Familial Cancer
DOI 10.1007/s10689-014-9702-y

ORIGINAL ARTICLE

Deletion of exons 1–3 of the *MEN1* gene in a large Italian family causes the loss of menin expression

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Abstract Multiple endocrine neoplasia type 1 (MEN1) syndrome is an autosomal dominant disease, characterized by parathyroid adenomas, endocrine gastroenteropancreatic tumors and pituitary adenomas, due to inactivating mutations of the *MEN1* gene (chromosome 11q13). *MEN1* mutations are mainly represented by nonsense, deletions/insertions, splice site or missense mutations that can be detected by direct sequencing of genomic DNA. However, MEN1 patients with large heterozygous deletions may escape classical genetic screening and may be misidentified as phenocopies, thereby hindering proper clinical surveillance. We employed a real-time polymerase chain reaction application, the TaqMan copy number variation assay, to evaluate a family in which we failed to identify an *MEN1* mutation by direct sequencing, despite a clear clinical diagnosis of MEN1 syndrome. Using the TaqMan copy number variation assay we identified a large deletion of the *MEN1* gene involving exons 1 and 2, in three affected family members, but not in the other nine family members that were to date clinically unaffected. The same genetic alteration was not found in a group of ten unaffected subjects, without family history of endocrine tumors. The *MEN1* deletion was further confirmed by multiplex ligation-dependent probe amplification, which showed the deletion extended from exon 1 to exon 3. This new approach allowed us to correctly genetically diagnose three clinical MEN1 patients that were previously considered as MEN1 phenocopies. More importantly, we excluded the presence of genetic alterations in the unaffected family members. These results underline the importance of using a variety of available biotechnology approaches when pursuing a genetic diagnosis in a clinically suggestive setting of inherited endocrine cancer.

Keywords MEN1 · Gene deletions · Quantitative PCR · Genetic analysis · MLPA

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Introduction

Multiple endocrine neoplasia type 1 (MEN1) syndrome is an autosomal dominant disease, characterized by parathyroid adenomas, endocrine gastroenteropancreatic tumors and pituitary adenomas, due to inactivating mutations of the *MEN1* gene on chromosome 11q13. *MEN1* mutations are scattered within and around the menin open reading frame and are mainly represented by nonsense, deletions/insertions, splice site or missense mutations which can be detected readily by direct sequencing of genomic DNA [1, 2]. *MEN1* germline mutations are identified in 70 % of the

Published online: 13 February 2014

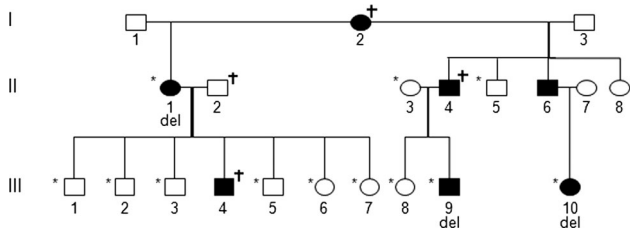

Fig. 1 Pedigree of the MEN1 kindred. *Closed* and *open symbols* represent symptomatic and asymptomatic individuals, respectively. * = tested patients; † = deceased family members; *del* = patients presenting the deletion encompassing exon 1–3

familial forms and in 10 % of sporadic cases, while in almost 20 % of clinically affected patients *MEN1* gene analysis fails to detect germline mutations (cases that are termed MEN1 phenocopies) [3]. Indeed, 5–25 % of MEN1 patients do not harbour mutations that can be identified by sequencing in the *MEN1* gene coding region, but have whole or partial gene deletions. Mutations in the promoter, 5′-untranslated and 3′-untranslated regions have rarely been screened for systematically [4–9].

The recent Endocrine Society clinical practice guidelines for MEN1 recommend performing multiplex ligation-dependent probe amplification analysis (MLPA) for the detection of exonic deletions in patients in whom a *MEN1* mutation is not identified within the coding region and splice sites [1]. Alternatively, long-range polymerase chain reaction (PCR) amplification (LRPA) can be employed [10].

We here describe a *MEN1* deletion identified by employing a new method, based on a quantitative PCR assay, in a large Italian family with three family members clinically presenting MEN1, previously diagnosed as MEN1 phenocopies.

