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Early onset acromegaly associated with a novel deletion in CDKN1B 5'UTR region

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Abstract:	Genetic alterations frequently are involved in the development of a pituitary adenoma in young age. We here characterize the functional role of a deletion in CDKN1B 5'-UTR region (c.-29_-26delAGAG) identified in an acromegalic patient that developed a growth hormone (GH) pituitary adenoma during childhood. Our results show that the identified novel heterozygous deletion in the CDKN1B 5'-UTR region associates with a reduction in CDKN1B mRNA levels, a predicted altered secondary mRNA structure, and a reduced CDKN1B 5'-UTR transcriptional activity in vitro. The patient displayed loss of heterozygosity in the same CDKN1B 5'-UTR region at tissue level and the 5'UTR region containing the deleted sequence encompasses a GRE. These findings indicate that the identification of functional alterations of newly discovered genetic derangements need to be fully characterized and always correlated with the clinical manifestations.
Response to Reviewers:	Reviewer #2: I still have a single point: Concerning citation # 6 (Occhi, G., Trivellin, G., Ceccato, F., De Lazzari, P., Giorgi, G., Dematté, S., Grimaldi, F., Castello, R., Davì, MV., Arnaldi, G., Salviati, L., Opocher, G., Mantero, F., Scaroni, C.: Prevalence of AIP mutations in a large series of sporadic Italian acromegalic patients and evaluation of CDKN1B status in acromegalic patients with multiple endocrine neoplasia. Eur J Endocrinol. 163(3), 369-376 (2010)) does not report a variation in the 5'UTR region in a patient with acromegaly. This is done by

	another work of the same group you refferred in #9. So at the bottom of page 2 you should just include reference 9. The sentence should therefore become "The latter has been found as carrying different variants in patients with acromegaly [9]." We agree with this change, that has been performed in the revised version of our manuscript.

Early onset acromegaly associated with a novel deletion in *CDKN1B* 5'UTR region

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ABTRACT

Genetic alterations frequently are involved in the development of a pituitary adenoma in young age. We here characterize the functional role of a deletion in *CDKN1B* 5'-UTR region (c.-29_-26delAGAG) identified in an acromegalic patient that developed a growth hormone (GH) pituitary adenoma during childhood. Our results show that the identified novel heterozygous deletion in the *CDKN1B* 5'-UTR region associates with a reduction in *CDKN1B* mRNA levels, a predicted altered secondary mRNA structure, and a reduced *CDKN1B* 5'-UTR transcriptional activity in vitro. The patient displayed loss of heterozygosity in the same *CDKN1B* 5'-UTR region at tissue level and the 5'UTR region containing the deleted sequence encompasses a GRE. These findings indicate that the identification of functional alterations of newly discovered genetic derangements need to be fully characterized and always correlated with the clinical manifestations.

INTRODUCTION

Pituitary adenomas are the most frequent intracranial tumors with a prevalence of 77 – 94 cases/100.000 inhabitants, equally distributed among sexes and ages [1, 2]. The development of a pituitary adenoma in young age is frequently associated with genetic alterations, including multiple endocrine neoplasia type 1 (MEN1), Carney complex (CNC), familial isolated pituitary adenomas (FIPA), and MEN type 4 (MEN4). The latter is caused by mutations in the Cyclin Dependent Kinase Inhibitor 1B (*CDKN1B*) gene, encoding for the CDK inhibitor $p27^{Kip1}$[3, 4], that are rare in

the settings of FIPA [5, 6], but cause MEN4 [3]. Mutations in the *CDKN1B* gene are associated with the development of multiple endocrine tumors, displaying highly variable phenotypes that share features with both MEN1 and MEN2 syndromes in rats and show a MEN1-like phenotype in humans [4, 7]. Different types of pituitary adenomas have been described in MEN4 patients, including GH-secreting [3, 8, 9], ACTH-secreting [8, 10] and non functioning pituitary adenomas [11]. On the contrary, *CDKN1B* mutations/rearrangements have not been reported in sporadic settings [6, 8, 12].

We here present a patient with a recurrent GH-secreting pituitary adenoma, which occurred at a very young age, and whose putative genetic causes have been intensively investigated.

