



Multiple Endocrine Neoplasia Type 1 (*MEN1*) 5'UTR Deletion, in *MEN1* Family, Decreases Menin Expression

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ABSTRACT

Multiple endocrine neoplasia type 1 (*MEN1*) is an autosomal dominant disorder characterized by the occurrence of parathyroid, pancreatic and pituitary tumors, and is due to mutations in the coding region of the *MEN1* gene, which encodes menin. We investigated a family with identical twins that had *MEN1*, with different *MEN1* tumors. DNA sequence analysis of the *MEN1* coding region had not identified any abnormalities and we hypothesized that deletions and mutations involving the untranslated regions may be involved. Informed consent and venous blood samples were obtained from five family members. Sanger DNA sequencing and multiplex ligation-dependent probe amplification (MLPA) analyses were performed using leukocyte DNA. This revealed a heterozygous 596bp deletion ($\Delta 596\text{bp}$) between nucleotides –1087 and –492 upstream of the translation start site, located within the *MEN1* 5' untranslated region (UTR), and includes the core promoter and multiple cis-regulatory regions. To investigate the effects of this 5'UTR deletion on *MEN1* promoter activity, we generated luciferase reporter constructs, containing either wild-type 842bp or mutant 246bp *MEN1* promoter, and transfected them into human embryonic kidney HEK293 and pancreatic neuroendocrine tumor BON-1 cells. This revealed the $\Delta 596\text{bp}$ mutation to result in significant reductions by 37-fold ($p < 0.0001$) and 16-fold ($p < 0.0001$) in luciferase expression in HEK293 and BON-1 cells, respectively, compared to wild-type. The effects of this 5'UTR deletion on *MEN1* transcription and translation were assessed using qRT-PCR and Western blot analyses, respectively, of mRNA and protein lysates obtained from Epstein-Barr-virus transformed lymphoblastoid cells derived from affected and unaffected individuals. This demonstrated the $\Delta 596\text{bp}$ mutation to result in significant reductions of 84% ($p < 0.05$) and 88% ($p < 0.05$) in *MEN1* mRNA and menin protein, respectively, compared to unaffected individuals. Thus, our results report the first germline *MEN1* 5'UTR mutation and highlight the importance of investigating UTRs in *MEN1* patients who do not have coding region mutations. © 2020 The Authors. *Journal of Bone and Mineral Research* published by Wiley Periodicals LLC on behalf of American Society for Bone and Mineral Research (ASBMR).

KEY WORDS: GENETIC ANALYSIS; MONOZYGOTIC TWINS; NEUROENDOCRINE; PARATHYROID-RELATED DISORDERS; PROMOTER ACTIVITY

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Introduction

Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant disorder characterized by the combined occurrence of parathyroid, pancreatic and pituitary tumors.^(1,2) The majority (>95%) of MEN1 patients will develop parathyroid tumors, 35% to 75% of patients will develop neuroendocrine tumors (NETs) of the pancreas (PNETs), <30% will have pituitary adenomas, and <25% will develop other endocrine tumors such as adrenal adenomas and carcinomas, and NETs of the thymus and lungs.⁽¹⁾

The majority (>90%) of MEN1 patients have germline mutations affecting the coding region of the *MEN1* gene^(2–4) (Fig. 1), which is located on chromosome 11q13.⁽⁵⁾ The *MEN1* gene spans 9 kilobases (kb) of genomic DNA that contain the 10 exons which encode a ubiquitously expressed 610 amino acid tumor suppressor protein, menin (NM_130799.2). The coding region of the *MEN1* gene spans exons 2 to 10, while exon 1, the 5' region upstream of exon 2 and the 3' region of exon 10 are non-coding⁽⁶⁾ (Fig. 1A). The 1.4 kb region upstream of exon 2 has been reported to have strong promoter activity⁽⁷⁾ (Supplemental Fig. 1). More than 1800 germline and somatic *MEN1* mutations have been reported in both familial and sporadic cases of MEN1,^(3,4,8) and these are scattered throughout the nine coding exons of the *MEN1* gene and show no genotype-phenotype correlation.^(2,4,8) Approximately 25% of the mutations are nonsense mutations, 40% are frameshift mutations, 20% are missense mutations, 10% are splice-site mutations,^(3,4) and 5% are rarer mutations, which include partial^(9–11) or complete deletions^(12,13) of the *MEN1* gene. Frameshift, nonsense, and splice-site mutations are predicted to result in a truncated menin protein that lacks functional domains, or complete loss of protein due to nonsense mediated mRNA decay.⁽⁴⁾ In contrast, missense mutations

may affect functionally important amino acid residues involved in protein interactions, transcriptional activity, or protein stability.^(4,8) To date, all reported mutations affect the 1830 bp protein-coding region.^(4,8) However, 5% to 10% of MEN1 patients do not have mutations within the coding region of the *MEN1* gene,^(3,4,8) suggesting that these patients may harbor mutations in the untranslated regions (UTRs) of the *MEN1* gene or they may represent phenocopies with mutations of other genes.⁽¹⁴⁾

Here, we report a deletion involving the minimal core promoter region within the *MEN1* 5'UTR (Fig. 1B) in a MEN1 family with identical twins, who had phenotypic differences⁽¹⁵⁾ (Fig. 2A).

Patients and Methods

Patients

The clinical details of the family, F/92 (Fig. 2A), with MEN1 have been previously reported.⁽¹⁵⁾ Briefly, the clinical findings of the three affected members were as follows. The father (I.1) of the identical twins (II.1 and II.2) had parathyroid adenomas, PNETs and, unusually for MEN1, a pheochromocytoma.⁽¹⁵⁾ One of the twins (II.1) was reported to have primary hyperparathyroidism (PHPT), due to parathyroid adenomas, at 16 years of age, and the other twin (II.2), who had a prolactinoma and presented with delayed puberty at the age of 15 years, was diagnosed to have PHPT at the age of 16 years.⁽¹⁵⁾ During the next 25 years, the clinical history of these twins has been as follows. Twin II.1 had a parathyroidectomy with autotransplantation of parathyroids into the forearm at age of 22 years. He had recurrent PHPT that required surgery four times for removal of parathyroid tumors in the neck and the transplanted parathyroids in the forearm,

