDH (forward primer, 5'-AACGGATTTGGTTCGTATTGGGC-3' and reverse primer, 5'-TCGCTCCTGGAAGATGGTGATG-3'). All reactions were in the initial exponential amplification phase and the cycle at which each sample crossed the threshold (C_t) was recorded for *CDKN1B* and GAPDH. The $\Delta\Delta C_t$ method was used for the calculation of the different amounts of mRNA (32). Negative controls were included in all PCR reactions and assays were carried out in triplicate.

Student's *t*-test was used for statistical analysis.

Results

CDKN1B sequencing in index cases

There were 15 index cases negative for MEN1 germline mutations and classified as MEN-like, that were considered as candidates for the p27 mutational screening. Among the 15 index cases, it was also possible to discard the presence of MEN1 large deletion/duplication by multiplex ligation-dependent probe amplification in 11 patients with high-quality DNA (Table 1). Among them, nine developed hyperparathyroidism (HPT) and pituitary adenoma (five GH-secreting, one ACTH-secreting, one prolactinoma, and two non-secreting), two had HPT and gastric carcinoid tumor, or insulinoma and bronchial carcinoid tumor, three were diagnosed with insulinoma and adrenal adenoma, and one patient was affected by a bronchial carcinoid tumor, a collagenoma and adrenal adenoma (see Table 1).

Among these 15 MEN-like probands, we detected the presence of a heterozygous GAGA deletion at 5'-UTR, c.-32_-29del in the patient ID7, a 69-year-old Spanish female affected by gastric carcinoid tumor and HPT (see Fig. 1A and B). Patients' clinical data are shown in Table 1. Sequence comparison of the 5'-UTR among human, mouse, and rat showed that these four nucleotides were highly conserved among all species analyzed, suggesting functional relevance of the sequence (Fig. 1C).

Functional effects of the 5'-UTR –32/–29 GAGA deletion

To functionally characterize the deletion detected in the patient ID7, we cloned the PCR fragment amplified from genomic DNA, corresponding to the –575 to –1

Figure 1 Germline mutation in the *CDKN1B* gene in a MEN1-like patient. (A) Left panel shows the genomic wild-type sequence of the region spanning nucleotides -32/-29 (underlined) in the *CDKN1B* gene of a healthy control; right panel shows the genomic sequence of patient ID7 with the presence of a 4 bp deletion encompassing nucleotides -32/-29 (arrows). (B) Sequences of the PCR fragments cloned from genomic DNA of the patient ID7. The upper panel shows the WT sequence (-32/-27 GAGA nucleotide underlined); the lower panel shows the sequence of the mutant fragment with deleted GAGA at nucleotides -32/-29 (MUT) (dashed line). (C) Homology between the different 5'-UTR sequences of *CDKN1B* orthologs in the mouse, rat, and human. Full colour version of this figure available via <http://dx.doi.org/10.1530/EJE-11-0929>.

nucleotides, into the PCR-Topo Cloning Vector. The sequencing of the cloned PCR product confirmed the presence of a heterozygous GAGA deletion as shown in Fig. 1B.

Transcription from *CDKN1B* is initiated predominantly from a single site which is conserved in the human and mouse genes (29, 30, 31). Initiation at this site produces a 5'-UTR of 472 nucleotides in the human *CDKN1B* mRNA and 502 nucleotides in the mouse *Cdkn1B* mRNA. In addition, several minor transcription start sites were identified for both the mouse and human genes (29, 30, 31). In addition to the capability to drive transcription of a downstream reporter gene, the 5'-UTR region of human *CDKN1B* contains a U-rich element that is involved in regulating *CDKN1B* mRNA stability and/or translation efficacy (33). In fact, several mRNA-binding proteins were found to interact with the *CDKN1B* 5'-UTR and to regulate its expression (34).

