Germline mutation landscape of multiple endocrine neoplasia type 1 using full gene next-generation sequencing

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Abstract

Background: Loss-of-function germline *MEN1* gene mutations account for 75–95% of patients with multiple endocrine neoplasia type 1 (MEN1). It has been postulated that mutations in non-coding regions of *MEN1* might occur in some of the remaining patients; however, this hypothesis has not yet been fully investigated.

Objective: To sequence for the entire *MEN1* including promoter, exons and introns in a large MEN1 cohort and determine the mutation profile.

Methods and patients: A target next-generation sequencing (tNGS) assay comprising 7.2 kb of the full *MEN1* was developed to investigate germline mutations in 76 unrelated MEN1 probands (49 familial, 27 sporadic). tNGS results were validated by Sanger sequencing (SS), and multiplex ligation-dependent probe amplification (MLPA) assay was applied when no mutations were identifiable by both tNGS and SS.

Results: Germline *MEN1* variants were verified in coding region and splicing sites of 57/76 patients (74%) by both tNGS and SS (100% reproducibility). Thirty-eight different pathogenic or likely pathogenic variants were identified, including 13 new and six recurrent variants. Three large deletions were detected by MLPA only. No mutation was detected in 16 patients. In untranslated, regulatory or in deep intronic *MEN1* regions of the 76 MEN1 cases, no point or short indel pathogenic variants were found in untranslated, although 33 benign/likely benign and three new VUS variants were detected.

Conclusions: Our study documents that point or short indel mutations in non-coding regions of *MEN1* are very rare events. Also, tNGS proved to be a highly effective technology for routine genetic *MEN1* testing.

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Introduction

Multiple endocrine neoplasia type 1 (MEN1, OMIM #131100) is a rare and complex inherited cancer syndrome transmitted with autosomal dominant inheritance that

predisposes individuals to the development of more than 20 endocrine and non-endocrine tumors. Despite variable frequencies in different series, parathyroid tumors

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(82–100%) leading to hyperparathyroidism (HPT), in addition to pituitary (PIT) (19–65%) and pancreatoduodenal endocrine tumors (PET) (41–100%), are the most prevalent endocrine tumors in MEN1. It is estimated that 50% of patients carrying germline mutations in the *MEN1* tumor suppressor gene will develop at least one MEN1-related tumor up to 20 years of age and almost all cases will present clinical disease at 50 years. Absence of correlation between genotype and phenotype as well as a wide intra-familial clinical variability have been reported in MEN1 cases (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11). Presently, the major causes of death are malignant PETs and thymic carcinoid tumors and patients have a shorter life expectancy (12, 13). Compared to familial MEN1, sporadic cases are usually older at the time of diagnosis, live longer and exhibit no more than two MEN1-related tumors (14).

MEN1 (OMIM # 613733) is a 10-exon gene with 1830bp in the coding region that encodes the 610 amino acid protein menin, which interacts with at least 12 proteins and regulates transcription, genome stability, cell division and proliferation (5, 6, 8). Germline heterozygous *MEN1* mutations are responsible for 75–95% of MEN1 phenotypes (5, 6, 8, 15, 16, 17, 18, 19). Importantly, up to 5–25% of patients may not harbor *MEN1* mutations in the coding region, 10% of cases are due to *de novo* *MEN1* mutations and 5–10% are phenocopies (5). Rarely (3.5%), *MEN1*-negative carriers with MEN1-like phenotypes may harbor germline mutations in the *CDKN2B/p15*, *CDKN2C/p18*, *CDKN1A/p21*, *CDKN1B/p27* and *AIP* genes (20, 21, 22).

Most *MEN1* mutations are frameshift and nonsense (56–64%), which predict truncated menin-protein missing at least one out of the three nuclear localization signals located at the C-terminal segment (19, 23). Thus, menin becomes unable to suppress transcription factors, leading to predisposition for tumor formation. Missense variants represent one quarter of the different mutations types, whereas splice-site and in-frame indels are also represented and frequently interfere in menin-protein interactions (5, 8, 19, 23). In turn, gross deletions correspond to 2.5% of the different mutation types, occur in 1% of MEN1 cases and are usually investigated using assays as multiplex ligation-dependent probe amplification (MLPA) (19, 23).

The identification of *MEN1* germline mutation is highly useful because it confirms the clinical diagnosis of the MEN1 index case, defines the carrier and non-carrier status of at-risk family members, rules out non-carriers from long-term periodic follow-up and allows prenatal and preimplantation genetic diagnosis. Therefore, *MEN1*

testing substantially optimizes genetic counseling, surveillance and therapeutic strategies (1, 2, 3, 4, 5, 6, 8, 9, 10, 11, 12, 24). Early genetic diagnosis promotes early clinical diagnosis and treatment of MEN1-related tumors that has a consistent positive impact on morbidity, quality of life and survival rate (1, 2, 3, 4, 5, 6, 7, 8, 13, 24). In contrast, detrimental consequences of delayed diagnosis should not be underestimated (25).

Current MEN1 guidelines recommend *MEN1* mutation testing for cases far beyond the classical MEN1 phenotype and symptomatic or asymptomatic first-degree relatives (1, 5). Genetic testing should be also offered for several sporadic cases with MEN1-related tumors, such as multiglandular parathyroid disease, gastrinoma and multiple PET at any age; parathyroid hyperplasia (<40 years); recurrent HPT; several atypical MEN1-related phenotypes (5) and, more recently, PIT macroadenoma identified at a young age, adding a substantial number of cases to be genetically tested (26). This goal should be pursued as genetic diagnosis provides an opportunity for earlier treatment, avoiding comorbidities as those associated with HPT, PIT macroadenomas or PETs (3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 24, 25, 26, 27).

MEN1 testing has been usually performed using both Sanger sequencing (SS) and MLPA assays (5, 8, 9, 13, 23, 28, 29, 30). Although genetic testing should be ideally performed in literally all cases fulfilling current criteria, SS technology has limitations to achieve this purpose. Thus, the *MEN1* gene is relatively large and lacks mutational hotspots making SS a burdensome, time-consuming, laborious and expensive technique. Moreover, in MEN1 patients with no mutation in the coding region of the *MEN1* gene, it has been postulated the existence of *MEN1* mutations in introns far from intron/exon boundaries, untranslated or regulatory regions (5, 8, 10, 13, 18, 19, 23, 28, 29, 30). However, SS may not usually cover such gene regions, preventing systematic studies on this topic (5, 8, 10, 13, 18, 19, 23, 28, 29, 30). Therefore, novel, higher-throughput and greater capacity of data generation, robust and affordable strategies as NGS technologies are required (31, 32, 33, 34). NGS is feasible in clinical practice today and different NGS strategies have been adopted for hereditary cancer syndromes, such as WGS, WES and tNGS sequencing (31, 32, 33, 34).