The patient, a 30-year-old woman, came to our observation due to previous diagnosis of acromegaly. At the age of 5 years the patient presented with excessive growth velocity and therefore underwent clinical investigation that disclosed GH hypersecretion due to a pituitary adenoma. At the age of 6 years the patient underwent trans-nasal-sphenoidal adenomectomy with histological diagnosis of pituitary macroadenoma with positive immunohistochemistry for GH and a Ki-67<1%. Despite the lack of pituitary adenoma recurrence, IGF-1 levels were high and therefore Bromocriptine therapy was started. Biochemical control was not achieved, and medical therapy was changed to somatostatin analog (SSA) s.c. without benefit. Therefore, at the age of 7 years, the patient underwent external fractionated radiotherapy (29 sessions with total administered dose of 4640 Gy). Nevertheless, high GH and IGF-1 levels persisted and therapy with SSA was started again. Since then, the patient had been treated in the last 20 years with SSA due to the lack of GH suppression under glucose load and to increased IGF-1 levels when medical therapy was withdrawn. The patient developed gallbladder stones and central hypothyroidism.

Because of the young age at disease onset, a genetic predisposition was hypothesized and the presence of germ-line MEN1 and AIP mutations was investigated, with negative findings. In the search for other possible genetic causes and because of the lack of clinical signs and symptoms of CNC, *CDKN1B* was analyzed for the presence of mutations. We found a c.-29_-26delAGAG in the *CDKN1B* 5'-UTR region of the patient and failed to identify the same variant in 20 normal subjects and in 10 acromegalic patients. This deletion occurs next to a previously described *CDKN1B* deletion in the 5'-UTR region [13]. The latter has been found as carrying different variants in patients with acromegaly [9]. The aim of this study is to characterize the functional role of the c.-29_-26delAGAG in *CDKN1B* 5'-UTR region.

MATERIALS AND METHODS

DNA extraction and sequencing

Genomic DNA was extracted using a QIAamp DNA Mini Kit (Qiagen, Milano, IT). *CDKN1B* gene coding regions and intron-exon boundaries were amplified by Polymerase Chain Reaction (PCR) by using the thermal cycler GeneAmp PCR System 9700 (Life Technologies, Milano, IT), by applying the following thermal cycling conditions: incubation at 96°C for 3 min; 35 cycles at 94°C for 30 s, 66°C for 1 min and 72°C for 1 min with a final extension at 72°C for 7 min. Each reaction mixture was prepared using GOTaq green master mix (Promega, Milano, IT). PCR primers employed to amplify the 5'UTR region (5'UTR p27 for and 5'UTR p27 rev) and the coding sequence (exons 1 – 3) are described in Table 1. Direct sequencing was performed as reported [14].

DNA isolation from formalin-fixed, paraffin-embedded tissue sections

Formalin-fixed, paraffin-embedded (FFPE) tissue sections from the patient's pituitary adenoma were incubated with xylene to dissolve paraffin, and DNA was isolated by using the QIAamp DNA FFPE Tissue Kit procedure (Qiagen).

Prediction of mRNA *CDNK1B* secondary structure

Secondary structure analysis of the 5'-UTR (-575/-1) *CDKN1B* mRNA was performed by using the web application www.rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi.

cDNA synthesis and relative qPCR

RNA was isolated from peripheral blood leukocytes of the patient and of healthy controls. RNA quality and quantity were evaluated by using the Experion automated electrophoresis system (Bio-Rad, Milano, Italy). RNA samples with a RNA Quality Indicator between 9.4 and 10 and a 28S/18S rRNA ratio >1.7 were processed. For cDNA synthesis, RNA was reverse-transcribed using Superscript First Strand Synthesis System for RT-PCR (Life Technologies) with random hexamers, according to the manufacturer's instructions. The amount of *CDKN1B* mRNA was quantified by relative qPCR using Taq Man Gene Expression assay Hs01597588_m1 (Life Technologies). The qPCR assay was performed on 100 ng of reverse transcribed RNA per replicate and each reaction mixture was prepared using 1X TaqMan Gene Expression Master Mix, 1X TaqMan Gene Expression Assay, for a total reaction volume of 20µl. The samples were assessed in triplicate on the 7900HT FAST Real-Time PCR System (Life Technologies) and the program reaction was: 1 cycle at 95°C for 10 min, 40 cycles at 95°C for 15 s and 60°C 1 min. Each qPCR reaction included

a negative control (NTC). The reaction specificity was verified by amplicon sequencing (data not shown).

