

# ***MEN1* in pancreatic endocrine tumors: analysis of gene and protein status in 169 sporadic neoplasms reveals alterations in the vast majority of cases**

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## **Abstract**

Pancreatic endocrine tumors (PETs) may be part of hereditary multiple endocrine neoplasia type 1 (MEN1) syndrome. While *MEN1* gene mutation is the only ascertained genetic anomaly described in PETs, no data exist on the cellular localization of MEN1-encoded protein, menin, in normal pancreas and PETs. A total of 169 PETs were used to assess the i) *MEN1* gene mutational status in 100 clinically sporadic PETs by direct DNA sequencing, ii) immunohistochemical expression of menin in normal pancreas and 140 PETs, including 71 cases screened for gene mutations, and iii) correlation of these findings with clinical–pathological parameters. Twenty-seven PETs showed mutations that were somatic in 25 patients and revealed to be germline in 2 patients. Menin immunostaining showed strong nuclear and very faint cytoplasmic signal in normal islet cells, whereas it displayed abnormal location and expression levels in 80% of tumors. PETs harboring *MEN1* truncating mutations lacked nuclear protein, and most PETs with *MEN1* missense mutations showed a strong cytoplasmic positivity for menin. Menin was also misplaced in a significant number of cases lacking *MEN1* mutations. In conclusion, the vast majority of PETs showed qualitative and/or quantitative alterations in menin localization. In 30% of cases, this was associated with *MEN1* mutations affecting sequences involved in nuclear localization or protein–protein interaction. In cases lacking *MEN1* mutations, the alteration of one of the menin interactors may have prevented its proper localization, as suggested by recent data showing that menin protein shuttles between the nucleus and cytoplasm and also affects the subcellular localization of its interactors.

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## **Introduction**

Pancreatic endocrine tumors (PETs) arise sporadically or in the context of hereditary cancer syndromes (Capelli *et al.* 2009), the most common of which is multiple endocrine neoplasia type 1 (MEN1) syndrome,

an autosomal dominant disorder caused by germline alterations of the *MEN1* gene. The syndrome is characterized by the combination of neoplastic–hyperplastic lesions affecting the parathyroids, enteropancreatic endocrine tissues, and the anterior pituitary gland.

Less frequently, patients can develop tumors in other endocrine and non-endocrine tissues (Marx *et al.* 1999, Pannett & Thakker 1999).

Despite extensive studies, the genetic background of PET is still poorly understood. The most common anomaly also encountered in sporadic PETs is *MEN1* gene inactivation due to mutation of one allele and loss of the second allele (Capelli *et al.* 2009). Indeed, allelic losses at chromosome 11q13, which includes the locus of *MEN1* gene (Larsson *et al.* 1988), are found in 46% of sporadic PETs (Speel *et al.* 1999, Rigaud *et al.* 2001), while the reported frequency of *MEN1* mutations is lower, thus suggesting that additional oncosuppressor genes lie on this chromosomal arm (Eubanks *et al.* 1994, Chakrabarti *et al.* 1998, Gortz *et al.* 1999, Jonkers *et al.* 2006). *MEN1* gene encodes for menin, a 610 amino acid protein (Chandrasekharappa *et al.* 1997, Lemmens *et al.* 1997) that is ubiquitously expressed in adult tissues (Chandrasekharappa *et al.* 1997) and located prevalently in the nucleus via three nuclear localization signals (NLS1, NLS2, and NLSa) in its C-terminal fragment (Guru *et al.* 1998, La *et al.* 2006). Cavallari *et al.* (2003) provided the first description of the immunohistochemical expression pattern of menin in human pancreas using indirect immunofluorescence (IF) on frozen tissues, showing that the protein is mainly localized into the nucleus of all pancreatic cell types. They also analyzed seven pancreaticoduodenal endocrine tumors showing variable expression levels of menin compared to normal tissue. However, no data exist on the subcellular localization of the protein associated with its mutations.