So far, the few available NGS studies evaluating the *MEN1* gene have applied WES or WGS approaches, confirming the presence of occasional somatic *MEN1* mutations in several sporadic tumors beyond biallelic *MEN1* inactivation in parathyroid tissues from MEN1

patients (35, 36, 37, 38, 39). Germline *MEN1* mutations were analyzed in three studies using WES, in a large primary hyperparathyroidism family and in five MEN1 cases (38, 39, 40). Also, a 22 genes tNGS panel including *MEN1* was recently applied to investigate somatic mutations in sporadic pancreatic neuroendocrine tumors (41). In addition, an eight-gene tNGS panel was applied to search germline mutations in young patients with pituitary adenoma (42). However, despite tNGS being cheaper and faster than WES and WGS, there is no previous systematic study using this technology as a genetic diagnostic tool in MEN1 syndrome (8, 9, 13, 43). Worthwhile, independent of which NGS technology is used, caution should be exercised to avoid errors and misinterpretations that may carry negative clinical impacts (44, 45).

In the present study, we applied tNGS to our MEN1 cases to investigate the full *MEN1* gene including coding and non-coding regions, searching for mutations possibly occurring in introns far from intron/exon boundaries and untranslated and regulatory regions. Also, we compared tNGS and SS data to validate the former technology for *MEN1* testing in MEN1.

Patients and methods

Patients

The study was approved by the Local Ethical Committee (CAPPesq), and written, fully informed consent was given by all patients or their parents. Both tNGS and SS assays were conducted in peripheral blood leukocyte DNA samples obtained from 76 apparently unrelated index cases with a clinical diagnosis of MEN1. Thirty-one affected men (mean age, 40 ± 15 years; 7–69 years) and 45 women (mean age, 41 ± 11 years; 19–67 years) were enrolled, and the overall mean age at diagnosis was 40 ± 13 years (7–69 years).

The clinical diagnosis of MEN1 was defined as previously established (1, 5, 27). Familial MEN1 was characterized by the combined presence of a MEN1 index case and at least one first-degree family member with one or more main MEN1-related tumors (HPT, PET, PIT). Sporadic MEN1 was defined as the presence of an isolated case with the clinical diagnosis of MEN1 and unsatisfactory/unknown or negative MEN1 familial history. Probands were considered apparently unrelated when they clearly stated no to be related to any other patient enrolled in this study.

Classification of variants

MEN1 gene variants were classified following criteria of the American College of Medical Genetics and Genomics (ACMG), which characterize variants as benign, likely benign, variants of uncertain significance (VUS), likely pathogenic and pathogenic based on clinical, computational, segregation and population data (46, 47). In addition, to reinforce segregation data, the segregation equation $N=(1/2)^m$, as defined by Jarvik (48), was supported, where N means the probability of the observed variant occur by chance (no pathogenic) and m defines the number of meiosis of the variant that is informative for segregation, excluding the index case. The criteria to pathogenicity (P) are dependent on segregation data coming from either one family (a) or two or more analyzed families (b). For these groups, different cutoffs define a grading of evidence levels of pathogenicity: supporting evidence (a, $P \leq 1/8$; b, $P \leq 1/4$), moderate evidence (a, $P \leq 1/16$; b, $P \leq 1/8$) and strong evidence (a, $P \leq 1/32$; b, $P \leq 1/16$) (48).

In MEN1 patients presenting germline mutations, the vast majority (>96%) of cases will harbor a *MEN1* mutation (1, 2, 3, 4, 5, 6, 7, 8, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 43). Thus, we determined the *MEN1* as a target in NGS and SS.

Sanger sequencing

In our tertiary academic hospital, we have been routinely offering DNA genetic diagnosis for MEN1 patients since 2004 using SS and, more recently, multiplex ligation-dependent probe amplification (MLPA) (3, 4, 18, 49, 50, 51). Beyond *MEN1*, other genes as *CDKN2B/p15*, *CDKN2C/p18*, *CDKN1A/p21*, *CDKN1B/p27* and *AIP* have been analyzed in our patients with MEN1-like conditions, sporadic MEN1-related tumors and familial acromegaly (52, 53, 54).

For SS, amplicons were obtained through PCR of the complete coding DNA sequence (CDS) (± 50 bp upstream/downstream) using eight primer pairs. Amplicons were purified with Illustra ExoProStar 1-step (GE Healthcare) and sequenced using Big Dye terminator v3.1 (Thermo Fisher). Capillary electrophoresis (CE) was conducted in an ABI 3130xl automatic sequencer (Thermo Fisher). After data acquisition, AB1 files were analyzed using CodonCode Aligner version 5.0 (CodonCode, MA, USA) using as references the *MEN1* gene sequence (ENSG00000133895 or NG_008929.1) and transcript

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MEN1-001 (ENST00000312049 or NM_130799.2), as this transcript codes for the main functional isoform of *MEN1*.

NGS libraries and sequencing

For NGS, the targeted sequence was amplified by long-range PCR (Long PCR Enzyme mix 5U/μL, Thermo Fisher) using one primer pair to amplify the entire *MEN1* gene (coding regions, introns, untranslated and regulatory regions). The primer sequences were forward, TAACAGACACTGATACCCAGCTAAAGC and reverse, GGGATACGAAGGAGAGGAACTAGG (both 5'–3'). The amplicon size obtained was 7239bp (GRCh37/hg19 chr11:64571249-64578487), following the manufacturer's instructions.

Supplementary data (see section on [supplementary data](#) given at the end of this article) shows detailed data on cycling conditions and quantification of amplicons, preparation of libraries, sequencing using MiSeq Illumina platform and softwares applied to data processing, generation of pipelines to provide running quality metrics and inspection of reads and comparative analysis of tNGS with SS. Also, The impact and effects of variants were analyzed using seven different databases: dbSNP (<http://www.ncbi.nlm.nih.gov/SNP/>), Leiden Open Variation Database (LOVD) (<http://www.lovd.nl/3.0/home>), Ensembl (<http://www.ensembl.org/index.html>), Catalogue of Somatic Mutations in Cancer (COSMIC) (<http://cancer.sanger.ac.uk/cosmic>), the Human Gene Mutation Database (HGMD) (<http://www.hgmd.cf.ac.uk/ac/index.php>), GnomAD (<http://gnomad.broadinstitute.org>) and ABraOM (<http://abraom.ib.usp.br/>). In parallel, predictive algorithms used for *in silico* analysis and clinical databases consulted are found in Supplementary data.

We preliminarily applied a five-gene panel to a strict subset of nine available *MEN1*-negative carriers, following the same protocol previously defined and optimized for the *MEN1* tNGS assay, aiming to investigate germline mutations in *CDKN2B/p15*, *CDKN2C/p18*, *CDKN1A/p21*, *CDKN1B/p27* and *AIP* genes. As for the *MEN1* tNGS assay, the panel covered the full open reading frame of the five genes using specific long-range PCR (Supplementary data).

Multiplex ligation-dependent probe amplification (MLPA)

Amplicon-based tNGS and also SS are not able to detect gross deletions. Thus, MLPA was provided to *MEN1*-negative carriers. For this analysis, we applied the MLPA kit P244, targeting *MEN1*, *AIP* and *CDKN1B* genes.