Materials and methods

Patients

We studied a large Italian family with 21 members, 17 of whom are living, including three subjects showing a clear MEN1 phenotype (see Fig. 1; Table 1). Among living family members, 12 consented to blood withdrawal for genetic testing and signed an informed consent, including the three affected patients. Among the four family members who were not alive, three died from malignant tumors not classically associated with the MEN1 phenotype (exocrine pancreatic cancer (n = 2); laryngeal cancer (n = 1)), while the fourth family member died from metastatic spread of a bronchial carcinoid. This latter subject also had a history of primary hyperparathyroidism, a prolactin-secreting pituitary adenoma, papillary thyroid carcinoma, bilateral adrenal hyperplasia, with a clinical MEN1 phenotype similar to the other affected family members (see Table 1). Genetic testing of this subject was not possible.

Table 1 Clinical phenotype and history of the three affected family members

II-1 (F, 74 years)	
56 years	Left parathyroid adenoma
58 years	Right parathyroid adenoma
	PRL-secreting pituitary macroadenoma
62 years	Left adrenal gland macronodular hyperplasia
65 years	Pancreatic glucagonoma
III-9 (M, 48 years)	
38 years	ACTH-secreting pituitary adenoma
42 years	Multiple parathyroid adenomas
48 years	Pancreatic neuroendocrine carcinoma with lymphnode metastases
	Bilateral diffuse adrenal gland macronodular hyperplasia
III-10 (F, 23 years)	
15 years	Left parathyroid adenoma
	PRL-secreting pituitary microadenoma
23 years	Left adrenal gland macronodular hyperplasia
	Pancreatic insulinoma

DNA isolation and direct sequencing

Genomic DNA (gDNA) was isolated from each subject's whole blood by using the QIAamp DNA Blood Mini Kit (QIAGEN, Milano, Italy) on the QIAcube automated system (QIAGEN). DNA from somatic cells was isolated by using the QIAamp DNA FFPE Tissue Kit (QIAGEN) from paraffin-embedded parathyroid adenoma and pancreatic neuroendocrine carcinoma from patient III-9. At least 100 ng of DNA were used for each application.

Direct DNA sequencing of the *MEN1* coding region and intron–exon boundaries using sequence-specific primers was performed as described previously [11–13]. A pool of normal human gDNAs from six male and six female subjects was employed as control for genetic analysis (referred to as "normal control"). Similarly, a group of eight unrelated patients undergoing *MEN1* genetic analysis were studied.

TaqMan® gene copy number assays

The pre-designed TaqMan® gene copy number assays (Hs01998305_cn; Hs02189358_cn; Hs01221989_cn; Hs03796573_cn; Hs03794510_cn; Hs03785674_cn; Hs00736968_cn; Hs03768072_cn; Hs03793578_cn; Hs03800380_cn; Hs03773377_cn; Hs01778293_cn; Hs01920054_cn; Hs01699461_cn; Hs01957966_cn; Hs00823207_cn; Life

Technologies, Milano, Italy), covering the *MEN1* sequence almost completely, were used to detect gene copy number in quadruplicate gDNA samples.

A sample not containing DNA template (no template control, NTC) was used as negative control, while normal control with a known copy number for the gene of interest was used as calibrator in quadruplicate. Briefly, 5 ng of gDNA were amplified by adding target-specific forward and reverse primers and the genotyping master mix, containing AmpliTaq Gold® DNA Polymerase (Life Technologies), according to the manufacturer's instructions. FAM™ dye-labeled MGB probes covering the MEN1 encoding sequence and the flanking regions were used. Samples were then analyzed on the ABI PRISM® 7900 Sequence Detection Systems (Life Technologies), using PCR cycling conditions according to user's manual (40 cycles = 95 °C for 15 s and 60 °C for 60 s). The assays were run in duplex with VIC dye-labeled TaqMan copy number reference assays. These reference genes are known to be present in two copies in a diploid genome, regardless of the copy number of the target of interest and are used to normalize sample input and minimize the variation between the targets of the test and reference assays. The TaqMan copy number reference assay (RNase P) containing the reference sequence-specific forward and reverse primers and the VIC® dye-labeled TAMRA™ probes was employed. Data from 7900HT were analyzed by SDS software and then exported on copy caller software as described by manufacturer (see TaqMan® copy number assays protocol applied biosystem and CopyCaller™ Software User Guide Applied Biosystem).