The best reference genes were chosen by using the GeNorm program, as previously reported [14]. All samples were normalized against four different housekeeping genes: ACTB (β-actin), GUS (β-Glucuronidase), CYC (Cyclophylin) and HuPO (human acidic ribosomal protein). HuPO was used as reference gene (4326314E) [15]. The amplification efficiency (E) was calculated based on the slopes of the standard curves for our sample and reference gene (E= 99%).

The ΔΔCt method and RQ manager 1.2.1 (Life Technologies) were used to calculate the mRNA amounts. Data analysis was performed by SDS 2.4 software (Life Technologies).

Immunohistochemistry

Immunohistochemistry (IHC) was performed using a monoclonal anti-p27 antibody (BD Biosciences, CA, USA), as already reported [3].

Plasmid constructs

The *CDKN1B* 5'UTR region (-821/-1 to the start site of translation) displaying (5'-UTR-DEL) or not (5'-UTR-WT) the deletion (c.-29_-26delAGAG) was cloned into the pCR 2.1 vector (Life Technologies) by using the TA Cloning Kit (Life Technologies). Ligation reaction was performed by using T4 DNA Ligase Express Link (Life Technologies).

DNA was then amplified by PCR using primers containing a BglII and a NcoI sequence (5'UTR p27 BglII and 5'UTR p27 NcoI, see Table 1). After enzymatic digestion with BglII and NcoI restriction enzymes (Euroclone, Milano, IT), the 5'-UTR-WT and 5'-UTR-DEL *CDKN1B* sequences were cloned in the pGL4.10 [luc2] vector (Promega) containing the firefly luciferase reporter gene. The following three constructs were generated: pGL4 basic (promoterless), pGL4-5'-UTR-WT, and pGL4-5'-UTR-DEL.

Cell culture

GH3, MCF-7, AtT-20/D16v-F2 and Hela cells were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA) and cultured as previously described [11, 15, 16]. The human cervix adenocarcinoma cell line, Hela, was cultured in the DMEM high glucose medium (Gibco by Life Technologies) enriched with 10% FBS.

Transfection and luciferase gene reporter assays

GH3, MCF-7, and AtT-20/D16v-F2 cells were transfected with a 100 ng/well pGL4 construct (pGL4 basic, pGL4-5'-UTR-WT, or pGL4-5'-UTR-DEL) by using Lipofectamine LTX PLUS Reagents (Life Technologies). The cells were co-transfected with pRL-TK vector (Promega) in triplicates. Luciferase activity assays were performed by using the Dual-Glo Luciferase Assay System (Promega). The readings were carried out on EnVision Multilabel Reader (Perkin Elmer, Monza, IT).

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were carried out using the SimpleChIP® Enzymatic Chromatin IP Kit Agarose Beads #9002 (Cell Signaling Tecnology, Danvers, Massachusetts, USA) after incubating Hela cells with or without 10^{-6} M Dexamethasone (Sigma-Aldrich, Saint Louis, MO, USA) for 24 h. Primer sequences for amplification of *CDKN1B* 5'-UTR region encompassing the c. -29_-26delAGAG (5'-UTR p27_ChIP for and 5'-UTR p27_ChIP rev) are described in Table 1. The following PCR reaction program was employed: 95 °C for 5 min, 34 cycles at 95 °C for 30 s, 59°C for 30 s and 72°C for 30 s with a final extension at 72°C for 5 min.

Statistical analysis and informed consent

Data were expressed as mean ± standard error of the mean (SEM). We used the paired or unpaired Student t test to evaluate individual differences between means. P values <0.05 were considered significant. Patient gave written informed consent for molecular analysis and data collection.

RESULTS

***CDKN1B* sequencing results**

A germ-line heterozygous deletion in the *CDKN1B* gene 5'-UTR region (c.-29_-26delAGAG) was found in the patient (Figure 1B) and in her mother, who has always been asymptomatic. The father displayed a wild type *CDKN1B* gene 5'-UTR region.