MLPA probes covered each one of the ten exons of the *MEN1* gene including the upstream region. DNA fragment analysis was performed in the same ABI 3130xl CE device (Thermo Fisher) using ROX 500 (Thermo Fisher) as a DNA ladder. Running and injections parameters were used according to the manufacturer's instructions. After DNA fragment electrophoresis, FSA files were analyzed with MRC-Holland's own software, Coffalyser (MRC-Holland, Amsterdam, The Netherlands). The gross deletions identified by MLPA were considered as such only if two consecutive ligation probes were involved. Additionally, sequences obtained by NGS/SS excluded the presence of SNVs on probe ligation sites as they may generate false positive results by MLPA. Also, hemizygous was reinforced by absence of heterozygous SNVs and lower average read depth observed in NGS sequences in deleted regions detected by MLPA.

Statistical analysis

Statistical analysis was performed using IBM-SPSS software for Windows, version 20.0. Categorical data were described as number of tumors, presence/absence of germline mutation or presence/absence of familial history and association between these characteristics was verified using the chi-squared test. Data with a normal distribution as age at the time of diagnosis had their values expressed as means ± standard deviation and comparisons between these categories were provided using Student's *t*-test. As both tNGS and SS assays were performed using DNA from the same blood sample, the percentage agreement statistic and Cohen's kappa test were applied to verify the reliability of tNGS compared to the gold standard SS. A *P* value <0.05 was considered statistically significant.

Results

Clinical features

The prevalence of main *MEN1*-related tumors in the 76 *MEN1* patients was, respectively: HPT, 99% (75/76); PET, 86% (65/76) and; PIT, 78% (59/76). Most patients (47/76; 62%) presented all three main *MEN1*-related tumors. The remaining 29 patients (29/76; 38%) with two main *MEN1*-related tumors presented the following tumor associations: HPT/PET (17/76; 22%), HPT/PIT (11/76; 15%) and PET/PIT (1/76; 1%).

Familial *MEN1* was clinically diagnosed in 49 patients (49/76, 64%) whereas the remaining were classified as sporadic cases (27/76; 36%) (Fig. 1). Age at diagnosis of

the 49 familial cases was 39 ± 14 years, while for the 27 sporadic cases it was 41 ± 14 years ($P=0.496$).

Pathogenic *MEN1* variants

Frequency in familial and sporadic *MEN1* cases

In our series, heterozygous *MEN1* germline variants were documented in 60 out of 76 *MEN1* patients (79%): 57 cases were detected by tNGS and SS (75%), whereas three others (4%) harbored large gene deletions documented by MLPA (Fig. 1). Nine cases (9/60; 15%) with typical *MEN1* phenotype (familial or simplex cases) harbored variants classified as likely pathogenic, according

to criteria from ACMG. Subsequently, after the probability of segregation equation $N=(1/2)^m$ was applied, three likely pathogenic variants were reclassified as pathogenic. Overall, considering the combined criteria (ACMG and SE), 79% (60/76) of patients harbored a pathogenic/likely pathogenic *MEN1* variant.

In the familial *MEN1* subset, 44 cases (44/49; 90%) were mutation carriers: 41 (84%) were detected by tNGS/SS and three by MLPA assay (6%). After *MEN1* testing, we uncovered 16 newly inherited *MEN1* cases out of 27 (59%) apparently sporadic cases, while the remaining 11 were *MEN1*-negative carriers (Fig. 1).

In total, 60 *MEN1* carriers were documented (75%), and 16 (five familial, 11 sporadic) were *MEN1*-negative cases. As expected, the prevalence of germline pathogenic/likely pathogenic *MEN1* variants was higher in familial (44/49; 90%) than in sporadic (16/27, 59%; $P=0.002$) cases. The 16 mutation-negative cases were older than the 60 patients harboring pathogenic/likely pathogenic *MEN1* variants (38 ± 14 years vs 46 ± 12 years; $P=0.033$). Most *MEN1*-negative index cases (12/16; 75%) had no more than two *MEN1*-related tumors, in comparison with *MEN1*-positive index cases (20/60, 33%; $P=0.003$).

Figure 1

Flow chart applied to genetic diagnosis of 76 unrelated *MEN1* index cases using Targeted Next Generation Sequencing (tNGS) and multiplex ligation-dependent probe amplification (MLPA) assays. *MEN1*, multiple endocrine neoplasia type 1; F-*MEN1*, Familial *MEN1*; S-*MEN1*, sporadic *MEN1*; CDKIs, cyclin-dependent kinase inhibitors; NGS assay was based on long-range PCR amplification of the full *MEN1* open reading frame using MiSeq Illumina platform; MLPA assay was performed to investigate gross deletions in the *MEN1*, *AIP* and *CDKN1B/p27* genes in tNGS/*MEN1* mutation-negative patients; nine *MEN1*-negative cases by tNGS and *MEN1/AIP/p27*-negative by MLPA were screened to germline mutations by tNGS addressed to CDKIs (*CDKN2B/p15*, *CDKN2C/p18*, *CDKN1A/p21*, *CDKN1B/p27*) and *AIP* genes; circle with full line, positive *MEN1*-mutation patients identified by *MEN1*-NGS or *MEN1*-MLPA assays; circle with dashed line, *MEN1* mutation-negative patients by NGS or MLPA assays (*MEN1*, *CDKN1B/p27* and *AIP*); triangle, patients without germline mutation identified in full open reading frames of the *MEN1*, *CDKN2B/p15*, *CDKN2C/p18*, *CDKN1A/p21*, *CDKN1B/p27* and *AIP* genes by tNGS and with no gross deletions in *MEN1*, *CDKN1B/p27* and *AIP* by MLPA assay.

Mutation types

Overall, 38 different *MEN1* variants were identified (Fig. 2). Ten pathogenic and three likely pathogenic *MEN1* variants were novel (13/37; 35%), as they were absent from eight available databases (Table 1). All variant types detected by tNGS were also verified by SS and vice versa. Large gene deletions, as expected, were not detected by both methods. Combining results from tNGS/SS and MLPA assays, most variants were frameshift (21/60; 35%), followed by in splice-site (14/60; 23%), nonsense (11/60; 18%), missense (7/60; 12%), in-frame indel variants (4/60; 7%) and large deletions (3/60; 5%) (Fig. 2 and Table 1).

Most (62%) of the 13 new pathogenic/likely pathogenic variants were predicted to code truncated proteins generated from frameshift deletions/insertions (6/13) or nonsense variant (2/13), followed by three in-frame insertion/deletion and one intronic and one missense point mutation.

Analysis of *MEN1* variants

In our cohort, we identified six (6/41; 15%) different missense variants and one of them was new. We again applied the criteria adopted by ACMG (46, 47) combined with the probability equation of segregation by chance (48)

Figure 2

Mutations were identified in intron-exon frontiers and coding region of the *MEN1* gene in 60 out of our cohort of 76 unrelated MEN1 index cases by both tNGS and SS (57 cases), and MLPA assay (3 cases). Truncating mutations (frameshift and nonsense) (NGS and SS) and gross *MEN1* deletions (MLPA assay) are represented below diagram while non-truncating (missense and in-frame) and splicing mutations are located above; #, new mutations reported in the present study; ##, mutations previously reported from our cohort (8, 18); shadow region corresponds to non-coding regions of the *MEN1* gene.

to classify pathogenicity of the four new variants (missense and in-frame) and confirm five missense variants and one in-frame previously reported as pathogenic (Table 2).