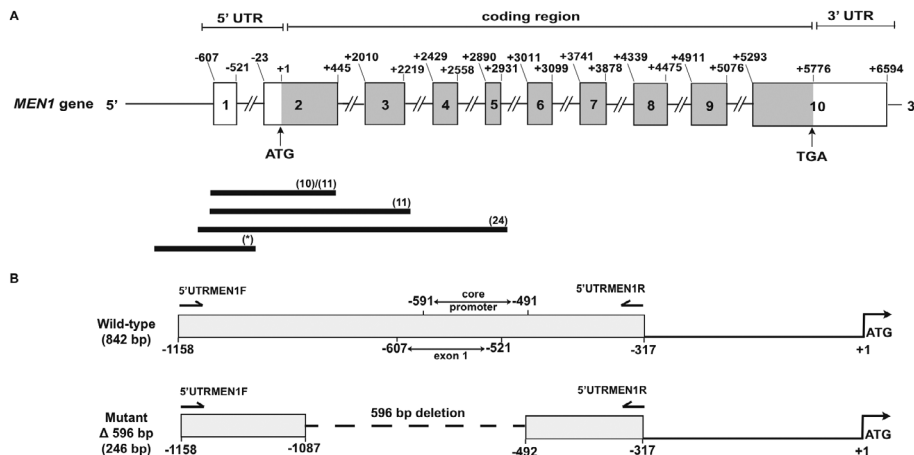
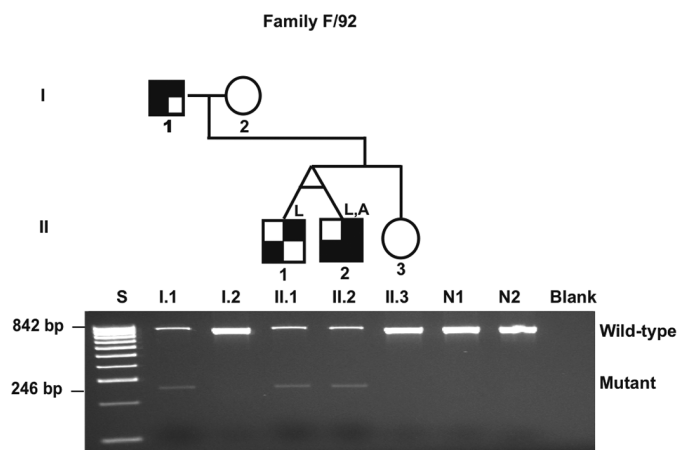


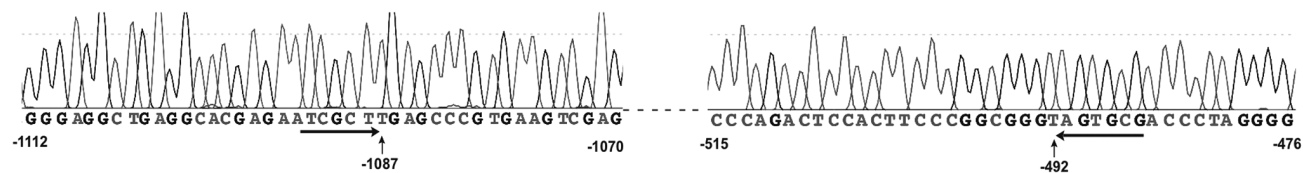
Fig 1. Schematic representation of the *MEN1* gene and *MEN1* 5'UTR. (A) Schematic representation of the *MEN1* gene (*MEN1* reference sequence ENST00000312049.10) which consists of 10 exons (represented by boxes). The introns are represented by broken lines connecting the boxes. The coding region (gray) of the *MEN1* gene spans exons 2 to 10 while the 5' region upstream of exon 2, exon 1 and the 3' region of exon 10 are non-coding (white). The nucleotide positions of the translation start site (ATG), the translation stop site (TGA) and the intron-exon boundaries are indicated relative to the translation start site. Previously reported large germline deletions of the *MEN1* gene that affect the protein-coding and promoter regions, including the 5'UTR and the translation start-site (ATG), are shown underneath by black lines with the references reporting these shown in parenthesis and the asterisk refers to this report.^(10,11,24) (B) Schematic representation of the wild-type 842 bp *MEN1* 5'UTR from nucleotides –1158 to –317 (shown as a gray box) upstream of the translation start site (ATG), and the mutant 246 bp *MEN1* 5'UTR which lacks 596 bp from nucleotides –1087 to –492 (represented by a broken line). The positions of the oligonucleotide primers 5'UTRMEN1F and 5'UTRMEN1R, exon 1, the minimal core promoter and the translation start site are shown.

A



B

Unaffected showing wild-type *MEN1* 5'UTR



Affected showing mutant *MEN1* 5'UTR

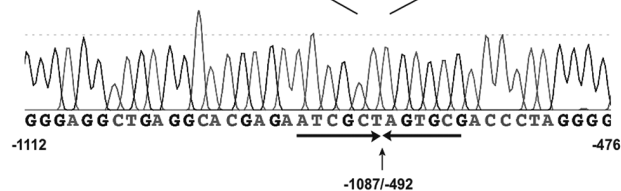


Fig 2. Identification of a 596 bp deletion in the *MEN1* 5'UTR in an *MEN1* kindred. (A) Family F/92 with *MEN1*. Upper panel shows the clinical features. Squares-males; circles-females; open symbols-unaffected; filled upper right quadrant-primary hyperparathyroidism (parathyroid tumors); filled lower right quadrant-pituitary tumors; filled lower left quadrant-PNETs; filled upper left quadrant-other tumors; L-lipoma; and A-angiofibroma. Some of the clinical details of the family have been previously reported.⁽¹⁵⁾ Individual I.1 also had a phaeochromocytoma, which are reported to occur in <1% of *MEN1* patients.⁽¹⁾ Lower panel shows the results of PCR amplification of leukocyte DNA using oligonucleotide primers 5'UTRMEN1F and 5'UTRMEN1R (Fig. 1B), which yielded a 842 bp wild-type fragment in two unaffected members of the family (I.2 and II.3) and two unrelated normal controls (N1 and N2), but an additional smaller 246 bp fragment which co-segregated with *MEN1* in the three affected individuals (I.1, II.1 and II.2). S: 100-bp DNA marker ladder. (B) DNA sequence chromatograms of the proximal (-1112 to -1070) and distal (-515 to -476) regions adjacent to the 596 bp deletion are indicated relative to the translation start site in an unaffected (I.2) and affected (I.1) individual, showing the wild-type *MEN1* 5'UTR and the mutant *MEN1* 5'UTR, respectively.

between ages 27 and 41 years, after which he became hypoparathyroid and required treatment with oral alfacalcidol. At the age of 25 years he was found to have a 9 mm PNET and at 30 years of age he had hypoglycaemia due to an insulinoma that was treated by a partial pancreatectomy. There has been no recurrence of the hypoglycemia but he has developed multiple pancreatic microadenomata of ~3 mm size. Pituitary imaging studies indicate that he has not developed a pituitary tumor, and his pituitary hormones remain within the normal range. He developed a lipoma at the age of 28 years, which was excised.