For this reason, to investigate whether the deleted 5'-UTR sequence detected in the patient ID7 affected the expression of *CDKN1B* mRNA and/or protein, we decided to clone the minimal regulatory region of the

CDKN1B gene that has been shown to efficiently drive transcription of a downstream reporter gene (30, 31, 35, 36) and measure the effects on transcription exerted by the mutant allele. For this purpose, the 575 bp sequence in the *CDKN1B* 5'-UTR was PCR amplified from wild-type or mutant genomic DNAs and inserted upstream of the firefly luciferase reporter gene in the promoterless pGL3 vector, generating wild-type (5'-UTR-WT) and deleted (5'-UTR-MUT) constructs (Fig. 2A). These constructs were transfected into HeLa cells and luciferase activity was determined 48 h after transfection. As shown in Fig. 2B, cells transfected with luciferase cDNA under the control of the 5'-UTR-MUT showed a significant reduction (almost 40%; P value <0.0024) in the luciferase activity, compared with cells transfected with the 5'-UTR-WT construct. Analysis by real-time quantitative RT-PCR indicated that the steady-state levels of luciferase mRNA produced by the 5'-UTR-MUT construct were less abundant ($\sim 32\%$) than those produced by 5'-UTR-WT (Fig. 2C). Similar results were obtained when transfected cells were deprived of serum, as shown in Fig. 2D.

The 5'-UTR of both human and mouse *CDKN1B* genes have been shown to contain an internal ribosome entry site (IRES) (37, 38). The ribosome entry window for the IRES-dependent translation is located between nucleotides -50 and -36 upstream of the p27 translation start codon (for the sake of clarity, we set the A nucleotide of the ATG translation start codon as $+1$), within a U-rich region that forms a large single-stranded loop (33, 38, 39). We performed a secondary structure prediction of the 5'-UTR-WT ($-575/-1$) sequence using a web-based application from www.tbi.univie.ac.at/~ivo/RNA/ (40, 41). As shown in Fig. 3, the 575 nucleotides of 5'-UTR in the wild-type *CDKN1B* (5'-UTR-WT) contain a secondary stem and loop structure that includes the element $-32/-29$ GAGAGA. This loop contains a U-rich region that has previously been shown to interact with mRNA-binding proteins and to modulate ribosome recruitment (19, 38, 39). The ribosome entry window for IRES-initiated translation is between nucleotides 36 and 50 (indicated by stars in Fig. 3) (38). Conversely, the secondary structure prediction of the 575-nucleotide-long 5'-UTR of the mutant patient ID7 (5'-UTR-MUT) shows a different secondary structure, with the destruction of the predicted stem and loop. This observation suggested that the $-32/-29$ GAGA may impair the translation efficacy of mutant *CDKN1B* gene.

We further investigated whether the sequence comprising the $-32/-29$ GAGA element is important for the control of transcription and/or translation efficiency of *CDKN1B* mRNA. First, we performed an *in vitro* coupled transcription–translation assay. With this aim, we introduced the *CDKN1B* 5'-UTR-WT or the 5'-UTR-MUT sequences upstream of the AUG initiation codon of luciferase cDNA into the pcDNA3 vector under the transcriptional control of the T7 promoter, as shown

Figure 2 Transcription rate mediated by the wild-type or mutant *CDKN1B* promoter/5'-UTR region. (A) Schematic diagram of pGL3 promoterless constructs carrying wild-type sequence (5'-UTR-WT) or the $-32/-29$ GAGA deletion (5'-UTR-MUT). (B) Luciferase assay. HeLa cells were transfected with constructs encoding luciferase under the control of *CDKN1B* 5'-UTR-WT or 5'-UTR-MUT respectively. After 48 h of transfection, the cells were harvested and luciferase activity was determined and normalized to the co-transfected Renilla. Luciferase activity was expressed as relative values to those generated by the empty vector pGL3 as mean of three replicates. $*P < 0.0024$. (C) Q-RT-PCR analysis of luciferase mRNA expression mediated by the wild-type or mutant *CDKN1B* 5'-UTR region. HeLa cells were transfected with constructs (pGL3 promoterless) encoding luciferase under the control of *CDKN1B* 5'-UTR-WT or 5'-UTR-MUT. After 48 h of transfection, the cells were harvested and luciferase mRNA was quantified and normalized to the co-transfected Renilla in real-time PCR. Values were expressed relative to those generated by the pGL3-5'-UTR-WT as mean of three replicates. (D) Luciferase assay. pGL3 promoterless constructs encoding luciferase under the control of *CDKN1B* 5'-UTR-WT or 5'-UTR-MUT were transfected into HeLa cells in the presence or absence of serum. After 48 h, the cells were harvested and luciferase activity was determined and normalized to the co-transfected Renilla as above. Luciferase activity was expressed as relative values to those generated by the empty vector pGL3 as mean of three replicates.