Multiplex ligation-dependent probe amplification

Deletions within the *MEN1* gene were assessed in gDNA samples using the SALSA MLPA probemix P017-C1 MEN1 (version 15) (MRC-Holland, Amsterdam, The Netherlands). Reference and control probe-pairs that were specific to unrelated genetic regions were also included in the probe-mix. All probes had amplification products from 142 to 373 nucleotides in length and had an annealing temperature >70 °C as per the RAW probe program (MRC-Holland, Amsterdam, The Netherlands). PCR products were analyzed on an AB3130 XL capillary electrophoresis apparatus (Applied Biosystems, Lennik, Belgium). Copy number quantification involved normalization of the peak area of the *MEN 1*-specific MLPA probe by dividing it by the combined areas of the control probes. This ratio was compared with the similar ratio obtained from control DNA. Deletion was observed when the wild-type signal was reduced by 35–50 % for each *MEN 1*-specific probe.

Immunohistochemistry

Immunohistochemistry to detect menin reactivity was performed by employing the goat polyclonal anti-menin antibody (N-19 sc-8201; Santa Cruz Biotechnology, Heidelberg, Germany), as previously described [14, 15]. Normal parathyroid tissue was employed as positive control, while negative controls were performed by omission of the primary antibody.

Results

MEN1 mutation studies

Direct *MEN1* sequencing failed to identify any mutation. On the contrary, as shown in Fig. 2a, b, TaqMan® gene copy number assays using specific probes for *MEN1* exon 1 assessed that the three affected patients (II-1, III-9 and III-10) displayed half of the gDNA quantity as compared to the normal control (NC). In contrast, the nine family members that were clinically normal and without MEN1 features at the time of investigation, as well as the eight unrelated patients, displayed a normal gDNA copy number for exon 1. Similar results were found for the assay using a probe specific for exon 2 (Fig. 2c). On the contrary, the assay using a probe specific for *MEN1* regions different from exon 1 and exon 2 (exon 11 is shown in Fig. 2d) showed the same gDNA quantity for all the examined subjects. Therefore, these results indicate that *MEN1* exons 1 and 2 are deleted in the three affected patients, but not in the unaffected family members, as well as in the unrelated patients.

Accordingly, MLPA analysis confirmed the presence of exon 1 and 2 deletions at the *MEN1* locus in the three affected family members, but not in the unaffected family members (Fig. 3). In addition, MLPA analysis also showed the presence of a deletion of exon 3 in the same subjects that was not detected by the TaqMan copy number assay, since the TaqMan probe set did not cover exon 3.

In addition, the TaqMan copy number assay was performed on pancreatic neuroendocrine carcinoma from an affected patient. This showed a copy number of 0 for exons 1 and 2, while the other exons covered by the commercial probes had a copy number of 2, which indicates that the loss of heterozygosity was limited to only part of the MEN1 gene.

Immunohistochemical study of menin

MEN1 deletions in MEN1-related tumors are likely to result in a reduced (or absent) menin staining. As shown in Fig. 4, menin staining was absent in the pancreatic