Detection of loss of heterozygosity (LOH) in the patient's pituitary adenoma

The *CDKN1B* 5'-UTR region encompassing the deleted sequence was amplified by PCR from DNA isolated from FFPE pituitary adenoma sections, by using the 5'UTR p27_ChIP for and 5'UTR p27_ChIP rev primers, which amplify a 160 bp fragment. *CDKN1B* 5'UTR region amplification from the DNA of the patient's pituitary adenoma shows a single 156 bp band on a

10% acrylamide gel. On the contrary, we obtained two bands of 160 bp and 156 bp, respectively, from the patient's germ-line DNA, demonstrating LOH in the patient's pituitary adenoma (Online Resource 1). These findings have been confirmed by direct sequencing .

CDKN1B mRNA secondary structure prediction and expression

As shown in Figure 1C, the wild-type *CDKN1B* 5'-UTR region (575 nucleotides) displays a 'stem and loop' secondary structure, that includes the -29/-26 AGAG element. This region contains a 'U-rich' sequence which interacts with mRNA-binding proteins and modulates ribosome recruitment [17-19], being involved in *CDKN1B* mRNA stability regulation. The deleted *CDKN1B* 5'-UTR region has a different predicted secondary structure (Figure 1D) as compared to the wild-type *CDKN1B* 5'-UTR, indicating that the c.-29_-26delAGAG modifies the predicted 'stem and loop' structure of the 5'UTR region.

CDKN1B mRNA expression levels were analyzed by qPCR in peripheral blood T lymphocytes of the patient and we found a 70% reduction in *CDKN1B* mRNA expression as compared to 3 normal unrelated subjects. ($p<0.01$).

$p27^{Kip1}$ expression in patient's pituitary tumor

IHC shows that $p27^{Kip1}$ protein staining is reduced and mainly cytoplasmic in the patient's pituitary adenoma (Figure 1F) as compared to a representative GH-secreting pituitary adenoma tissue from a *CDKN1B* deletion-negative patient (Figure 1E), where $p27^{Kip1}$ protein staining is higher and predominantly nuclear.

Functional characterization of the c.-29_-26delAGAG in *CDKN1B* 5'-UTR region

The luciferase gene reporter assays showed that pGL4-5'-UTR-DEL luciferase activity was significantly reduced in all the evaluated cell lines (GH3, MCF7, AtT-20/D16v-F2) as compared to pGL4-5'-UTR-WT luciferase activity ($p<0.01$) (Online Resource 2).

Identification of a Glucocorticoid response element (GRE) in the *CDKN1B* 5'-UTR region

Glucocorticoids induce $p27^{Kip1}$ protein expression in lymphoid and non-lymphoid cells [20]. To explore the hypothesis that the *CDKN1B* 5'-UTR region encompassing the deleted sequence contains a Glucocorticoid Response Element (GRE) a ChIP assay was performed. We found that the *CDKN1B* 5'-UTR region was amplified from the DNA immunoprecipitated with an anti-GR antibody, both before and after dexamethasone treatment of HeLa cells (Online Resource 3). In addition, the band obtained after PCR from the immunoprecipitated DNA isolated from HeLa cells

treated with dexamethasone is stronger, indicating that treatment with glucocorticoids induces GR-DNA interaction at the investigated *CDKN1B* 5'-UTR region. These results are validated by the presence of adequate positive controls, such as a good quality chromatine (input sample), correctly immunoprecipitated by the Anti-H3 antibody, but not by an anti-rabbit antibody. The positive controls are not modified by treatment with dexamethasone, indicating that the employed experimental conditions guarantee the success of immunoprecipitation.

DISCUSSION

Our study underlines the importance of evaluating the functional effects of genetic alterations identified in light of specific clinical features in a case of recurrent GH-secreting pituitary adenoma, which occurred at a very young age.

Indeed, our results show that the identified novel heterozygous deletion in the *CDKN1B* 5'-UTR region, consisting of c.-29_-26delAGAG, causes a reduction in *CDKN1B* mRNA levels, a predicted altered secondary mRNA structure, and a reduced *CDKN1B* 5'UTR region transcriptional activity in vitro. We also found a LOH in the same *CDKN1B* 5'-UTR region at tissue level, possibly accounting for the reduced $p27^{Kip1}$ protein levels in the pituitary adenoma of the affected patient and the lack of clinical manifestations in the patient's mother, who displayed the same germ-line deletion in heterozygosity. Moreover, the 5'UTR region containing the deleted sequence encompasses a GRE.