In the other twin (II.2), the prolactinoma was treated with cabergoline and the PHPT by partial parathyroidectomy and with autotransplantation of parathyroids into the forearm, at 24 years of age. He suffered from recurrent PHPT that required parathyroidectomy and three further neck explorations between the ages of 30 and 40 years, following which he is normocalcemic. His only medication is cabergoline and he is normo-prolactemic. At the ages of: 34 years, he was found to have a 7 mm non-functioning PNET at 34 years; 36 years a facial angiofibroma; and at 39 years had excision of an abdominal lipoma. He has not had

pancreatic surgery and remains well. Blood samples for DNA sequence analysis of the *MEN1* gene were obtained from these three affected members (I.1, II.1 and II.2; Fig. 2A) and two unaffected relatives (I.2 and II.3; Fig. 2A). Informed consent was obtained from individuals, using protocols approved by the local and national ethics committees (MREC/02/2/93).

Genetic analysis

Leukocyte DNA was extracted and used to amplify by the polymerase chain reaction (PCR) the coding regions and intron-exon boundaries of the *MEN1* gene, using 15 primer pairs that span the nine coding exons of the *MEN1* gene and the 18 intron/exon boundaries utilizing conditions, as described.⁽¹⁴⁾ DNA sequence analysis of the gel-purified PCR products was performed using the BigDye Terminator Cycle Sequencing Kit (Life Technologies, Paisley, UK) and an ABI automated capillary sequencer (Applied Biosystems, Loughborough, UK), as described.⁽¹⁶⁾ Sequences were compared with the *MEN1* Ensembl reference sequence ENSG00000133895. Multiplex ligation-dependent probe amplification (MLPA) analysis was used to analyze variations in *MEN1* gene copy number. An MLPA kit P244-C1 (MRC Holland, Amsterdam, The Netherlands) was used to detect deletions or duplications of exons of *MEN1*, as reported.⁽¹⁷⁾ The DNA sequence of the 5'UTR of the *MEN1* gene was determined using specific PCR primers (forward, 5'UTRMEN1F: 5'-AAAATTAGGC GGGAGTGGTG-3'; and reverse 5'UTRMEN1R: 5'-CCGGGGC TGTAAGTCTGT-3') (Sigma-Aldrich, Cambridge, UK) (Fig. 1B; Supplemental Fig. 1). The frequency of the *MEN1* 5'UTR deletion was assessed in: 1284 patients who were clinically diagnosed to have MEN1; and in 74,180 participants, who comprised affected and unaffected family members from all diagnostic categories within the rare disease arm of the 100,000 (100K) Genomes Project (100KGP). Data from the 100KGP were accessed using a custom shell script and bcftools (v1.9). The set of 74,180 structural variant call files (vcf), which had been called by a combination of Manta and Canvas algorithms, were filtered and scanned for deletions that intersect *MEN1* and the immediately upstream region (chr11:64,803,000–64,814,000, B38).

Cell lines

Human embryonic kidney HEK293 cells (CRL-1573, obtained from ATCC (LGC Standards, Middlesex, UK) were maintained in Dulbecco's Modified Eagle medium: nutrient mixture F-12 with GlutaMax (DMEM/F-12, Glutamax) (Life Technologies), supplemented with 10% heat-inactivated fetal calf serum (FCS), as described.⁽¹⁶⁾ Human metastatic pancreatic neuroendocrine tumor (BON-1) cells were maintained in Dulbecco's Modified Eagle medium: nutrient mixture F-12 (DMEM/F-12) (Life Technologies), supplemented with 10% heat-inactivated FCS, as described.⁽¹⁸⁾ Epstein-Barr-virus (EBV) transformed lymphoblastoid cells were established and maintained for eight or fewer passages in Roswell Park Memorial Institute (RPMI) 1640 medium (Life Technologies), supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 20% heat-inactivated FCS, as described.⁽¹⁹⁾ All cells were incubated at 37°C, 5% CO₂ and 95% humidity.

Dual luciferase reporter activity assays

Luciferase reporter constructs, containing either wild-type or mutant *MEN1* 5'UTR, were cloned into the pGL4.10 vector (Promega, Southampton, UK), which contains the Firefly luciferase reporter gene (LUC).⁽²⁰⁾ BON-1 and HEK293 cells, at 50% to

70% confluence, were transiently transfected with 0.5 µg/well of luciferase reporter constructs and 0.05 µg/well of *Renilla* luciferase (pRL-TK) co-reporter vector (Promega) using Lipofectamine 2000 reagent (Invitrogen, Leicester, UK), as reported.⁽²⁰⁾ Cells were lysed after 48 hours and luciferase reporter activity assays were performed using the Dual-Luciferase Reporter Assay System (Promega) and a Veritas Microplate Luminometer (Turner BioSystems, Southampton, UK), as previously described.⁽²⁰⁾ Firefly luciferase reporter activity was normalized against *Renilla* luciferase expression.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from EBV transformed lymphoblastoid cells using the RNeasy Mini Kit (Qiagen, Manchester, UK), as previously reported.⁽¹⁹⁾ RNA concentration and quality was determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Loughborough, UK), and RNA integrity determined by agarose gel electrophoresis. Up to 1 µg of total RNA was converted to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen), as previously reported.⁽²⁰⁾ qRT-PCR reactions were performed using QuantiTect *MEN1* and *GAPDH* primer assays (Qiagen) and QuantiTect SYBR Green PCR Kit (Qiagen), on a Rotor-Gene Q Cycler (Qiagen), as previously described.⁽²⁰⁾ The relative expression of the target *MEN1* cDNA, normalized to the housekeeper *GAPDH* mRNA, was determined using described methods.⁽²¹⁾

Western blot

Total protein was extracted from EBV transformed lymphoblastoid cells using 500 µL of ice-cold NP40 lysis buffer (150mM NaCl, 50mM Tris (pH 8.0); 1% Triton X-100 [vol/vol], and 1× Protease inhibitor tablet (Roche, Hertfordshire, UK), maintained in constant agitation at 4°C for 30 min, centrifuged for 10 min at 10,000 rpm (7840 g) and the supernatant collected. Protein concentration was determined using the Bradford assay, as previously described.⁽²⁰⁾ Protein samples were prepared in 4× Laemmli loading dye (BioRad, Hertfordshire, UK), boiled at 95°C for 5 min and resolved using SDS-PAGE gel electrophoresis. Samples were transferred onto PVDF membrane (PerkinElmer, Cambridge, UK), blocked in 5% non-fat dried milk powder (commercially available as Marvel) dissolved in PBS and incubated with 1:1000 rabbit anti-menin antibody (AB-303203; Abcam, Cambridge, UK) or 1:2500 rabbit anti-GAPDH (AB-9485; Abcam) in 5% milk/PBS-T overnight. Membranes were subsequently incubated with anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies (SC-2004; Santa Cruz Biotechnology, Dallas, TX, USA) and visualized using ECL Western blotting substrate (BioRad) on a Chemidoc XRS+ system (BioRad) and densitometry analysis performed using Image J software (NIH, Bethesda, MD, USA; <https://imagej.nih.gov/ij/>), as previously described.⁽²⁰⁾

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM) and statistical analysis was performed using one-way ANOVA in which the Bonferroni correction for multiple testing was applied.⁽²⁰⁾ A value of $p < 0.05$ was considered significant for all analyses.