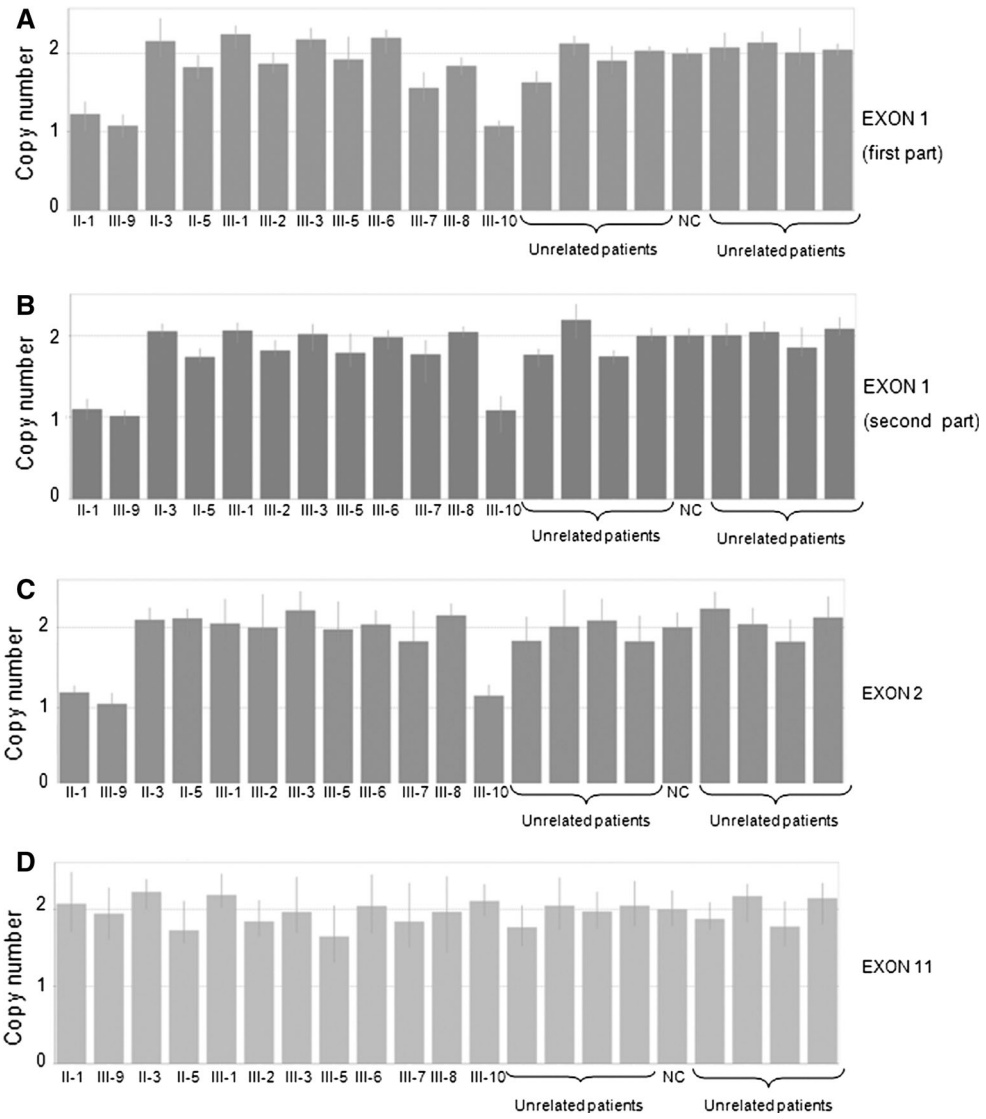

Fig. 2 MEN1 exon 1 and exon 2 copy number assay. Exon 1–11 of the MEN1 gene were amplified by using TaqMan® gene copy number assays, as described in the "Materials and methods". **a** MEN1 exon 1 (first part) copy numbers in affected (II-1, III-9, and III-10) and unaffected family members (II-3, II-5, III-1, III-2, III-3, III-5, III-6, III-7, III-8), as well as in the NC and in eight unrelated patients. **b** MEN1 exon 1 (second part) copy numbers in affected (II-1, III-9, and III-10) and unaffected family members (II-3, II-5, III-1, III-2, III-3, III-5, III-6, III-7, III-8), as well as in the NC and in eight unrelated patients. **c** MEN1 exon 2 copy numbers in affected (II-1, III-9, and III-10) and unaffected family members (II-3, II-5, III-1, III-2, III-3, III-5, III-6, III-7, III-8), as well as in the NC and in eight unrelated patients. **d** MEN1 exon 11 copy numbers in affected (II-1, III-9, and III-10) and unaffected family members (II-3, II-5, III-1, III-2, III-3, III-5, III-6, III-7, III-8), as well as in the NC and in eight unrelated patients