Our findings strengthen the association of *CDKN1B* 5'UTR variants with a peculiar clinical phenotype, i.e. GH-secreting pituitary adenomas, as previously reported [6, 9]. Furthermore, a recent study identified a GAGA (-32/-29) germline heterozygous deletion in the *CDKN1B* 5'-UTR in a patient affected by gastric carcinoid tumor and hyperparathyroidism, providing evidence that this germline deletion alters *CDKN1B* transcription and *CDKN1B* mRNA levels [13], similarly to what here reported. Indeed, we found a 26-68% reduction in the transcriptional activity of the deleted *CDKN1B* 5'UTR region in different cell lines. These results show that the reduction in *CDKN1B* 5'UTR region transcriptional activity is not tissue-specific, since it has been found in cell lines originating from different tissues and species. In addition, we found a 70% reduction in *CDKN1B* mRNA expression in the patient's leukocytes as compared to normal controls. The latter finding, however, could also be due to mRNA decay of the c.-29_-26delAGAG carrier allele in vivo. All together, our results and those from Malanga et al. suggest that the *CDKN1B* 5'UTR region from nucleotide -32 to nucleotide -26 with respect to the translation start site is relevant for the regulation of *CDKN1B* transcription. These results are in keeping with the finding that the human *CDKN1B* 5'-UTR contains a U-rich element involved in regulating *CDKN1B* mRNA

stability and translation efficacy [17-19]. And indeed the identified c.-29_-26delAGAG modifies the predicted *CDKN1B* mRNA 'stem and loop' secondary structure, possibly impairing ribosome entry and subsequent mRNA transcription. In addition, $p27^{Kip1}$ protein levels are reduced and the protein is mainly cytoplasmic in the patient's pituitary adenoma as compared to a GH-secreting pituitary adenoma from a patient lacking the c.-29_-26delAGAG. It has been previously reported that, when in the nucleus, $p27^{Kip1}$ binds and inhibits cyclin/CDK complexes, acting as a cell cycle inhibitor [7]. On the contrary, there is evidence that a cytoplasmic localization impairs its function as a cell cycle inhibitor and correlates with high tumor grade [21]. Therefore, the finding that in our patient's pituitary adenoma $p27^{Kip1}$ is mainly cytoplasmic, together with a reduced protein amount, suggests that this protein might exert a reduced oncosuppressor function. These findings are in keeping with the clinical aggressiveness of the GH pituitary adenoma in this patient, who needed to undergo two surgeries and external radiation therapy without reaching disease control.

It has been previously demonstrated that glucocorticoids induce $p27^{Kip1}$ protein expression in lymphoid and non-lymphoid cells [22], indicating that the *CDKN1B* 5'-UTR region encompasses a GRE. Indeed, we could amplify the *CDKN1B* 5'-UTR region from DNA immunoprecipitated with an anti-GR antibody in a ChIP assay. The immunoprecipitated DNA region contains the -29/-26 AGAG sequence, that was found to be deleted in our patient. These results provide indirect evidence as to the presence of a GRE in the deletion site, suggesting that the deleted *CDKN1B* 5'-UTR region might be less responsive to glucocorticoids, with further possible functional consequences.

These findings indicate that the identification of functional alterations of newly discovered genetic derangements need to be fully characterized and always correlated with the clinical manifestations. However, the presence of other mutations (somatic or germline) cannot be excluded, possibly contributing to the development of an aggressive and early onset acromegaly in our patient.

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CONFLICT OF INTERESTS

EdU received consulting fees from Novartis and Pfizer. MCZ received consulting fees from Novartis and Genzyme. The other Authors have nothing to disclose and have no conflict of interest.

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LEGENDS TO FIGURES

Figure 1: **Germline mutation in patient's *CDKN1B* gene, *CDKN1B* mRNA secondary structure**

A) Wild-type sequence (Wild type) of the *CDKN1B* 5'-UTR region with the -29/-26 AGAG nucleotides underlined.