in Fig. 4A (pcDNA3-5'-UTR-WTLuc and pcDNA3-5'-UTR-MUTLuc respectively). Subsequently, both pcDNA3-5'-UTR-WTLuc and the pcDNA3-5'-UTR-MUT Luc constructs were subjected to *in vitro* coupled transcription–translation in a system of rabbit reticulocytes. After completing the reaction, the activity of the newly synthesized, functionally active luciferase was

Figure 3 (A) Secondary structure prediction for the 575 bp 5'-UTR-WT region of *CDKN1B* gene. (B) Secondary structure prediction for the 575 bp 5'-UTR-MUT region of *CDKN1B* gene. Deleted regions are indicated by the arrow and brackets. The minimum free energy (MFE) for each structure is indicated at the bottom. See text for details.

measured with a luminometer. As shown in Fig. 4B, it is notable that there was a significant reduction (of ~40%; P value=0.0032) in the luciferase activity when the *CDKN1B* 5'-UTR-MUT was placed upstream of the luciferase coding region compared with the same construct containing 5'-UTR-WT. A parallel analysis by quantitative real-time RT-PCR of the luciferase mRNA produced by the pcDNA3-5'-UTR-WTLuc and the pcDNA3-5'-UTR-MUTLuc constructs, respectively,

demonstrated that the mRNA abundance transcribed by the 5'-UTR-MUT construct was ~70% of the mRNA abundance transcribed by the 5'-UTR-WT construct, with a reduction of about 30% (Fig. 4C).

To measure the contribution of the 5'-UTR sequence to the transcription and/or translation of *CDKN1B* mRNA in the living cells, we inserted the full-length 5'-UTR-WT and the deleted 5'-UTR-MUT sequences upstream of the luciferase reporter gene in the pGL3-promoter plasmid, which contains the SV40 promoter element (Fig. 5A). These constructs were then transfected into HeLa cells and the luciferase activity or luciferase mRNA levels were measured after 48 h respectively. As shown in Fig. 5B, after normalizing luciferase activity for the internal Renilla control, the average of luciferase activity shown by the pGL3-promoter-5'-UTR-MUT plasmid was consistently lower (~40%) compared with that shown by pGL3-promoter-5'-UTR-WT plasmid at the same time point (P value <0.0018). Conversely, the analysis of luciferase mRNA levels by quantitative real-time RT-PCR demonstrated that the mRNA abundance transcribed by the 5'-UTR-MUT construct was reduced by only 30% compared with that transcribed by the 5'-UTR-WT construct (Fig. 4C).

Altogether, these results suggested that the deleted -32/-29 GAGA region exerted a critical control on transcription and/or translation of *CDKN1B* mRNA. In particular, the finding that compared with 5'-UTR-WT, the reduction in the activity of the luciferase driven by

Figure 4 Coupled transcription/translation analysis of wild-type or mutant *CDKN1B* promoter/5'-UTR region. (A) Schematic diagram of pcDNA3 Luc constructs carrying wild-type sequence (5'-UTR-WT) or the -32/-29 GAGA deletion (5'-UTR-MUT). (B) Luciferase assay. The level of functional luciferase expression was measured by a Luminometer as a mean of three replicates and expressed relative to pcDNA3-5'-UTR-WTLuc. * P <0.0032. (C) *In vitro* transcription of *CDKN1B* 5'-UTR luciferase mRNA. The luciferase RNA transcripts were generated by *in vitro* transcription using 1 µg Stul linearized plasmids and T7 RNA polymerase. Right panel, densitometry data obtained from IVT RNA bands by ImageJ Software (free software provided by NIH, MD, USA).