The new missense variant *p*.Leu41Pro classified as likely pathogenic segregated in four affected at-risk relatives and it was absent in seven other asymptomatic at-risk family members (Table 1, case 1; Table 2). This variant was located at a highly conserved gene region in humans and animals, in the JunD-NM23H1-RPA2-SMAD3-interacting domains.

All patients with in-frame variants presented typical features of MEN1 with multiglandular tumor involvement. Three in-frame variants (two new) were classified as likely pathogenic and one as pathogenic.

The new in-frame pathogenic variant c.354_356delGAA (codon 119) found in one *de novo* case (case 10) was initially classified as likely pathogenic. It was redefined as pathogenic as it occurred in a repetitive region of exon 2 and its mutated sequence was exactly the same generated by 'warm spot' *MEN1* mutation c.358_360delAAG described in the subsequent codon 120 (16, 19).

Considering the three patients with likely pathogenic variants, two of them were familial (cases 8 and 56, Tables 1 and 2) and another was a simplex MEN1 case. The latter patient presented a new in-frame variant in exon 5 due to a consecutive duplication of the normal sequence GGCTGCTCT (position 794-802), which resulted in a repetition of the corresponding amino acids (c.802_803insGGCTGCTCT; *p*.Trp265_Leu267dup) (Table 1, case 20; Table 2). The latter variant was identified in a 43-year-old patient with typical MEN1 phenotype

presenting with primary HPT, non-functioning PETs, gastrinoma, bilateral nodular adrenocortical hyperplasia, collagenomas and facial angiofibromas. Familial history showed a brother with urolithiasis and an aunt with breast carcinoma. At-risk family members were invited to come to the hospital, but remained unavailable. Thus, segregation analysis of the variant could not be performed yet (Table 2). This variant was located at NM23H1-NMHCIIA-FANCD2-HDAC1-interacting domains.

The *p*.Leu414Pro missense variant (Table 1, case 28; Table 2), classified as likely pathogenic by ACMG criteria, was absent in six healthy siblings. The index case was aged 28 years and presented a typical MEN1 phenotype characterized by HPT (multiglandular disease), macroprolactinoma, collagenomas and non-functioning adrenal tumor. The latter patient was previously reported by us harboring a pathogenic variant, based on changing of the aliphatic amino acid leucine for the non-aliphatic proline amino acid in a highly conserved region (18).

Overall, analyzing the five different variants previously reported, four of them were initially classified (*p*.I147F, *p*.Leu413Arg, *p*.L414P, *p*.D418Y) as likely pathogenic and *p*.H139Y as pathogenic, based on previous *in vitro* studies (55). In turn, the strength of familial segregation changed the classification of three variants (*p*.I147F, *p*.Leu413Arg, *p*.D418Y) from likely pathogenic to pathogenic (Table 2).

Introns far from exon/intron boundaries, untranslated and regulatory regions

Notably, no mutation was verified by tNGS in introns far from exon/intron boundaries, untranslated or

Table 1 Pathogenic germline variants identified in coding regions and intron-exon frontiers of the *MEN1* gene in 57 unrelated MEN1 cases, by targeted NGS and Sanger sequencing.

ID	Sex/age ^a	Phenotype	Exon	Codon	Mutation	Protein change	HGMD	dbSNP	Effect/ACMG/SE	Reference
1	M/69	F-MEN1	2	41	c.122T>C	p.L41P	NA	NI	MS ^d /LP/ILP	This study (18)
2	F/37 ^a	F-MEN1	2	68	c.201_201delC	p.A68Pfs*51	CD075463	NI	FS/P	(16)/(28)
3	M/47 ^a	F-MEN1	2	83-84	c.249_252delGTCT ^c	p.I85Sfs*33	CD075467/CD972304	rs587776841	FS/P	(16)/(28)
4	F/34	F-MEN1	2	83-84	c.249_252delGTCT ^c	p.I85Sfs*33	CD075467/CD972304	rs587776841	FS/P	(16)/(28)
5	M/51	F-MEN1	2	83-84	c.249_252delGTCT ^c	p.I85Sfs*33	CD075467/CD972304	rs587776841	FS/P	(16)/(28)
6	F/30	F-MEN1	2	83-84	c.249_252delGTCT ^c	p.I85Sfs*33	CD075467/CD972304	rs587776841	FS/P	(16)/(28)
7	F/30	F-MEN1	2	83-84	c.249_252delGTCT ^c	p.I85Sfs*33	CD075467/CD972304	rs587776841	FS/P	(16)/(28)
8	M/54	F-MEN1	2	89-95	c.266_286del21	p.L89_A95del	NI	NI	IF/LP/ILP	(18)
9*	M/36	F-MEN1	2	106	c.315_316insCCCTC	p.Y106Pfs*12	NI	NI	FS/P	This study
10*	F/20	S-MEN1	2	119	c.354_356delGAA	p.Lys119del	NI	NI	IF ^d /LP/ILP	This study
11	F/52 ^a	F-MEN1	2	126	c.377G>A	p.W126*	CM981253	NI	NS/P	(10)
12	M/57	S-MEN1	2	129	c.385_385delC	p.L129Sfs*56	NI	NI	FS/P	This study
13	M/28	F-MEN1	2	139	c.415C>T	p.H139Y	CM970921	NI	MS/P	(15)
14	F/49 ^a	F-MEN1	2	144	c.431_432delinsAA	p.F144*	CX055790	NI	NS/P	(66)
15	F/48 ^a	F-MEN1	2	147	c.439A>T	p.I147F	CM074341	NI	MS/LP/ILP	(18)
16	F/37	F-MEN1	3	183	c.548G>A	p.W183*	CM981259	rs794728650	NS/P	(17)
17	F/36 ^a	F-MEN1	3	186	c.558delT	p.F186Lfs*38	CD035821	NI	FS/P	(67)
18	M/19 ^a	F-MEN1	3	210-211	c.628_631delACAG ^c	p.T210Sfs*13	CD972310	NI	FS/P	(16)
19	F/50 ^a	F-MEN1	3	218	c.652_652delC	p.R218Gfs*6	NI	NI	FS/P	This study
20	M/42	S-MEN1	5	265-267	c.802_803insGGCTGCTCT	p.Trp265_Leu267dup	NI	NI	IF ^d /LP/ILP	This study
21	M/33	F-MEN1	6	302	c.903_909delCTACCAC	p.Y302Rfs*64	NI	NI	FS/P	This study
22	F/26 ^a	F-MEN1	7	318	c.951_952insC	p.I318Hfs*49	NI	NI	FS/P	(18)
23	M/27	F-MEN1	7	339	c.1015C>T	p.Q339*	CM053978	NI	NS/P	(68)
24	F/48 ^a	F-MEN1	8	363	c.1087G>T	p.E363*	NI	NI	NS/P	This study
25	F/51 ^a	S-MEN1	8	378	c.1133delA	p.E378Gfs*67	CD075465	NI	FS/P	(18)
26	F/38 ^a	F-MEN1	9	413	c.1238T>G	p.L413R	CM074346	NI	MS/LP/ILP	(18)
27	M/45	F-MEN1	9	413	c.1238T>G	p.L413R	CM074346	NI	MS/LP/ILP	(18)
28	M/27 ^a	S-MEN1	9	414	c.1241T>C	p.L414P	CM010342	NI	MS ^d /LP	(18)
29	M/33	F-MEN1	9	415	c.1243C>T ^c	p.R415 ^c	CM970937	NI	NS/P	(17)
30	M/42	S-MEN1	9	415	c.1243C>T ^c	p.R415 ^c	CM970937	NI	NS/P	(17)
31	M/43 ^a	F-MEN1	9	418	c.1252G>T	p.D418Y	CM981277	NI	MS/LP/ILP	(69)
32	M/28	F-MEN1	9	423	c.1268G>A	p.W423*	CM004419	NI	NS/P	(70)
33	M/55 ^a	S-MEN1	10	461-468	c.1382_1404del23	p.E461Gfs*62	NI	NI	FS/P	(71)
34	M/7 ^a	F-MEN1	10	516	c.1546_1547insC ^c	p.R516Pfs*15 ^c	C1972640	rs767319284	FS/P	(15)
35	F/35 ^a	S-MEN1	10	516	c.1546_1547insC ^c	p.R516fs*15 ^c	C1972640	rs767319284	FS/P	(15)
36	M/54	F-MEN1	10	516	c.1546_1547insC ^c	p.R516fs*15 ^c	C1972640	rs767319284	FS/P	(15)
37*	F/42 (DN)	F-MEN1	10	516	c.1546_1547insC ^c	p.R516fs*15 ^c	C1972640	rs767319284	FS/P	(15)
38	M/38 ^a	F-MEN1	10	519	c.1554_1578del25	p.P519Efs*32	NI	NI	FS/P	This study
39	M/60 ^a	S-MEN1	10	522	c.1564_1565delGT	p.V522Rfs*8	NI	NI	FS/P	This study
40	F/19	S-MEN1	10	527	c.1579C>T	p.R527*	CM970942	rs104894261	NS/P	(16)
41	F/42	F-MEN1	10	527	c.1579C>T	p.R527*	CM970942	rs104894261	NS/P	(16)
42	F/33	F-MEN1	10	557	c.1669A>T	p.K557*	NI	NI	NS/P	This study
43	F/30 ^a	F-MEN1	Intron 3	-	c.654+1G>T	-	CS982266	rs794728622	SS/P	(72)
44	M/17	F-MEN1	Intron 3	-	c.654+1G>T	-	CS982266	rs794728622	SS/P	(72)
45	M/43	F-MEN1	Intron 3	-	c.654+1G>T	-	CS982266	rs794728622	SS/P	(72)
46	F/29	F-MEN1	Intron 3	-	c.654+1G>T	-	CS982266	rs794728622	SS/P	(72)
47	F/52	F-MEN1	Intron 3	-	c.654+1G>T	-	CS982266	rs794728622	SS/P	(72)