B) Deleted sequence (Deleted) of the *CDKN1B* 5'-UTR region with the c.-29_-26delAGAG. (red box).

C) Predicted secondary structure of the wild type 5'-UTR (-575/-1) *CDKN1B* mRNA performed by using the web application: www.rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi.

D) Predicted secondary structure of the deleted 5'-UTR (-575/-1) *CDKN1B* mRNA.

E) Immunohistochemical staining for $p27^{Kip1}$ in a representative GH-secreting pituitary adenoma tissue from a *CDKN1B* deletion-negative patient, showing prevalent nuclear $p27^{Kip1}$ immunoreactivity.

F) Immunohistochemical staining for $p27^{Kip1}$ in the patient's pituitary adenoma, showing prevalent cytoplasmic $p27^{Kip1}$ distribution.

Online Resource 1: Detection of loss of heterozygosity (LOH) in the patient's pituitary adenoma

Somatic DNA from the patient's pituitary adenoma was amplified for the *CDKN1B* 5'-UTR region encompassing the deleted sequence and has been separated on a 10% acrylamide gel.

M = DNA Molecular Weight Marker VIII (Roche, Milano, IT). # = germline DNA amplification for *MEN1* exon 7 gene from the DNA isolated form the patient's pituitary adenoma. + = positive control for *CDKN1B* 5'-UTR region amplification from germ-line DNA (unaffected patient). B = *CDKN1B* 5'-UTR region amplification from the patient's germ-line DNA. PA = *CDKN1B* 5'-UTR region amplification from the DNA isolated form the pituitary adenoma of our patient. NTC = no template control.

Online Resource 2: Functional characterization of the c.-29_-26delAGAG in *CDKN1B* 5'-UTR region

Luciferase gene reporter assay was performed with three cells lines (GH3, MCF7, and AtT-20/D16v-F2) transfected with either pGL4 basic (promoterless, grey bars), pGL4-5'-UTR-WT (white bars), or pGL4-5'-UTR-DEL (black bars). Data represent the mean of three independent experiments and are expressed as mean ± SEM luciferase activity % vs. pGL45'-UTR-WT luciferase activity. **p<0.01 vs. pGL45'-UTR-WT luciferase activity.

Online Resource 3: ChIP assay

HeLa cells were treated without (control) or with 10^{-6}M Dexamethasone (+ DEX) and chromatin extracts were immunoprecipitated with specific antibodies: normal Rabbit IgG antibody (Anti-Rabbit Ab, negative control), anti-acetyl-histone H3 antibody (Anti- H3 Ab, positive control), Glucocorticoid Receptor antibody (Anti-GR Ab). Input: input sample, resenting a control for chromatin loading. Purified DNA was analyzed by standard PCR as described in the Materials and methods section. M = DNA Molecular Weight Marker VIII. NTC = no template control.