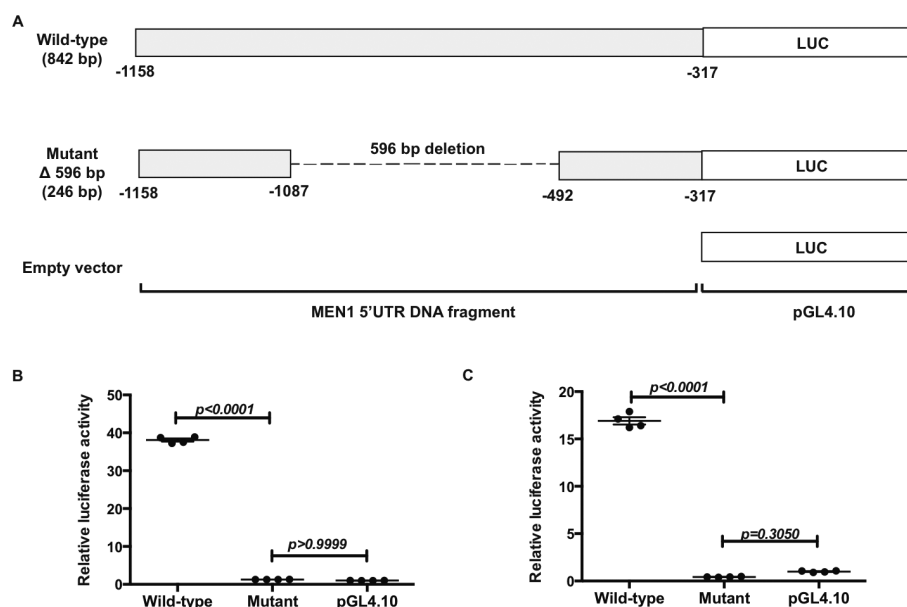


Fig 3. Effects of the 5'UTR Δ 596bp deletion on *MEN1* promoter-driven luciferase activity. (A) Schematic representation of luciferase reporter constructs containing either the wild-type 842 bp *MEN1* 5'UTR or the mutant Δ 596bp *MEN1* 5'UTR cloned into the pGL4.10 vector, upstream of the Firefly luciferase reporter gene (LUC). The empty promoter-less pGL4.10 luciferase reporter vector was included as a negative control and was used to normalize luciferase expressions. (B,C) Relative luciferase expression in cells following transient transfection with either wild-type or mutant Δ 596bp *MEN1* promoter-driven luciferase reporter constructs. (B) HEK293 cells. (C) BON-1 cells. The 596 bp deletion in the *MEN1* promoter caused a reduction in luciferase reporter expression *in vitro*. Data are represented as mean \pm SEM, $n = 4$.

Results

Identification of a 596 bp deletion in the *MEN1* 5' UTR

Mutational analysis of leukocyte DNA from individual II.2, using conventional Sanger DNA sequencing of the *MEN1* coding region (exons 2 to 10) and intron/exon boundaries, did not identify any pathogenic variants. However, a heterozygous deletion of two probe sites within the 5'UTR of the *MEN1* gene was identified using MLPA gene copy number analysis (Supplemental Fig. 1). The presence of this deletion was confirmed using PCR amplification across the two MLPA probe sites (Fig. 1B; Supplemental Fig. 1), which produced a 842 bp wild-type fragment and 246 bp mutant fragment. Only the 842 bp wild-type fragment was observed in the two unaffected members of the family (I.2 and II.3) and two unrelated normal controls (N1 and N2) (Fig. 2A). In contrast, the 246 bp mutant fragment was observed, and co-segregated with *MEN1* in the three affected individuals (I.1, II.1 and II.2) from the family (Fig. 2A). Sanger DNA sequencing of this region of the *MEN1* 5'UTR further confirmed the deletion to be 596 bp in size, and to be located between nucleotides -1087 and -492 upstream of the *MEN1* gene translation start site (c. $-590_{-24}+29$ del; Fig. 2B). In addition, this deleted region contained the whole of exon 1 (nucleotides -607 to -521 ; c. -110_{-24}), the minimal core promoter region (nucleotides -591 to -491 ; c. $-94_{-24}+30$) and multiple cis-regulatory and initiator elements (located between nucleotides -700 to -592 ; c. -203_{-95}) (Fig. 1B; Supplemental Fig. 1). This 5'UTR 596 bp deletion was not found in 75,464 individuals, which comprised 74,180 participants of the 100KGP, 135 *MEN1* patients who had pathogenic *MEN1* variants, and 1149 patients with *MEN1*-associated tumors, thereby

demonstrating an allele frequency of 0% for this deletion in these cohorts.

The 596 bp deletion in the *MEN1* 5'UTR reduces *MEN1* promoter activity *in vitro*

The 596 bp deleted region (Δ 596bp) of the *MEN1* 5'UTR contained the minimal core promoter and multiple regulatory and initiator elements (Supplemental Fig. 1), and we therefore examined its likely functional effects on *MEN1* promoter activity using *in vitro* luciferase assays by transient expression of wild-type *MEN1* 5'UTR or mutant Δ 596bp *MEN1* 5'UTR luciferase reporter constructs (Fig. 3A) in HEK293 and BON-1 cells. Luciferase expression from the mutant Δ 596bp *MEN1* 5'UTR luciferase construct was 37-fold lower ($p < 0.0001$) than the wild-type *MEN1* 5'UTR luciferase construct in HEK293 cells, but was not significantly different to the control, promoter-less pGL4.10 empty vector (wild-type = 38.09 ± 0.42 ; mutant = 1.28 ± 0.01 ; pGL4.10 = 1.00 ± 0.01 ; $n = 4$; $p < 0.0001$; Fig. 3B). Similarly, luciferase expression from the mutant Δ 596bp *MEN1* 5'UTR luciferase construct was 16-fold lower ($p < 0.0001$) than the wild-type *MEN1* 5'UTR luciferase construct, but was not significantly different from the control promoter-less pGL4.10 empty vector in BON-1 cells (wild-type = 16.91 ± 0.38 ; mutant = 0.43 ± 0.02 ; pGL4.10 empty vector = 1.00 ± 0.04 ; $n = 4$; $p < 0.0001$; Fig. 3C). Thus, in both cell lines, the mutant Δ 596bp *MEN1* 5'UTR deletion caused a decrease in luciferase expression to the basal levels observed in cells transfected with the control promoter-less vector, thereby suggesting that the 5'UTR deletion abolishes transcription and translation.