(Continued)

Table 2 Classification of ten in-frame/missense *MEN1* variants (4 new variants) and nine other new null variants using criteria adopted by The American College of Medical Genetics and Genomics (ACMG), combined with probability of segregation of a given variant to occur by chance (SE).

ID	Variant/effect	Phenotype	Familial segregation		In silico impact prediction tools	Populational databases	Families number with the variant	Other reported missense variants in the same codon (PM5)	Functional data	Classification (ACMG+SE)
			(number of <i>MEN1</i> carriers/ no carrier)	(SE*, final value)						
1	c.122T>C (p.L41P)/MS	F-MEN1	PP1-PM (4/7) (a: 1/16)	(0/3)	PP3	PM2	1 (present study)	–	PP2, PP4	LP=>LP
13	c.415C>T (p.H139Y)/MS	S-MEN1	– (0/3)	–	PP3	PM2	2 (present study; (15) ^a)	PM5 (p.H139D/P/Q/R) ^d	PP2, PP4, PS3	P
15	c.439A>T (p.I147F)/MS	F-MEN1	PP1-PS (16/15) (a: 1/65 536)	–	PP3	PM2	1 (18) ^b	–	PP2, PP4	LP=>P**
26	c.1238T>G (p.L413R)/MS	F-MEN1	PP1-PS (3/4) (b: 1/64)	–	PP3	PM2	2 (18) ^b	PM5 (p.L413P) ^e	PP2, PP4	LP=>P**
27	c.1238T>G (p.L413R)/MS	F-MEN1	PP1-PS (3/2) (b: 1/64)	–	PP3	PM2	2 (18) ^b	PM5 (p.L413P) ^e	PP2, PP4	LP=>P**
28	c.1241T>C (p.L414P)/MS	S-MEN1	– (0/6)	–	PP3	PM2	1 (18) ^b	PM5 (p.414Q/R) ^f	PP2	LP
31	c.1252G>T (p.D418Y)/MS	F-MEN1	PP1-PS (5/4) (a: 1/32)	–	PP3	PM2	2 (present study; 69 ^g)	PM5 (p.418H/N) ^g	PP2, PP4	LP=>P**
8	exon2:c.266_286del21/IF	F-MEN1	PP1-PM (5/3) (a: 1/16)	–	PP3	PM2	1 (Toledo et al. (18) ^b)	–	PM4, PP4	LP
10	c.354_356delGAA/IF	S-MEN1	PP1-PP (1/3)	–	PP3	PM2	1 (present study)	–	PM4, PM6, PP4	LP=>P***
20	c.802_803insGGCTGCTCT/IF	S-MEN1	–	–	–	PM2	1 (present study)	–	PM4, PP4	LP
56	c.825-30_830delinst/IF	F-MEN1	PP1-PM (5/8) (a, 1/16)	–	PP3	PM2	1 (present study)	–	PM4, PP4	LP
9	c.315_316insCCTC/FS	S-MEN1	PP1-PP (2/3)	–	PP3	PM2	1 (present study)	–	PV51, PM6, PP4	P
12	c.385_385delC/FS	S-MEN1	– (1/1)	–	PP3	PM2	1 (present study)	–	PV51, PP4	P
19	c.652_652delC/FS	F-MEN1	PP1-PM (5/4) (a, 1/16)	–	PP3	PM2	1 (present study)	–	PV51, PP4	P
21	c.903_909delCTACAC/FS	F-MEN1	PP1-PM (4/5) (a, 1/8)	–	PP3	PM2	1 (present study)	–	PV51, PP4	P
24	c.1087G>T/NS	F-MEN1	PP1-PP (2/2)	–	PP3	PM2	1 (present study)	–	PV51, PP4	P
38	c.1554_1578del25/FS	F-MEN1	PP1-PP (3/1) (a, 1/4)	–	PP3	PM2	1 (present study)	–	PV51, PP4	P
39	c.1564_1565delGT/FS	S-MEN1	– (1/0)	–	PP3	PM2	1 (present study)	–	PV51, PP4	P
42	c.1669A>T/NS	F-MEN1	PP1-PS (7/2) (a, 1/64)	–	PP3	PM2	1 (present study)	–	PV51, PP4	P
55	c.784-2A>C/SS	F-MEN1	PP1-PP (4/2) (a, 1/8)	–	PP3	PM2	1 (present study)	–	PV51, PP4	P

The criteria to pathogenicity (P) depend on segregation data coming from one family (a) or two or more families (b). For these groups, different cutoffs define a grading of evidence levels of pathogenicity: PP, supporting evidence (a, $P \leq 1/8$; b, $P \leq 1/4$); PM, moderate evidence (a, $P \leq 1/16$; b, $P \leq 1/8$); PS, strong evidence (a, $P \leq 1/32$; b, $P \leq 1/16$).