Menin is likely to work as a scaffold recruiting either positive or negative regulators of transcription to control expression of genes that are involved in different cell functions (La *et al.* 2004). Indeed, among others, menin interacts with the transcription factor JunD suppressing its growth promoting activity (Agarwal *et al.* 1999, 2003, Kim *et al.* 2003), and with Smad3 inhibiting transforming growth factor- $\beta$  (TGF- $\beta$ ) signaling pathway (Kaji *et al.* 2001). Additional interactions include those with the metastasis suppressor NM23 that is involved in DNA replication (Ohkura *et al.* 2001), and the transcription factor p65/nuclear factor- $\kappa$ B (NF- $\kappa$ B) (Heppner *et al.* 2001). These properties, together with the fact that *MEN1* mutations are of the loss of function type and that the wild-type allele is lost in tumors derived from MEN1-affected patients ('second hit'), identify menin as a tumor suppressor acting through either JunD and TGF- $\beta$  central pathways or more complex effects through multiple interactions.

Menin also associates with a histone methyltransferase complex that methylates lysine 4 of histone H3, an epigenetic event associated with transcriptional activation (Yokoyama *et al.* 2004). In line with such a role, it has been reported that menin directly regulates the expression of the cyclin-dependent kinase inhibitors p27<sup>kip1</sup> and p18<sup>Ink4c</sup>. The loss of function of menin leads to the down-regulation of these inhibitors thereby causing a deregulation of cell growth (Milne *et al.* 2005).

Furthermore, menin is also involved in the regulation of transcription of different genes such as *hTERT*, *IGFBP2*, and *CASPASE8* (Schnepp *et al.* 2004), and interacts with proteins involved in the maintenance of genome stability (Jin *et al.* 2003, Sukhodolets *et al.* 2003).

In this study, we analyzed a large series of apparently sporadic PETs with the aim to i) assess the frequency and site of mutations in the *MEN1* gene, ii) compare *MEN1* gene status with immunohistochemical stain for menin, and iii) correlate this data with clinical-pathological information.

## Materials and methods

### Patients and tissues

All samples were obtained from patients who underwent either explorative or radical surgery. Informed consent for analysis of normal and neoplastic tissues was obtained from patients, in accordance with the approval of the Verona University and Hospital Trust's Ethical Committee. The *MEN1* mutational study was performed on 100 PETs comprising 75 non-functioning and 25 functioning tumors (16 insulinoma, 4 gastrinoma, 2 somatostatinoma, 2 VIPoma, and 1 glucagonoma; Table 1 and Supplementary Table 1, see section on supplementary data given at the end of this article).

**Table 1** The 100 pancreatic endocrine tumors included in the mutational analysis

Tumor type	Patients (n=100)	Sex (M/F)	Age (median)
Insulinomas			
WDET	14	7/7	36
WDEC	2	1/1	46
Other functioning tumors			
WDEC	9	3/6	61
Non-functioning tumors			
WDET	31	12/19	59
WDEC	44	20/24	54

WDET, well-differentiated endocrine tumor; WDEC, well-differentiated endocrine carcinoma; M, male; F, female.

Forty-one cases (31 non-functioning and 10 functioning) had already been analyzed for *MEN1* mutations in a previous study (Moore *et al.* 2001).

### Cell lines

The human PET cell lines were CM, BON, and QGP-1 (see Supplementary Table 2, see section on supplementary data given at the end of this article for further information). QGP-1 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS); CM cells were grown in RPMI 1640 supplemented with 5% FBS; BON cells were grown in DMEM and F12K (ratio 1:1) supplemented with 10% FBS. All the culture media were supplemented with 20 mM glutamine and 50 mg/ml of gentamicin sulfate (BioWhittaker, Treviglio, Italy). Cells were incubated at 37 °C with 5% CO<sub>2</sub>.