Figure 5 Transcription and translation rates mediated by the wild-type or mutant *CDKN1B* promoter/5'-UTR region. (A) Schematic diagram of pGL3 promoter constructs carrying wild-type sequence (5'-UTR-WT) or the -32/-29 GAGA deletion (5'-UTR-MUT). (B) Luciferase assay. HeLa cells were transfected with pGL3-promoter-5'-UTR-WT or pGL3-promoter-5'-UTR-MUT. After 48 h of transfection, the cells were harvested and luciferase activity was determined and normalized to the co-transfected Renilla. Luciferase activity was expressed as relative values to those generated by the empty vector pGL3 as mean of three replicates. * $P \leq 0.0018$. (C) Q-RT-PCR analysis of luciferase mRNA expression mediated by the wild-type or mutant promoter/5'-UTR region. HeLa cells were transfected with pGL3-promoter-5'-UTR-WT or pGL3-promoter-5'-UTR-MUT constructs. After 48 h of transfection, the cells were harvested and luciferase mRNA was determined and normalized to the co-transfected Renilla in real-time PCR. Values were expressed relative to those generated by the pGL3 promoter 5'-UTR-WT as mean of three replicates.

5'-UTR-MUT was slightly more consistent than the reduction in mRNA levels, suggesting that the -32/-29 GAGA deletion in the 5'-UTR region of *CDKN1B* may impair both transcription and translation.

Expression of *CDKN1B* mRNA in peripheral blood leukocytes of patient ID7

All these observations prompted us to evaluate whether the presence of the GAGA deletion affects the expression level of *CDKN1B* mRNA in the patient ID7 *in vivo*. With this aim, we analyzed *CDKN1B* mRNA expression in the mRNA extracted from the peripheral blood leukocytes of patient ID02S514 and from several healthy donors. As shown in Fig. 6, quantitative RT-PCR revealed a statistically significant difference between the average *CDKN1B* mRNA expression of healthy donors ($n = 5$) and the level of *CDKN1B* mRNA in the patient ID7. The GAGA deletion resulted in a threefold reduction in mRNA levels compared with healthy controls (P value = 0.024), indicating that the mutant *CDKN1B* genotype was associated with decreased expression of *CDKN1B* mRNA. Since protein

samples were unavailable from this patient, we could not determine the effects of the mutation on the protein level, both in the blood and tumor of patient ID7.

Discussion

Recently, a syndrome termed MENX with features overlapping MEN1 and MEN2 in the rat was attributed to a homozygous inactivating mutation of the Cdk inhibitor *CDKN1B* gene. This finding has prompted us to search *CDKN1B* mutations in *MEN1*- and *RET*-negative patients showing a MEN-like phenotype in the human. To date, six germline mutations have been identified leading to the definition of a new type of MEN-like syndrome in the human, named MEN4, though the phenotypic features are still undefined due to the small number of patients reported (1, 2, 3, 4, 5). Most *CDKN1B* mutations reported so far associated with MEN4 syndrome are inactivating mutations that lead to prematurely truncated protein (TGG > TAG at codon 76 (p.W76X)) (4) or to a premature stop at codon 69 caused by a 19 bp duplication (2), to a protein predicted to be 60 amino acids longer due to a change in the stop codon (TAA to CAA; stop to Q) (3). In this study, we have identified a novel germline mutation in the *CDKN1B* gene in a proband affected by gastric carcinoid tumor and HPT, that was negative for mutations in *MEN1* and *RET*, being classified as a MEN-like syndrome. We provided evidence that the observed GAGA deletion encompassing nucleotides -32/-29 of

Figure 6 Q-RT-PCR analysis of *CDKN1B* expression in the MEN1-like patient and healthy controls. The Q-RT-PCR was performed on mRNA extracted from peripheral blood leukocytes. These results are representative of two independent experiments on total RNA. * $P \leq 0.05$.