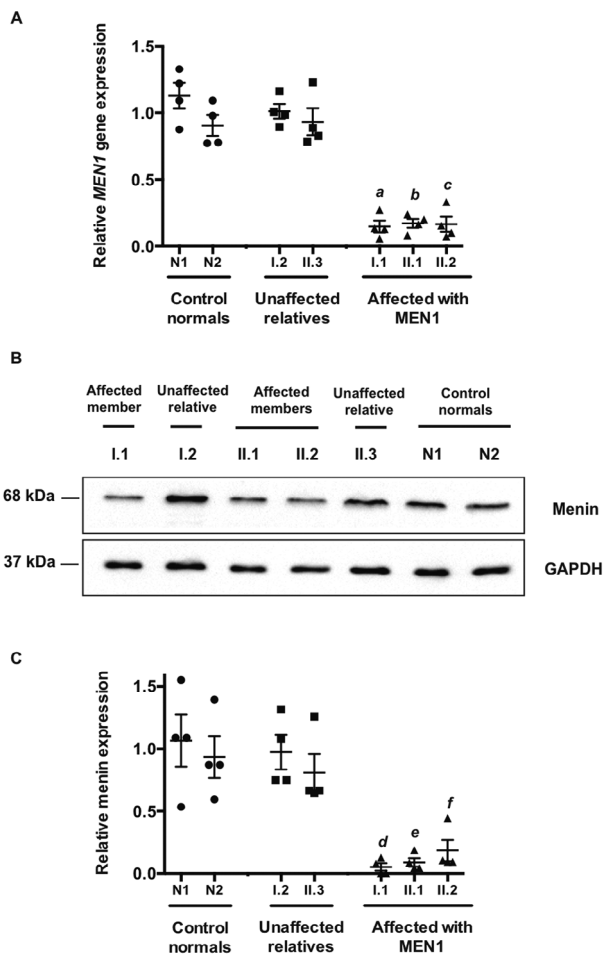


Fig 4. Effects of the 5'UTR Δ 596bp deletion on *MEN1* transcription and translation. (A) Relative *MEN1* mRNA expression from EBV transformed lymphoblastoid cells derived from three affected individuals (I.1, II.1 and II.2; Fig. 2A), two unaffected relatives (I.2 and II.3; Fig. 2A) and two unrelated normal controls (N1 and N2), normalized relative to *GAPDH* housekeeper. (B) Menin expression from EBV transformed lymphoblastoid cells derived from three affected individuals (I.1, II.1 and II.2; Fig. 2A), two unaffected relatives (I.2 and II.3 (Fig. 2A) and two unrelated normal controls (N1 and N2). GAPDH was used as the loading control. (C) Relative menin expression, normalized relative to GAPDH, was quantified using densitometry analysis. The 596 bp deletion in the *MEN1* 5'UTR caused a reduction in *MEN1* mRNA level and menin level in the affected individuals (Fig. 2A). Data are represented as mean \pm SEM, $n = 4$, $^a p < 0.0001$, $^b p < 0.0001$, $^c p < 0.0001$, $^d p < 0.0001$, $^e p < 0.0001$, $^f p = 0.0001$.

The 596 bp deletion in the *MEN1* 5'UTR reduces *MEN1* transcription and translation

Since the Δ 596bp segment of the *MEN1* 5'UTR resulted in reduced *MEN1* promoter activity *in vitro*, we investigated its effect on *MEN1* transcription and translation. Total mRNA and protein lysates obtained from EBV transformed lymphoblastoid cells of the three affected family members (I.1, II.1 and II.2; Fig. 2A), two unaffected relatives (I.2 and II.3; Fig. 2A), and two unrelated normal controls (N1 and N2) were used in qRT-PCR and Western blot analyses, respectively. This revealed an 84%