### *MEN1* gene mutational analysis

Genomic DNA was isolated after cryostat enrichment of frozen tissues to obtain a neoplastic cellularity of at least 90% (Achille *et al.* 1996) using DNeasy Blood and Tissue kit (Qiagen). Matched normal DNA was used to determine whether the mutations identified were somatic or germline. Genomic DNA from PET cell lines BON, QGP-1, and CM was isolated using the AllPrep DNA/RNA/Protein mini kit (Qiagen). The entire coding region (exons 2–10) and the adjacent splice junctions of the *MEN1* gene were amplified using primers reported in Supplementary Table 3, see section on supplementary data given at the end of this article by touch-down PCR. Cycling conditions were 94 °C for 3 min, 20 cycles at 94 °C for 20 s, 68 °C for 25 s (0.5 °C decrease for each cycle), followed by 15 cycles at 94 °C for 20 s, 55 °C for 20 s, and a final extension at 72 °C for 3 min. PCR purification and direct sequencing have been described (Balakrishnan *et al.* 2007). Sequence differences to the NCBI (<http://www.ncbi.nlm.nih.gov>) reference sequence (GeneID 4221) were identified via manual inspection of aligned electropherograms assisted by the Mutation Surveyor software package (SoftGenetics, State College, PA, USA). The genetic alteration identified was cross-referenced to variant information from the NCBI SNP database (<http://www.ncbi.nlm.nih.gov>), the Ensemble Genome Browser (<http://www.ensembl.org>), the Swiss-Prot (<http://ca.expasy.org>) and GenBank (<http://www.ncbi.nlm.nih.gov/Genbank>) databases, the COSMIC database (<http://www.sanger.ac.uk/genetics/CGP/cosmic>), and literature.

### Menin immunohistochemistry

Immunohistochemical staining for menin was performed on formalin-fixed paraffin-embedded (FFPE) tissue microarrays (TMAs) using the anti-menin commercial antibody A300-105A of the Bethyl Laboratories (Montgomery, TX, USA) at a dilution of 1:1000 for 1 h at room temperature. In order to verify the specificity of the immunostaining, the antibody was preadsorbed with the menin-blocking peptide (BP300-105, Bethyl Laboratories) in a saturating concentration (10 µg of peptide/ml) for 2 h before use. Heat-induced menin antigen retrieval was performed using microwave oven and 0.01 mmol/l (pH 6.0) citrate buffer for 30 min. All samples were processed using a sensitive 'Bond polymer Refine' detection system in an automated Bond immunostainer (Vision-Biosystem, Menarini, Florence, Italy). Sections incubated without the primary antibody served as a negative control.

TMAs contained 140 primary PETs, 38 matched metastasis (22 nodal and 16 liver), and 12 normal pancreas. The 140 PETs included 106 non-functioning and 34 functioning tumors (Supplementary Table 4, see section on supplementary data given at the end of this article). The construction of the TMAs was performed using a tissue arrayer (Beecher Instruments, Silver Spring, MD, USA) as described (Kononen *et al.* 1998). For most cases, at least three cores of 1 mm diameter per sample were analyzed. Slides were scanned with ScanScope GL System (Aperio Technologies, Vista, CA, USA) and visualized using ImageScope Software (Aperio Technologies). Three independent observers scored menin expression. The staining intensity was classified into four grades as follows: 0 (absent), 1 (weak), 2 (moderate), and 3 (strong), with separate scores for nucleus and cytoplasm. The quality of TMAs is testified by the detection of multiple antigens by immunohistochemistry (IHC) on serial sections, thus excluding the occurrence of significant protein degradation in these samples. Indeed, the very same TMAs used for this study have been extensively used for different immunohistochemical analysis, some of which have also been published (Missiaglia *et al.* 2010).