5'-UTR significantly impairs transcription and, possibly, translation of *CDKN1B* mRNA. It is worthy of mention that a previous study identified a mutation at the -7 position in the Kozak sequence (G to C) of *CDKN1B* in a patient with a parathyroid tumor and adrenal masses, suggesting that germline mutations that lead to impairment of transcription and/or translation of *CDKN1B* may be common in MEN1-like syndrome (3).

Transcription from *CDKN1B* is initiated predominantly from a single site that produces a 5'-UTR of 472 nucleotides in the human *CDKN1B* mRNA (29, 30, 31). Our results indicate that the GAGA deletion significantly impairs transcription *in vitro* and *in vivo*, since: i) studies of *in vitro* coupled transcription/translation of the *CDKN1B* 5'-UTR (-575/-1) carrying the c.-32_29del upstream a luciferase reporter gene was transcribed with significant minor efficiency compared with a construct carrying the wild-type *CDKN1B* 5'-UTR (-575/-1); ii) transient transfection experiments in HeLa cells showed that the GAGA deletion encompassing -32/-29 nucleotides in the 5'-UTR (-575/-1) of *CDKN1B* significantly impairs the transcription of a downstream luciferase reporter gene; and finally iii) the -32/-29 deletion mutant associates with a significant reduction in the amount of *CDKN1B* mRNA in the peripheral blood leukocytes of the patient.

In addition to the capability to drive transcription of a downstream reporter gene, the 5'-UTR region of human *CDKN1B* contains a U-rich element that is involved in regulating *CDKN1B* mRNA stability and/or translation efficacy (33). Computed analysis of *CDKN1B* 5'-UTR suggested that the c.-32-29del mutant destroys such secondary structure, thus impairing ribosome entry. Although not definitive, our data also indicate that the -32/-29 deletion may also impair the translation of *CDKN1B* mRNA, since we consistently observed that the reduction in the activity of luciferase driven by 5'-UTR-MUT was slightly more consistent than the reduction in mRNA levels.

This work also provides evidence that haploinsufficient expression of *CDKN1B* might be implicated in tumor predisposition in MEN-like patients. In fact, although we were not able to assess the level of p27 protein, we found that the heterozygous -32/-29 deletion mutant was associated with a significant reduction in the amount of *CDKN1B* mRNA in the peripheral blood leukocytes of the patient considered for this study. Accordingly, several lines of evidence indicate that even small changes in the amount of p27 protein can have dramatic phenotypic consequences. A drastic reduction of *CDKN1B* expression has been observed in almost 50% of all human cancers, usually correlating with histological aggressiveness and poor outcome (6, 7, 8, 9, 10). The analysis of animal models has demonstrated that the lack of p27 is able to promote tumor formation. These consequences can be attributed to the role of p27 as a mediator of anti-mitogenic

signals. In the absence of p27, cells exposed to signals that induce growth arrest fail to properly withdraw from the cell cycle, undergoing more mitotic divisions and neoplastic transformation. It is noteworthy that several genes in this cell-cycle regulating pathway have been implicated in endocrine tumors in man and mouse. First, both *MEN1* and *RET* apparently regulate the expression of *CDKN1B* and *p18*, suggesting that these Cdk inhibitors might serve as negative growth regulators in MEN-like tumors; *Cdkn1b*-null mice develop intermediate lobe pituitary adenomas (frequency 100%) (25, 26, 27); mice with various combinations of knockout of *CDKN1B* and *p18* show tumors of pituitary, thyroid C-cells, parathyroids, adrenal medulla, endocrine pancreas, testis, and duodenum, with symptoms that resemble MEN1 and MEN2 (42, 43); and finally, MENX rats that display extremely reduced/absent p27 expression in their tissues develop multiple endocrine tumors (1). In conclusion, these results extend previous studies on MEN4 and indicate that *CDKN1B* germline mutations may predispose to a human MEN4 condition, although they are fairly uncommon, and add novel evidence that alteration in the transcription/translation rate of *CDKN1B* mRNA might be the mechanism implicated in tumor susceptibility.

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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