Figure
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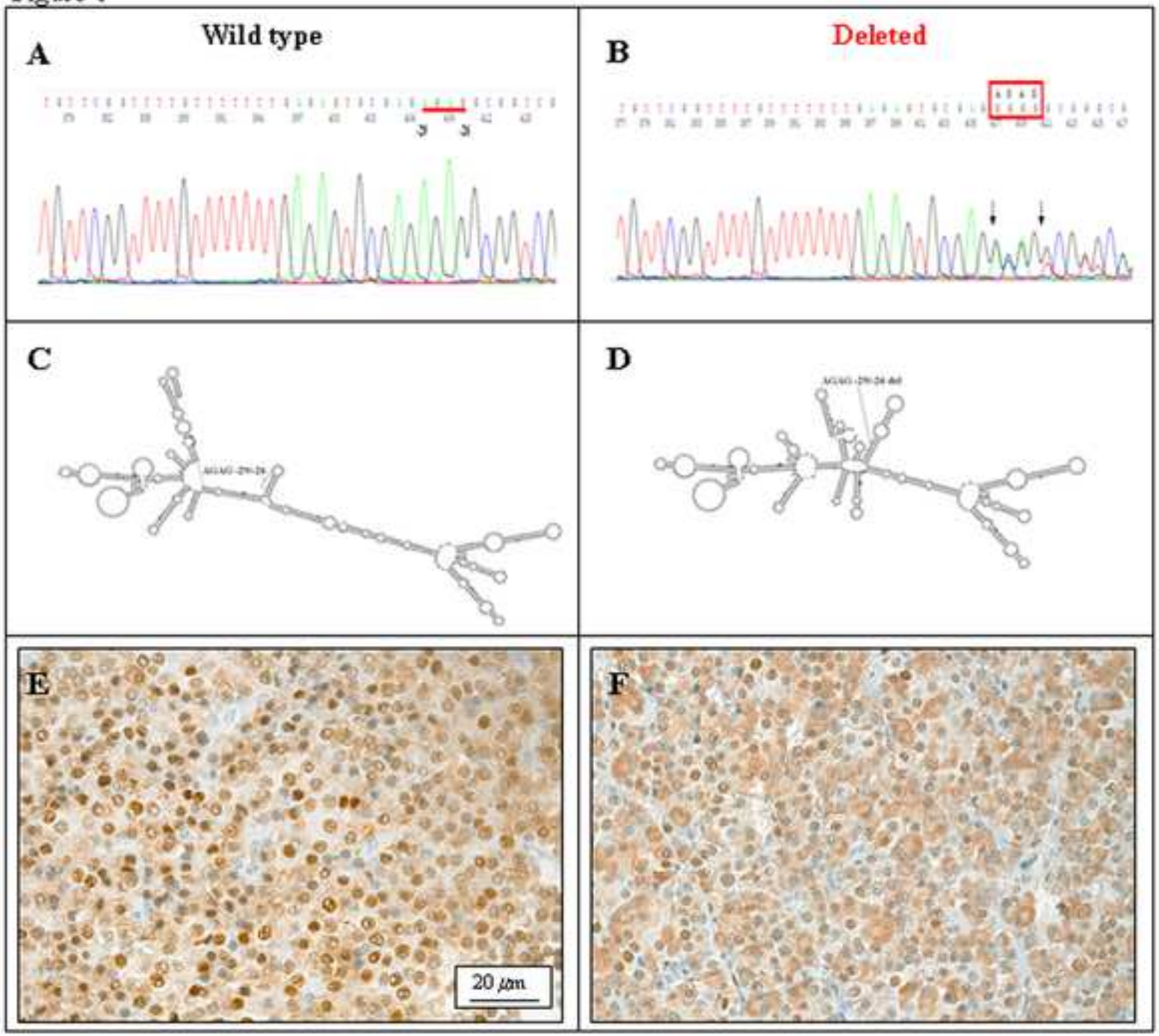
Figure 1



Table

Table 1: Primers employed to amplify *CDKN1B* promoter and coding regions

Name	**Sequence**
5'UTR p27_ChIP for	5'- TGTGTCTTTTGGCTCCGAGG - 3'
5'UTR p27_ChIP rev	5'- CTCCCGTTAGACACTCGCAC - 3'
5'UTR p27 for	5'- GAG GAG CGG GAG GGA GGT CG - 3'
5'UTR p27 rev	5'- CTAGGGCTCCCGTTAGACACT - 3'
p27ex1A for	5'- CGTCAGCCTCCCTTCCACCG - 3'
p27ex1A rev	5'- CTCTTCGTGGTCCACCGGGC - 3'
p27ex1B for	5'- GAGCCCTAGCCTGGAGCGGAT - 3'
p27ex1B rev	5'- GCGGGGCCCCAAACACATTCT - 3'
p27ex2 for	5'- CTGACTATGGGGCCAACTTC - 3'
p27ex2 rev	5'- GCCAGCAACCAGTAAGATCAG - 3'
p27ex3A for	5'- TGAACACTGGCTAAAGATAATTGCTATTTA - 3'
p27ex3A rev	5'- TGCCAGGTCAAATACCTTGTTTG - 3'
p27ex3B for	5'- GACCAAAGAACACAGCACAGAGGA - 3'
p27ex3B rev	5'- CTGGGGAGGGCAGTGAGGAT - 3'
5'UTR p27 BglII	5' GAG GAG CGG GAG GGA GAT CTG GGC TT 3'
5'UTR p27 NcoI	5' GAC ACT CGC ACG TTT GCC ATG GTT CTC 3'

Supplementary Material

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