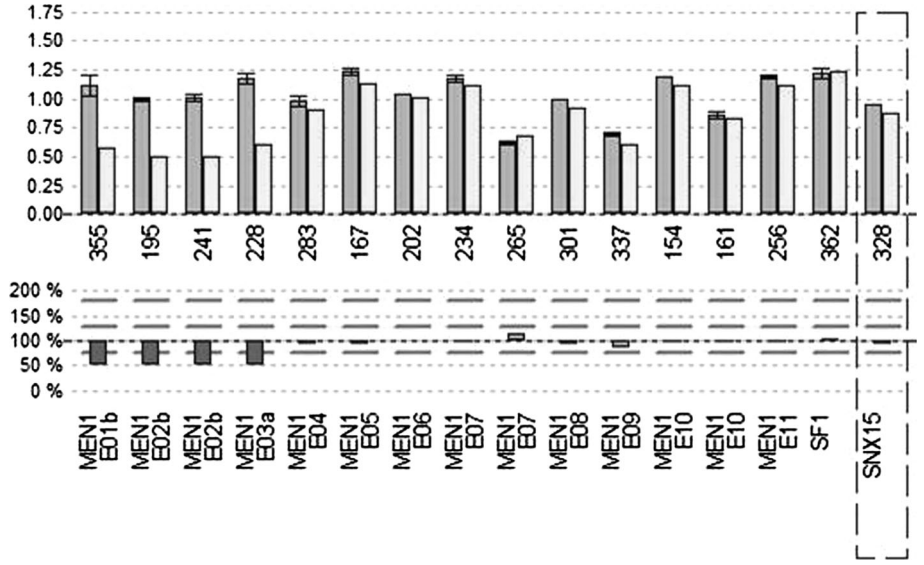

Fig. 3 Results of MLPA analysis of the MEN1 exons in the affected patients. A 50 % decrease in signal for exons 1, 2 (in both exon 2 probes) and 3 is seen in the *lower panel* of the figure. SF1 and SNX15 are unrelated reference genes that occur before and after the MEN1 gene region