^aOne isolated index case, without familial segregation (Agarwal et al. (15)); ^bcases 15, 26, 27 (with familial segregation) and 28 (isolated index case without familial segregation) were previously reported by us (Toledo et al. (18)); ^cfamilial MEN1 (Ozturk et al. (69)); ^dAgarwal et al. (15); ^eCebrian et al. (67); ^fClinVar database, Martin-Campos et al. (75); ^gAsteria et al. (76); ^hEllard et al. (66); ⁱClinVar database; ^jHeppner et al. (77); ^kWarner et al. (78); ^l**it was classified as likely pathogenic variant by ACMG and reclassified as pathogenic after segregation equation; ^m***it was reclassified as pathogenic as its resultant mutated sequence is the same of the warm spot variant c.358_360delAAG (16, 19).

F-MEN1, familial multiple endocrine neoplasia type 1; FS, frameshift; ID, identification of the patients; IF, in-frame; LP, likely pathogenic; MS, missense; acronyms and their definitions are informed exactly as established by ACMG (44); PV51, null variant (nonsense, frameshift, canonical +/–1 or 2 splice, sites initiation codon, single or multi-exon deletion) in a gene where loss of function (LOF) is a known mechanism of disease (MEN1 gene LOF=1, gene is extremely intolerant to LOF); NA, not available; NS, nonsense; P, pathogenic; PM1, variant is located in a mutational hot spot and/or in a critical and well-established functional domain without benign variation; PM2, absent from controls in populational databases as Exome Sequencing Project, 1000 genomes or ExAC; PM4, occurrence of protein length changes due to in-frame deletions/insertions in a non-repeat region or stop-loss variants; PM5, novel missense change at an amino acid residue where other different missense change was previously reported as pathogenic; PM6, assumed as *de novo* (without confirmation of paternity and maternity); PP1, variant cosegregation with disease in multiple affected family members in a gene clearly causing the disease; PP2, missense variant in a gene that has a low rate of benign missense variation and where missense variants are a common mechanism of disease; PP3, multiple lines of computational evidence (*in silico* analysis) support a deleterious effect on the gene or gene product; PP4, patient's phenotype or family history is highly specific for a disease with a single genetic etiology; PS3, presence of well-established *in vitro* or *in vivo* functional studies supporting a damaging effect on the gene; PS4, the prevalence of the variant in affected individuals is significantly increased in comparison with controls; SE*, segregation equation (45), defined as $N=(1/2)^m$, was supported where N means the probability of the observed variant occur by chance (no pathogenic) and m defines the number of meiosis of the variant that is informative for segregation, excluding index case; S-MEN1, sporadic MEN1; SS, splice site.

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Conversely, only seven polymorphisms were detected by SS in the present cases. Six of them occurred in *MEN1*-coding region and another in IVS1 region, as the designed primers covered the intron/exon boundary of this region. Comparing data from SS and tNGS, the latter technology yielded a significantly higher polymorphism detection rate (36 vs 7; $P < 0.05$).

Multiplex ligation-dependent probe amplification (MLPA)

MLPA assay was applied for the subset of 19 *MEN1*-negative patients (8 familial and 11 sporadic cases) (Fig. 1). Overall, gross *MEN1* deletions were identified in 4% (3/76) of all cases, in 5% of *MEN1*-positive patients (5/60), in 6% (3/49) of all familial *MEN1* cases and in 37% (3/8) of *MEN1*-negative familial *MEN1* cases (Fig. 2). Additionally, gross deletions were searched but not identified in the *AIP* and *CDKN1B* genes by MLPA assay in the 16 truly *MEN1*-negative cases.

Nine (three familial and six sporadic) available patients for testing out of the 16 *MEN1*-negative patients were studied. No case harbored mutations in the *CDKIs* or *AIP* genes (Fig. 1).

Discussion

tNGS to detect MEN1 mutations

Until recently, mutations in coding region and splicing sites of *MEN1* have been usually investigated by SS complemented by MLPA for large deletions. However, SS presents limitations in the analysis of the *MEN1* gene and besides it may not routinely cover the *MEN1* non-coding regions (5, 8, 10, 13, 18, 19, 23, 28, 29, 30, 56). In addition, it is highly challenging to perform *MEN1* testing in the extended number of cases fulfilling the current criteria for genetic analysis, using SS (5).

In the present study, we applied tNGS to our 76 cases as it is faster and cheaper than WES and WGS. We compared data from tNGS with SS and both technologies presented identically high accuracy, reproducibility and repeatability with no false positive. Thus, tNGS applied for the genetic diagnosis of *MEN1* was validated as a reliable test. A small percentage of false-negative results (5%) were seen in cases with gross *MEN1* deletions, we identified by MLPA. Also, the primer design and reaction optimization of tNGS proved the specificity of the *MEN1*-targeted long-range PCR methodology, associated with no occurrence

of primer cross-reactivity, dimer formation, allelic bias or dropout (34). Additional discussion on the adoption of long-range PCR as enrichment method, criteria to select the NGS platform and cost-effectiveness of tNGS may be found in Supplementary data.

Mutations in introns far from intron/exon boundaries or untranslated or regulatory regions

Worthwhile, tNGS allowed us to methodically investigate the full *MEN1* coding and non-coding regions in all 76 *MEN1* patients and no case harbored pathogenic/likely pathogenic variants in introns far from intron/exon boundaries, untranslated or regulatory gene regions. Thus, our present data indicated that point or short indel *MEN1* pathogenic/likely pathogenic variants in these gene regions are most probably very rare in *MEN1* patients. These findings contrast with the previous hypothesis suggesting that *MEN1* patients not harboring mutations in coding regions of the *MEN1* gene would carry mutations in non-coding gene regions (5, 8, 10, 13, 18, 19, 23).

Indeed, no previous systematic study investigating both the *MEN1* coding and non-coding regions was available, precluding further conclusions (8). Concordantly, reported mutations involving the promoter, 5' or 3' untranslated regions are represented by only a few large gross *MEN1* deletions not covered by SS and identified by different assays assessing gene copy number (8, 23, 28, 29, 30, 56). In addition, a single study using SS investigated a minimal promoter region in 23 *MEN1*-negative *MEN1* familial cases and revealed no *MEN1* germline mutations (30).

Furthermore, the possible occurrence of a second germline *MEN1* pathogenic/likely pathogenic variant in *MEN1* cases harboring *MEN1* mutations could be ruled out.

Recurrent MEN1 mutations

We found recurrent *MEN1* mutations, which could be due to independent mutational events favoring the existence of warm spot *MEN1* mutations in *MEN1* (8, 19, 23). In this line, we previously documented independent mutations in cases 26 and 27 by haplotype analysis (18). Alternatively, recurrent mutations may occur due to founder mutations (4, 28, 57, 58, 59). Concordantly, we reported the c.201_201delC (p.A68Pfs*51) founder mutation (case 2, Table 1) in a large *MEN1* family with 50 mutation carriers of the southeastern Brazil that came from Veneto, Italy in 1890 (4). However, haplotype analysis could not be

Table 3 Polymorphic germline variants identified by targeted NGS in non-coding and coding regions of the *MEN1* gene of 76 unrelated MEN1 index cases, classified by ACMG criteria.