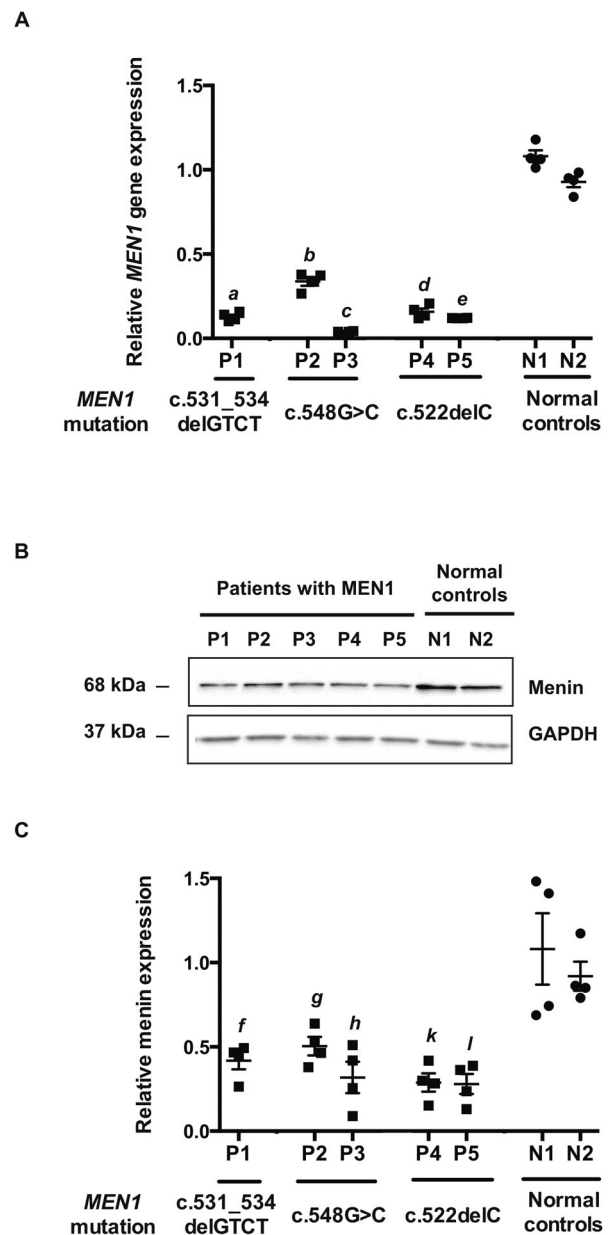


Fig 5. Effects of *MEN1* coding mutations on *MEN1* transcription and translation. (A) Relative *MEN1* mRNA expression from EBV transformed lymphoblastoid cells derived from five individuals affected with *MEN1* (P1, P2, P3, P4 and P5) and two normal controls (N1 and N2), normalized relative to *GAPDH* housekeeper. (B) Menin expression from EBV transformed lymphoblastoid cells derived from five individuals affected with *MEN1* (P1, P2, P3, P4 and P5) and two normal controls (N1 and N2). GAPDH was used as the loading control. (C) Relative menin expression, normalized relative to GAPDH, was quantified using densitometry analysis. The *MEN1* coding mutations caused a reduction in *MEN1* mRNA level and menin level in the affected individuals. Data are represented as mean \pm SEM, $n = 4$, $^a p < 0.0001$, $^b p < 0.0001$, $^c p < 0.0001$, $^d p < 0.0001$, $^e p < 0.0001$, $^f p = 0.0020$, $^g p = 0.0106$, $^h p = 0.0003$, $^k p = 0.0002$, $^l p = 0.0001$.

reduction in mean *MEN1* mRNA expression in the three affected patients (I.1 = 0.15 ± 0.04 ; II.1 = 0.17 ± 0.03 ; II.2 = 0.17 ± 0.06 ; mean = 0.16 ± 0.02 ; $n = 4$) compared to the two unaffected

relatives and two unrelated normal controls ($I.2 = 1.01 \pm 0.06$; $II.3 = 0.93 \pm 0.10$; $N1 = 1.13 \pm 0.10$; $N2 = 0.91 \pm 0.08$; mean = 1.00 ± 0.04 ; $n = 4$) (Fig. 4A; all $p < 0.05$). Moreover, there was an 88% reduction in mean menin protein expression in the three affected patients ($I.1 = 0.05 \pm 0.03$; $II.1 = 0.09 \pm 0.03$; $II.2 = 0.19 \pm 0.09$; mean = 0.11 ± 0.03 ; $n = 4$) compared to the two unaffected relatives and two unrelated normal controls ($I.2 = 0.97 \pm 0.14$; $II.3 = 0.81 \pm 0.15$; $N1 = 1.07 \pm 0.21$; $N2 = 0.93 \pm 0.17$; mean = 1.00 ± 0.08 ; $n = 4$) (Fig. 4B,C; all $p < 0.05$). However, the 5'UTR deletion, which resulted in one *MEN1* mutant allele, would be expected to be associated with an ~50% reduction in *MEN1* RNA and menin expression, but not the unexpected observed >50% reduction, which suggests that transcription and translation from the remaining wild-type *MEN1* allele in the EBV transformed lymphoblastoid cells of the *MEN1* patients, may also be affected. We postulated that this may involve a non-linear autoregulation of *MEN1* expression, similar to that reported upon overexpressing exogenous menin in *Men1*^{-/-} mouse embryonic fibroblast cells (MEFs),⁽⁷⁾ and that this may also occur in association with other *MEN1* mutations.

To investigate this hypothesis, we evaluated the effects of three previously reported *MEN1* coding region mutations^(22,23) on *MEN1* transcription and translation, using total mRNA and protein lysates, respectively, from EBV transformed lymphoblastoid cell lines of five patients and two (normal) unaffected controls (Fig. 5). For these studies, we selected three different types of *MEN1* mutations, which comprised: a frameshift (fs) 4 bp deletion (del) leading to a premature termination (Ter) (c.531_534delGTCT (p.(Ser178ArgfsTer6)))⁽²²⁾ in one patient (P1); a c.548G>C transversion leading to a missense substitution (p.(Trp183Ser)) in two related patients (P2 and P3)⁽²³⁾; and a fs 1 bp del leading to a premature Ter (c.522delC (p.(Leu175SerfsTer10))) in two related patients (P4 and P5).⁽²³⁾ qRT-PCR analyses, revealed an 84% reduction in mean *MEN1* mRNA expression in the five affected patients ($P1 = 0.13 \pm 0.01$; $P2 = 0.34 \pm 0.03$; $P3 = 0.04 \pm 0.00$; $P4 = 0.16 \pm 0.02$; $P5 = 0.12 \pm 0.00$; mean = 0.16 ± 0.02 ; $n = 4$) compared to the two normal controls ($N1 = 1.08 \pm 0.04$; $N2 = 0.93 \pm 0.03$; mean = 1.00 ± 0.04 ; $n = 4$) (Fig. 5A; all $p < 0.05$). Moreover, Western blot analyses revealed a 64% reduction in mean menin protein expression in the five affected patients ($P1 = 0.42 \pm 0.05$; $P2 = 0.50 \pm 0.05$; $P3 = 0.32 \pm 0.09$; $P4 = 0.29 \pm 0.05$; $P5 = 0.28 \pm 0.06$; mean = 0.36 ± 0.03 ; $n = 4$) compared to the two normal controls ($N1 = 1.08 \pm 0.21$; $N2 = 0.92 \pm 0.09$; mean = 1.00 ± 0.11 ; $n = 4$) (Fig. 5B,C; all $p < 0.05$). Thus, patients with *MEN1* mutations had significantly reduced *MEN1* and menin expression, suggesting that the marked decrease in *MEN1* expression may be a consequence of the effects of pathogenic *MEN1* variants on the non-linear autoregulation of the *MEN1* gene.

Discussion

We report the first *MEN1* mutation that does not involve an abnormality within the protein-coding region, but instead occurs in the promoter region and comprises a 596 bp deletion of the minimal core promoter within the *MEN1* 5'UTR (Figs. 1 and 2; Supplemental Fig. 1). This deletion, which co-segregates with *MEN1* in the family (Fig. 2), results in reduced *MEN1* transcription and translation of the encoded protein, menin (Fig. 4), and provides interesting insights in promoter misregulation. Thus, our findings highlight the importance of examining the 5'UTR when

investigating patients with *MEN1*, who do not have germline mutations in the protein-coding region of the *MEN1* gene, to establish a genetic diagnosis.