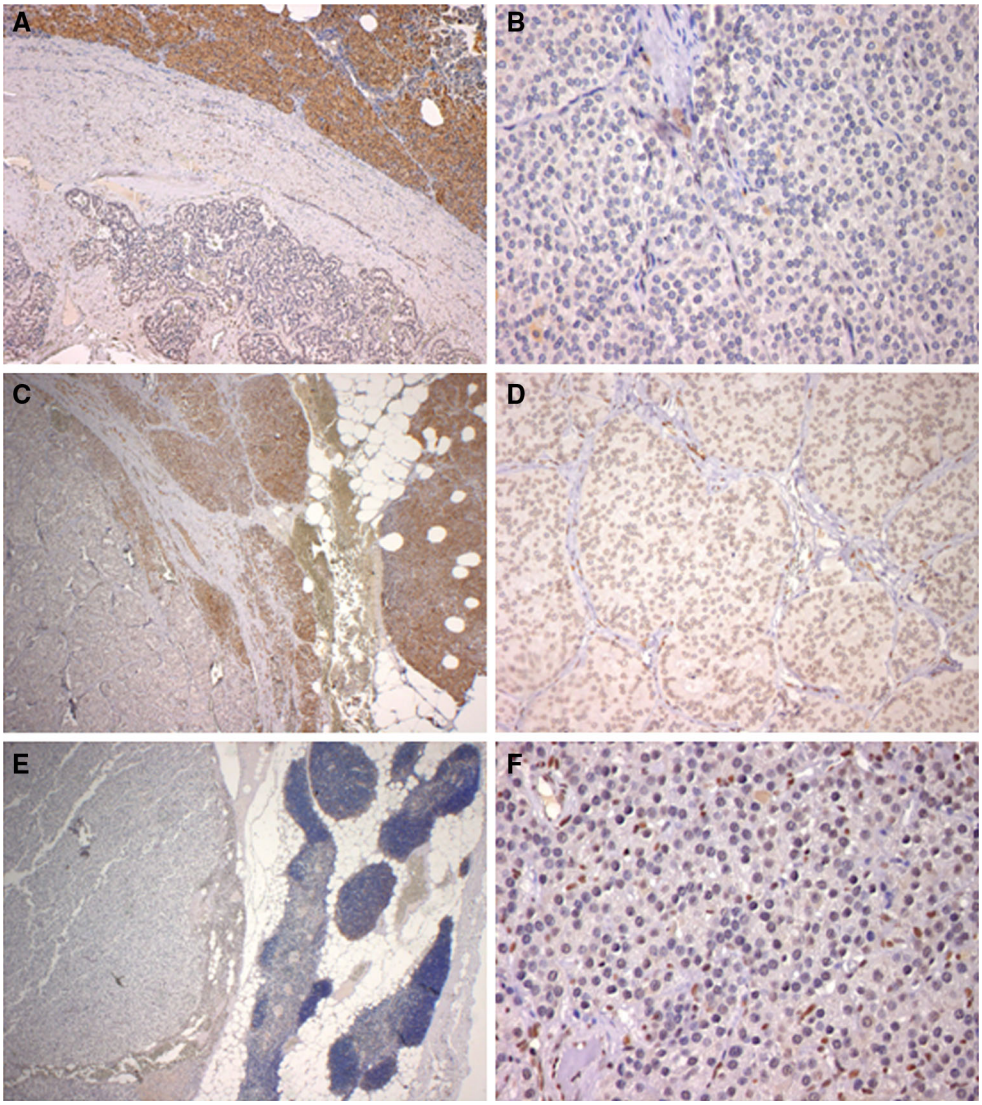

Fig. 4 Immunohistochemistry for menin. **a**, **b** Menin immunostaining of a representative area of the pancreatic glucagonoma (and adjacent normal pancreatic tissue) of patient II-1. Original magnification of ×200 for (**a**) and ×400 for (**b**). **c**, **d** Menin immunostaining of a representative area of the pancreatic neuroendocrine carcinoma (and adjacent normal pancreatic tissue) of patient III-9. Original magnification of ×200 for (**c**) and ×400 for (**d**). **e**, **f** Menin immunostaining of a representative area of parathyroid tumours (and adjacent normal thymic tissue) of patient III-10. Original magnification of ×200 for (**e**) and ×400 for (**f**)

glucagonoma of patient II-1 (Fig. 4a, b), as well as in the pancreatic neuroendocrine carcinoma of patient III-9 (Fig. 4c, d) and in a parathyroid tumour of patient III-10 (Fig. 4e, f).

Discussion

In this study, gross deletions in the *MEN1* gene were investigated by TaqMan copy number assay in a large family with three affected members showing MEN1 phenotype, who were negative for *MEN1* gene mutations on direct DNA sequencing. In these affected members we identified a gross deletion involving exon 1 and exon 2 of the *MEN1* gene, which was absent in the remaining nine living family members and in eight sporadic mutation-negative cases studied. The applied method allowed a definite diagnosis of MEN1 in these cases, with significant implications for both patients and their families. Indeed, the patients were correctly identified as *MEN1* mutation carriers (and not phenocopies) and their relatives as non-carriers. The latter classification allowed us to stop the intensive clinical follow-up they were undergoing, with a considerable psychological relief and sparing of clinical resources. While this approach was valuable in the current case, the TaqMan copy number assay may not be useful to

identify deletions located in *MEN1* gene regions different from the one detected in the presented family, that are not covered by this method. In addition, the latter cannot precisely identify the extent of the deleted exon 2 region (i.e. whether it extends to the whole exon 2 or to exon 3). This evidence is further underlined by the results of MLPA, showing that exon 3 is also deleted in the affected members of this family.

Previous published work reported alternative methods, such as MLPA, LRPA, reverse-transcription-PCR, gene dose assay and Southern blot, that allow the identification of *MEN1* deletions [16–23]. Our approach has some advantages as compared to other methodologies, such as Southern blot analysis [10], being much simpler and less labour-intensive similarly to MLPA, which is considered the gold standard for gene copy evaluation. While currently available commercial MLPA kits cover all exons of *MEN1*, additional methods, such as LRPA, may be required for complete gene evaluation. In addition, MLPA is highly sensitive to sample purity and to small changes in experimental conditions. Therefore, it should be performed in specialized reference centres that guarantee reliable results. These issues may hamper the routine clinical diagnostic application of such methods in non-specialized laboratories.