SNVs	Local	Total (n) (Het+ Hom)	% Het	dbSNP	COSMIC	1000 genomes, MAF	GnomAD (%)	AbraOM (%)	6500 exomes, MAF	ExAC (%)	ACMG classification
c.*307T>G	3'UTR	1	100	rs1804848	NI	G=0.9%	0.86	0.64	NI	NI	Benign
c.*185C>T	3'UTR	1	100	rs111895237	NI	T=0.5%	0.20	0.16	NI	NI	Benign
c.*306_*308delCTC	3'UTR	1	100	rs143341556	NI	DEL=8%	2	4.83	NI	NI	Benign
c.*302C>T	3'UTR	3	100	rs1804849	NI	T=1.2%	1.50	1.26	NI	NI	Benign
c.-35A>T	5'UTR	34	71	rs679946	NI	T=23%	32	37.97	NI	NI	Benign
c.-35A>C	5'UTR	7	100	rs679946	NI	C=20%	5.80	7.96	NI	NI	Benign
c.-6G>A	5'UTR	1	100	rs768088337	NI	A=0.2%	0.01	NI	NI	NI	Benign
c.-40G>C	5'UTR	21	100	rs552417059	NI	C=0.3%	0.34	NI	NI	NI	Benign
c.512G>A: p.R171Q	EXON 3	3	100	rs607969	23054	A=0.8%	1.22	1.72	A=1.5%	0.0008	Benign
c.597C>T: p.H204H	EXON 3	3	0	rs150512958	NI	T=0.1%	0.05	0.08	0.02%	0.06	Benign
c.1254C>T: p.D418D	EXON 9	40	83	rs2071313	NI	T=30%	38.50	31.44	T=39%	0.39	Benign
c.1299T>C: p.H433H	EXON 9	67	9	rs540012	NI	T=2%	99.20	97.70	T=2.5%	0.993	Benign
c.1508G>A: p.G503D	EXON 10	1	100	rs375804228	1188290	A=0.04%	0.05	NI	NI	0.0005	Benign
c.1624A>G: p.T541A	EXON 10	73	18	rs2959656	255213	A=15%	93	91.37	A=9%	0.94	Benign
c.-23-16C>G	IVS1	10	70	rs09606	NI	G=17%	16	10.00	NI	30	Benign
c.-23-24T>C (g.1139T>C)	IVS1	4	100	NI	NI	NI	NI	10.22	NI	NI	Benign
c.-23-273G>A (g.890G>A)	IVS1	2	100	NI	NI	NI	NI	NI	NI	VUS	
c.-23-277G>A (g.894G>A)	IVS1	3	33	NI	NI	NI	NI	NI	NI	NI	VUS
c.445+306G>A	IVS2	2	100	rs34047150	NI	A=0.6%	1	1.80	NI	NI	Benign
c.446-198G>A	IVS2	1	100	rs11607366	NI	A=0.6%	2.20	1.53	NI	NI	Benign
c.446-347G>A	IVS2	1	100	rs75989060	NI	A=0.7%	0.75	NI	NI	NI	Benign
c.445+401_445+402insTT	IVS2	20	100	rs11404218	NI	NI	NI	NI	NI	NI	Likely benign
c.445+539G>A	IVS2	13	92	rs67808744	NI	A=15%	10.50	NI	NI	NI	Benign
c.445+710delIT	IVS2	30	100	NI	NI	NI	NI	NI	NI	NI	Likely benign
c.445-691delIT (g.2505_2505delIT)	IVS2	1	100	NI	NI	NI	NI	NI	NI	NI	VUS
c.445+719G>A	IVS2	10	80	rs11231873	NI	A=13%	NI	NI	NI	NI	Benign
c.445+709_710delIT	IVS2	40	100	NI	NI	NI	NI	NI	NI	NI	Likely benign
c.445+183G>A	IVS2	14	86	rs624975	NI	A=17%	11	11.12	NI	NI	Benign
c.445+177G>A	IVS2	1	100	rs192961797	NI	A=0.3%	0.09	NI	NI	NI	Benign
c.445+401_445+402insT	IVS2	19	100	rs11404218	NI	NI	NI	NI	NI	NI	Likely benign
c.446-662A>G	IVS2	1	100	rs374393702	NI	NI	0.06	NI	NI	NI	Benign
c.912+283C>T	IVS6	72	16.67	rs504195	NI	C=16%	88.90	90.10	NI	NI	Benign
c.912+295_912+297delTGA	IVS6	66	18.2	rs2307769	NI	TGA=20%	87.00	90.45	NI	NI	Benign
c.1049+115A>G	IVS7	12	83.33	rs669976	NI	G=17%	11.70	10.84	NI	NI	Benign
c.1049+213delIT (g.5276_5276delIT)	IVS7	26	100	NI	NI	NI	NI	NI	NI	NI	Likely benign
c.1350+103G>C	IVS9	45	75.56	rs654440	NI	C=23%	31	38.51	NI	NI	Benign

In bold, new polymorphisms; italic, polymorphisms detected by both Sanger sequencing and NGS.

A, adenine; ACMG, American College of Medical Genetics and Genomics (47); C, cytosine; del, deletion; Ensembl, ENST00000312049; G, guanine; Het, heterozygosis; Hom, homozygosis; ins, insertion; IVS, intervening sequence; MAF, minor allele frequency; MEN1, multiple endocrine neoplasia type 1; n, number of patients; NGS, next-generation sequencing; NI, not identified; NM_130799.2; SNV, single nucleotide variation; UTR, untranslated regions; T, thymine.

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performed in the 22 remaining index cases, preventing adequate distinction between independent and founder mutations. Further careful genealogical, geographic and haplotype studies are planned to clarify this topic.

Pathogenic MEN1 germline variants

MEN1-tNGS

As expected, the overall prevalence of germline *MEN1* mutations was similar to other studies (5, 19, 23, 56). As anticipated, the prevalence of germline *MEN1* mutations was higher in familial than sporadic cases (5, 14), and the 16 newly uncovered inherited *MEN1* cases lead us to perform genetic screening of their at-risk relatives.

Missense variants represent up to 25% of the 576 mutations reported in *MEN1*, whereas in our cases a slightly lesser percentage was observed (14%) (23). The confirmation of pathogenicity of missense variants is challenging since functional studies of each of them may be infeasible. Most missense and in-frame variants, we found were initially classified as likely pathogenic using the ACMG criteria (46, 47). In turn, when we applied equation to calculate probability of at random occurrence of segregation, part of them were reclassified as pathogenic (48). However, the reclassification did not result in changes in the clinical management as no variant was considered benign, likely benign or variant of unknown significance.