Large germline deletions of *MEN1* that affect the protein-coding and promoter regions, including the 5'UTR, have been previously reported.^(10,11,24) These large deletions include: a deletion located upstream of the *MEN1* gene that results in loss of the translation start site and exons 2 to 5, in an Australian *MEN1* kindred⁽²⁴⁾; a deletion of exons 1 and 2 in a Spanish *MEN1* family⁽¹¹⁾; a deletion involving exons 1 to 2 in a Saudi *MEN1* family⁽¹⁰⁾; and a deletion of exons 1 to 3 in an Italian *MEN1* family.⁽¹¹⁾ The *MEN1* deletions reported in these families included both the *MEN1* 5'UTR as well as the ATG initiation codon, and part of the open reading frame (ORF), which could explain the loss of menin expression in the *MEN1* tumors, as demonstrated by studies of parathyroid tumor and PNETs from the Italian *MEN1* family.⁽¹¹⁾ However, the consequences of the sole loss of the minimal core promoter of the *MEN1* 5'UTR found in family F/92 (Fig. 2) on transcription and translation were difficult to predict as the region contains multiple cis-regulatory and initiator elements (Figs. 1 and 2; Supplemental Fig. 1). In addition, the 5'UTR region of *MEN1* transcripts have been shown to be highly variable, as transcription can be initiated simultaneously from multiple initiator elements present in the 5'UTR in the absence of a TATA box,^(7,25) although the heterogeneity of the 5' end of the *MEN1* transcripts does not affect the menin protein because all transcript variants are reported to be correctly spliced to exon 2 and therefore contain the same ORF.⁽²⁵⁾ The location of the minimal promoter and the multiple cis-regulatory regions have been determined by expressing multiple 5' and 3' serial deletions of a 2.0 kb *MEN1* gene promoter in different endocrine and non-endocrine cell lines and assessing responses by use of luciferase reporter assays.⁽⁷⁾ The regulatory elements were predicted to have potential binding sites (eg, the CCAAT box) for transcription factors such as SP1, which is normally present in TATA-less promoters to activate transcription through the direct interaction with basic transcription machinery factors.^(7,26,27) Furthermore, menin has also been reported to autoregulate transcription from the *MEN1* promoter,⁽⁷⁾ and our findings demonstrating that the loss of the minimal core promoter, due to the $\Delta 596$ bp *MEN1* 5'UTR in the *MEN1* family (Fig. 2), resulted in significantly decreased *MEN1* promoter activity (Figs. 3 and 4), is consistent with observations from these previous studies.⁽⁷⁾ However, the *MEN1* gene is under complex transcriptional regulation,⁽⁷⁾ and it seems possible that other transcription factor binding sites and regulatory elements within the 5'UTR of the *MEN1* gene (Supplemental Fig. 1) may also have roles that could explain the observed >50% reduction in *MEN1* RNA and menin expression in the patient derived EBV transformed cell lines. For example, the *MEN1* 5'UTR sequence contains two binding sites for paired box 5 (PAX5) (Supplemental Fig. 1), which is a master regulator of B-cell fate,⁽²⁸⁾ and EBV expresses a number of genes during infection, including the BamHI Z fragment leftward open reading frame 1 (*BZLF1*) gene that encodes a protein (Z), which is a transcription factor that directly interacts with PAX5 and inhibits the ability of PAX5 to activate promoters.⁽²⁹⁻³¹⁾ PAX5 may be acting as a repressor of *MEN1*, and its action may be lifted by EBV induced Z expression, which would result in an increase in menin expression in the wild-type *MEN1* expressing cell lines relative to the heterozygous *MEN1* expressing cell lines, thereby potentially contributing to the observed marked differences in *MEN1* expression (Figs. 4 and 5). In addition, menin and EBV proteins may have opposite actions on tumor protein

Table 1. *MEN1* Germline Mutations and *MEN1* Phenotypes Occurring in Monozygotic Twin Studies

Monozygotic twin studies (reference)	<i>Men1</i> mutation (location)	Predicted effect	Tumors (phenotypes)	
			Twin 1	Twin 2
(A) Flanagan and colleagues ⁽¹⁵⁾ and this study	c.-590_-24+29del (5'UTR)	Promoter misregulation	PHPT, INS, PNET(NF), L	PHPT, PRL, PNET(NF), L, A
(B) Palermo and colleagues ⁽³⁸⁾	c.1561_1571del (exon 10)	Thr521fs	PHPT ^a , GAS	PHPT ^a
(C) Concolino and colleagues ⁽³⁹⁾	c.292delC (exon 2)	Arg98fs	PHPT ^a , INS	AS
(D) Rix and colleagues ⁽⁴⁰⁾	c.1051T>C (exon 8)	Tyr351His	PHPT ^a , ACTH	PHPT ^a
(E) Tso and colleagues ⁽⁴¹⁾	c.1675A>T (exon 10)	Lys559Stop	PHPT ^a , INS	PHPT ^a , INS
(F) Namiyama and colleagues ⁽⁴²⁾	c.459delC (exon 3)	Asp153fs	PHPT ^a , PNET(NF)	PHPT ^a
(G) Bahn and colleagues ⁽⁴³⁾	None reported ^b	Unknown	PHPT, PRL, ACTH, GAS	PHPT ^a , PRL

MEN1 reference sequence ENST00000312049.10.

A = angiofibroma; ACTH = adrenocorticotrophic hormone secreting tumor; AS = asymptomatic; fs = frameshift; GAS = gastrinoma; INS = insulinoma; L = lipoma; NF = non-functioning; PHPT = primary hyperparathyroidism; PNET = pancreatic neuroendocrine tumor; PRL = prolactinoma; UTR = untranslated region.

^aReported as hyperparathyroidism.

^bStudy published 10 years prior to identification of *MEN1* gene in 1996.⁽⁹⁾

53 (p53) and retinoblastoma (Rb) activity,^(32,33) as expected of tumor suppressors and oncogenes, respectively, and investigations of the possible roles of these pathways and their components in controlling menin expression, may help to advance knowledge of the mechanisms regulating *MEN1* transcription and translation.

Reduced (or absent) *MEN1* transcription and translation could potentially be due to deletion of the wild-type *MEN1* allele, which would lead to a loss of heterozygosity (LOH), in the patient-derived EBV transformed cell lines. However, this would seem an unlikely possibility to explain the >50% reduction in *MEN1* and menin expression that was observed in association with the *MEN1* mutations in the EBV transformed cell lines from the patients (Figs. 4 and 5), because of the following three reasons: (i) the EBV transformed cell lines had undergone eight or fewer passages, thereby yielding a negligible likelihood of any secondary rearrangements; (ii) the probability of all eight patient-derived EBV transformed lymphoblastoid cell lines acquiring an identical second genetic abnormality with similar levels of mosaicism would be extremely low; and (iii) *MEN1* RNA and menin expression, which is still detected in EBV transformed lymphoblastoid cell lines from affected members of the *MEN1* family (Fig. 4), would not be observed if there was LOH of the wild-type *MEN1* allele together with the 596 bp *MEN1* promoter deletion that completely abolishes transcription and translation (Fig. 3). These observations indicate that the residual *MEN1* transcription of >15% (Figs. 4A and 5A) and menin expression of >10% to 35% (Figs. 4B,C and 5B,C) in these EBV lymphoblastoid cells likely comes from an intact wild-type allele.