The TaqMan copy number assay may represent an alternative method to identify *MEN1* deletions and/or duplications. As compared with MLPA, TaqMan copy number variation assays has comparable costs in terms of reagents, requires less time and specialized equipment/expertise to be performed. Indeed, the employed technique provides reliable results after a single step, represented by a quantitative PCR, following gDNA isolation. In addition, the employed assay can also detect deletions extending to the minimal *MEN1* promoter region, allowing the identification of deletions in regions that are excluded from the routinely performed investigations. However, none of the commercially available kits, concerning both TaqMan copy number assay and MLPA, completely cover the entire *MEN1* gene region. As noted above, the MEN1 MLPA kit demonstrated that the deletion extended to exon 3, which indicates that the probe-mix used in the TaqMan assay would require significant optimization to perform well across all coding regions. Following such optimization, both techniques could be useful when searching for a large MEN1 deletion/insertion.

In our series, the phenotype of the affected patients was similar. All of them had developed hyperparathyroidism, a pituitary adenoma (Cushing's disease and PRL-oma), a pancreatic endocrine tumor (glucagonoma, non functioning well-differentiated endocrine carcinoma, insulinoma) and bilateral adrenal macronodular hyperplasia, even if occurring at different times along their clinical history. Another large deletion similar to the one we describe here was reported by Bergman et al. [25] in a patient with classical MEN1 phenotype. However, it must be pointed out that in that patient the deletion began upstream of the gene and terminated before exon 6, obliterating the start codon, being much more extensive than the one described here. Therefore, the functional consequences, not explored by Bergman et al., as well as the clinical phenotype may not completely overlap. A more recent report [24] describes a Spanish family with ten family members showing a deletion encompassing *MEN1* exons 1 and 2, detected by MLPA. Among gene mutation carriers, eight family members displayed at least one MEN1 clinical manifestation (hyperparathyroidism in eight, prolactinomas in two and gastrinomas in three) after 12 years of follow-up. Our findings are slightly different, since our patients with an exon 1–3 deletion displayed the full MEN1 phenotype. Therefore, as with point mutations, clinical appearance among family members showing a similar genetic abnormality may differ [25], supporting the hypothesis that the phenotypes of the MEN1 cases with gross deletions are not significantly different from those with other *MEN1* mutations [10], that lack a direct genotype-phenotype correlation even though a higher risk of death secondary to a MEN1 tumor has been reported in patients with mutations affecting the JunD interacting domain [26]. Further studies on new families will be necessary to assess the lack of correlations between *MEN1* mutations and clinical manifestations of the disorder also in *MEN1* mutation carriers characterized by the loss of exons 1–3.

The majority of reported *MEN1* mutations, such as frameshift and nonsense mutations are predicted to result either in a truncated protein, with the consequent loss of functional domains or in loss of the translated protein because of nonsense-mediated mRNA decay [5]. Deletion of the first three exons likely determines the lack of MEN1 mRNA transcription and therefore no menin protein in the tumour samples, with important consequences at cellular level. In the tumoral tissue samples of an affected family member we could demonstrate somatic loss of *MEN1* exons 1 and 2, indicating the absence of wild-type *MEN1* gene exon 1 and exon 2. Therefore, we supposed that menin protein was not expressed in these tissues. And indeed, no menin immunostaining in the pathological tissues could be found, supporting the hypothesis that the deletion prevents menin transcription and translation. The molecular mechanisms that lead to such large germline deletions, however, remain to be elucidated [18].

Our work underlines that direct sequencing is not sufficient for a complete genetic analysis in patients with MEN1 phenotype, since, even if in rare cases, it cannot identify the underlying genetic alteration in all patients with a high clinical suspicion of MEN1. New approaches, such as real-time PCR and MLPA, may help us to achieve

a correct diagnosis also in patients not recognised by standard sequencing-based testing protocols.

Acknowledgments This work was supported by grants from the Italian Ministry of Education, Research and University (FIRB RBAP11884 M, RBAP1153LS, 2010TYCL9B_002), Fondazione Cassa di Risparmio di Ferrara, and Associazione Italiana per la Ricerca sul Cancro (AIRC) in collaboration with Laboratorio in rete del Tecnopolo "Tecnologie delle terapie avanzate" (LTTA) of the University of Ferrara; and by the Fonds d'Investissement de Recherche Scientifique (FIRS) of the Centre Hospitalier Universitaire (CHU) de Liège and Pfizer. S. A.

Conflict of interest The authors declare that they have no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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