MEN1-MLPA assay

A highly variable frequency of gross *MEN1* deletions has been reported (1–33%) (5, 8, 23, 28, 29, 30, 56). In our series, the prevalence of gross deletions (5%), considering the different mutation types, was similar to the larger reported series (<5%) including *MEN1* and *MEN1*-related phenotypes (8, 23, 28, 29, 30, 56). In addition, we showed occurrence of gross deletions in 6% of all familial *MEN1* cases and in 37% of the *MEN1*-negative familial *MEN1* cases, suggesting the importance of the positive familial history as predictive indicator for detection of gross *MEN1* deletions. Conversely, no gross deletion was seen in the 11 *MEN1*-negative sporadic cases. Further in deep studies on MLPA applied to *MEN1* are planned.

Likely pathogenic variants

All in-frame variants were initially classified as likely pathogenic. Concordantly, in-frame variants are likely

to cause deleterious effects on menin. One of our cases harbored a variant deleting codons 265–267. Two previous studies reported different in-frame deletions occurring at this same codon in two unrelated *MEN1* families (60, 61), and a definite segregation was shown in five members of a family (61). The association of typical *MEN1* clinical features in our patient, its classification as likely pathogenic by ACMG criteria, and the previously reported mutations in this highly conserved DNA region favor this variant's classification as likely pathogenic. Segregation analysis (SE) may reinforce the pathogenicity of the variant, as observed to missense variants in our study.

Non-pathogenic MEN1 germline variants

So far, 24 different *MEN1* polymorphisms have been reported in more than 1100 *MEN1* patients, 12 in the coding region (10 synonymous and 2 non-synonymous), nine in introns and three in untranslated regions (8, 19). The 36 different *MEN1* polymorphisms (3 VUS) we identified in the present study represent a substantial increase to those previously detected by SS (8, 19). Also, the prevalence of polymorphisms we verified (36/76; 47%) was substantially higher than that reported in the literature (24/1133; ~2%; $P < 0.05$) (19). Comparing our data with those reported by Lemos and Thakker (19), we detected 22 intronic polymorphisms vs nine reported by them (three-fold more), seven occurring in untranslated regions vs three, and 7 exonic polymorphisms vs 12, respectively. The finding of 4-fold more polymorphisms detected in non-exonic than in exonic regions clearly reflects the higher ability of tNGS to routinely detect variants in the non-coding region, compared to SS.

Of note, a better characterization of polymorphisms as benign, likely benign or VUS, may be valuable to clearly differentiate them from mutations for at least two reasons. One, variant misclassification may lead to inadequate clinical management and unnecessary surgery interventions (62). In addition, because disease-modifying polymorphisms in genes besides *MEN1* could influence *MEN1* phenotype (53, 63). Overall, our data provide strong evidence that the non-coding region of the *MEN1* gene has been scarcely sequenced so far.

The importance of the regional genomic repositories was here reinforced by the absence in six international databases of the polymorphism c.-23-24T>C (g.1139T>C) IVS1, which was present in 10% of the 609 Brazilian elderly controls from ABraOM and in our cases.

Non-mutation carriers

Compared with 60 mutation carriers, the 16 genuinely non-*MEN1* mutation carriers were older at the time of diagnosis, and most patients did not exhibit a third *MEN1*-related tumor, confirming recent data (14). Of note, five familial non-*MEN1* mutation carriers might be phenocopies or harbor so far unreported mutations.

Although one of our focus was to verify the robustness of *MEN1*-tNGS for the genetic diagnosis of *MEN1*, we also tested a multi-gene tNGS panel examining *CDKN1s* (*p15*, *p18*, *p21*, *p27*) and *AIP* genes in a limited number of available *MEN1*-negative carriers. No mutation was detected in *CDKN1s* and *AIP* genes, in agreement with the rarity of the association of these genes with *MEN1*-like phenotypes (20, 21, 22). Additionally, no gross deletions in *p27* and *AIP* were seen in these cases.

Non-mutation carriers might be due to *de novo* post-zygotic somatic mutations leading to mosaicism, mutations in so far undiscovered *MEN1*-related genes or random association of sporadic *MEN1*-related tumors.

Further studies are needed to clarify whether large multi-gene panels applied to *MEN1* in clinical practice would be helpful, accurate and cost-effective. Such panels may not only test *MEN1*, *CDKN1s* (*p15*, *p18*, *p21*, *p27*) and *AIP* genes but also *HRPT2*, *CASR*, *GNA11*, *AP2S1* and *GCM2* genes, as jaw tumor-HPT syndromes, familial hypocalciuric hypercalcemia and familial isolated HPT may mimic *MEN1* phenotypes (5, 8, 13, 64).

Recently, similar custom multi-gene tNGS panels have been applied for several endocrine tumor diseases, as pheochromocytoma/paraganglioma, pituitary adenoma and pancreatic neuroendocrine neoplasia (41, 42, 65). The effective validation of such panels using tNGS would have the potential to substitute the exhaustive, expensive gene by gene sequential analysis in *MEN1*-negative cases.

At present, WES or WGS have been mostly reserved to patients whose previous genetic analyses to all known *MEN1*-related genes, and after applying MLPA assay, resulted negative. Beyond WES and WGS, linkage analyses may be applied to informative families either for chromosomal mapping of *MEN1* region or to investigate potentially new *MEN1*-causing genes (8).

Although ACMG classification has not been applied to *MEN1* variants, we decided to follow it in an attempt to better characterize DNA variants we found in our patients. Thus, some variants in non-coding region were classified either as likely benign or VUS. This classification may impact clinical practice, as *MEN1*-negative patients

harboring a VUS should not be simply dismissed but clinically followed up periodically.

Potential limitations of the present study could be related to possible tumor mosaicism that could not be investigated in sporadic cases, the relatively limited sample size, and the presence of hypothetical founder mutations in the cases carrying repetitive mutations. However, these pitfalls did not prevent drawing conclusions from our data. Additional functional studies are needed to better characterize the three VUS we found in untranslated, regulatory or deep intronic *MEN1* regions by tNGS. Thus, up to now, we cannot convincingly rule out the hypothesis that pathogenic/likely pathogenic variants may occur in *MEN1* non-coding region. In cases they occur in patients harboring pathogenic variants, a potential modulating factor could be supported in future.

Our validated protocol based on tNGS long-range PCR showed to be highly effective to be implemented in the clinical practice for *MEN1* diagnostic testing, although further confirmatory data involving larger number of patients are welcomed. The decision to analyze other *MEN1*-related genes (gene panel) in clinical practice should be personalized considering phenotypic features, available methodologies and their regional cost-effectiveness.

In summary, we did not find any point or short indel mutations in the non-coding region of the *MEN1* in our 76 *MEN1* patients, which points to the rareness of such events. In addition, our data support that *MEN1* genetic testing based on a tNGS long-range PCR approach is a sensitive, affordable and robust method, with a high diagnostic yield, for detection of *MEN1* mutations. This opens the possibility of providing *MEN1* testing for a larger number of cases fulfilling the current criteria for *MEN1* testing. Ultimately, this potentially promising procedure may expand and improve prenatal and preimplantation genetic diagnosis, genetic screening and counseling, early diagnosis and therapeutic management of *MEN1*-related tumors, which may positively impact morbidity and mortality of patients with *MEN1* and *MEN1*-related phenotypes.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/EJE-18-0430>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this study.

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