Mutations affecting the 5'UTR of other tumor suppressor genes have also been reported in other endocrine cancer syndromes such as the hyperparathyroid-jaw tumor (HPT-JT) syndrome and MEN4, which are due to mutations of the cell division cycle 73 (*CDC73*) and cyclin-dependent kinase inhibitor 1B (*CDKN1B*) genes, respectively. Thus, a heterozygous insertion (c.-4_-11insG) in the *CDC73* 5'UTR occurring in *cis* with a loss of *CDC73* exons 4 to 10 in a family with HPT-JT, revealed it to impair luciferase reporter activity and reduce *in vitro* translation of the parafibromin protein.⁽³⁴⁾ However, it was the large intragenic deletion of exons 4 to 10 rather than the 5'UTR variant that was likely responsible for the phenotype in this family.⁽³⁴⁾ In another example involving a heterozygous 4-bp deletion (c.

-32_-29delGAGA) in the 5'UTR of the *CDKN1B* gene, the deletion was reported to significantly reduce luciferase reporter activity and decrease transcription of *CDKN1B* in the MEN4 patient's leukocytes.⁽³⁵⁾ However, another heterozygous 4-bp deletion (c.-456_-453delCCTT) in the 5'UTR of the *CDKN1B* gene and a point mutation (c.-469C>T) that affected the consensus Kozak sequence for initiation of translation of *CDKN1B* mRNA in MEN4 patients reduced the level of p27, encoded by *CDKN1B*, without affecting *CDKN1B* mRNA levels.^(36,37) These findings suggest that germline 5'UTR non-coding mutations that do not affect the ATG initiation codon can affect transcription and/or translation and can have a role in cancer susceptibility.

Both of the identical monozygotic twins in our study (Table 1, study A) had the 596 bp deletion (c.-590_-24+29del) affecting the *MEN1* 5'UTR, but one twin (II.1) had parathyroid adenomas, insulinoma, a non-functioning PNET, and a lipoma, whilst the other (II.2) had parathyroid adenomas, a prolactinoma, a non-functioning PNET, a lipoma, and an angiofibroma (Fig. 2A; Table 1).⁽¹⁵⁾ Such phenotypic variability in the *MEN1* syndrome is commonly observed and has also been reported to occur in monozygotic twins from six other studies (Table 1, studies B–G).^(38–43) Thus, of the seven studies (Table 1, studies A–G) of monozygotic twins, six (studies A, B, C, D, F and G) report differences in the occurrence of tumors between the identical twins, thereby indicating a lack of correlation between the phenotype and genotype in the majority (>85%) of twin pairs.^(15,38–40,42,43) Six of these seven studies (A, B, C, D, E and F, Table 1) reported an identified *MEN1* mutation, and three of these mutations comprised deletions of 1 bp to 10 bp and were predicted to result in frameshifts with premature truncations (Table 1, studies B, C and F); one resulted in a nonsense mutation (Lys559Stop, study E, Table 1); another lead to a missense mutation (Tyr351His, study D, Table 1); in addition to the 5'UTR deletion of 596 bp described here (Table 1, study A).^(15,38–42) Moreover, these six *MEN1* mutations are scattered throughout the 10 exons of the *MEN1* gene and highlight the lack of genotype-phenotype correlation in the *MEN1* syndrome, suggesting that genetic factors might not be the sole contributors affecting the expression of *MEN1* but that other non-heredity factors, such as epigenetics, may be involved. The one study (Table 1, study G) not reporting a *MEN1* mutation was published 10 years prior to the identification of the *MEN1* gene.⁽⁴³⁾

The morbidity and mortality amongst MEN1 patients has reduced considerably since the introduction of DNA testing in 1997, highlighting the importance of identifying patients at higher risk of developing MEN1 and to provide them with regular screening programs with early appropriate clinical interventions.^(4,8,44) There are over 1800 germline and somatic *MEN1* mutations that have been described in MEN1 patients, all of which affect the protein-coding part of the *MEN1* gene.^(4,8) However, there are also 10% to 20% of MEN1 patients in whom mutations have not been detected within the protein-coding region of the *MEN1* gene,^(4,8,44,45) and our results indicated that mutational analysis of non-coding regions of the *MEN1* gene is warranted in such patients. Thus, the detection of a *MEN1* mutation in such patients will help confirm the diagnosis of MEN1, and also identify those relatives who have inherited the *MEN1* mutation and are therefore at high risk of developing tumors that can be found earlier by screening programs that use plasma biochemical analysis and radiological imaging studies.⁽⁴⁴⁾

In summary, our study reports the first germline *MEN1* 5'UTR mutation, which causes promoter misregulation, in a family with MEN1 and highlights the importance of investigating UTRs in MEN1 patients who do not have coding region mutations.

Disclosures

The authors have nothing to disclose and all authors state that they have no conflict of interest.

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Authors' roles: KGK and AR performed and analyzed the experiments; HB and TC performed Sanger DNA sequencing and MLPA analyses; ATP and JCT accessed and undertook bioinformatics analysis on data from the 100KGP; 100KGP generated the dataset from affected and unaffected family participants; DEHF and TR identified the family and provided the clinical details; SGG provided the BON-1 cell line; KGK, MS, KEL and RVT conceived, designed and directed the studies; KGK, MS, KEL and RVT wrote the manuscript; all authors reviewed and approved the final version of the manuscript.

Author contributions: KGK: Conceptualization; data curation; formal analysis; investigation; validation; visualization; writing-

original draft; writing-review and editing. HB: Data curation; formal analysis; investigation; validation. TC: Data curation; formal analysis; investigation; validation. MS: Conceptualization; supervision; visualization; writing-original draft; writing-review and editing. ATP: Data curation; formal analysis; investigation; resources; software; validation. AR: Data curation; formal analysis; investigation; validation. SGG: Resources. TR: Data curation; resources. DF: Data curation; resources. Genomics England Research Consortium: Resources. JCT: Data curation; formal analysis; investigation; resources; software; validation. KEL: Conceptualization; supervision; visualization; writing-original draft; writing-review and editing. RVT: Conceptualization; funding acquisition; supervision; visualization; writing-original draft; writing-review and editing.

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