### Menin immunofluorescence

Tumor cell lines were seeded at a density of  $2 \times 10^4$  cells per well in 4-well chamber slides (Nalgene Nunc International Corp., Naperville, IL, USA) for 24 and 48 h. Cells were then rinsed three times with ice-cold PBS and fixed in ice-cold methanol for 10 min. The permeabilization was performed using 0.1%

Triton X-100 in PBS for 15 min at 20 °C. Menin was detected with the antibody A300-105A of the Bethyl Laboratories used at a dilution of 1:300. Bound antibody was detected with FITC-conjugated anti-rabbit antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Slides were analyzed on an Olympus BX61 microscope (Olympus, Hamburg, Germany) with the appropriate filter for FITC and a u.v. filter for the DAPI nuclear counterstain. The specificity of the IF pattern obtained was verified by preadsorbing the anti-menin antibody with the menin-blocking peptide (BP300-105, Bethyl Laboratories). All experiments have been repeated twice with comparable results.

### Protein extraction from FFPE tissue samples

For each extraction, three serial 15 µm sections (~25 mm<sup>2</sup> each) of FFPE PET tissue were used. After standard deparaffination of tissue sections (15 µm each), proteins were extracted as previously described (Becker *et al.* 2007), using the Qproteome FFPE tissue kit (Qiagen). Briefly, reference routinely hematoxylin/eosin-stained sections of the tissues were histologically verified, and areas of interest were indicated with a pen on the slides. Subsequently, the areas of interest were scratched from the slides with a needle. The microdissected tissue attached to the needle was transferred into the Qproteome FFPE tissue kit buffer. Proteins were extracted according to the protocol provided by the manufacturer and stored at -20 °C.

As a control for antibodies specificity, proteins were extracted from three previously described PET cell lines (Capurso *et al.* 2006, Cecconi *et al.* 2007) using the AllPrep DNA/RNA/Protein mini kit (Qiagen) according to the manufacturer's instructions.

### Immunoblotting

Total protein extracts from FFPE tissues (30 µg/lane) and cell lines (15 µg/lane) were subjected to SDS-PAGE (12% polyacrylamide gel), and blotting was performed on nitrocellulose membranes (Bio-Rad Laboratories) for 2 h at 60 V. The following antibodies were used: MEN1 (A300-105A, 1:5000, Bethyl Laboratories); menin (H-300, 1:400, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); β-actin (AC15, 1:5000, Sigma-Aldrich). Blots were developed using the ECLPlus Western Blot Detection System (Amersham/GE Healthcare Europe GmbH, Munich, Germany).

### Statistical analysis

Association of immunohistochemical staining with clinical-pathological variables was evaluated using Pearson's  $\chi^2$  tests or Fisher's test when appropriate for categorical variables; Kruskal-Wallis test or Wilcoxon test for continuous variables. All tests were considered significant when *P* value was <0.05. For all the calculation, the *R* statistical software package was used (<http://www.r-project.org>).

### Results

The mutational analysis of *MEN1* was executed on 100 primary neoplasms and 3 human PET cell lines. Immunohistochemical staining for menin was performed on 140 PETs, 71 of which coincided with those analyzed for mutations.

### *MEN1* gene mutational analysis in 100 PETs

Twenty-seven PETs were found to harbor non-synonymous *MEN1* mutations (27%; Table 2 and Fig. 1). Of these, 25 were somatic and 2 were germline (Table 2, numbers 14 and 16) as assessed by the analysis of peripheral blood DNA. Mutations were 8 missense (30%), 3 nonsense (11%), 11 deletions (41%), 1 small insertion (3%), and 4 splice site mutations (15%). Each mutated tumor carried a different *MEN1* mutation.

Direct DNA sequencing of the 41 cases already investigated by single-strand conformation polymorphism and conformation-sensitive gel electrophoresis confirmed all the nine alterations previously identified (see Table 2 for details; Moore *et al.* 2001).

Among the 25 somatic mutations, 3 were in insulinomas (19%) and 22 in non-functioning PETs (30%). With regard to sporadic non-functioning PETs, which are the most representative tumor subgroup in our series, 13/44 (29%) malignant tumors and 9/31 (29%) benign tumors were mutated. Moreover, the genetic alterations identified were cross-referenced with variant information from databases and literature, and interestingly only one mutation (number 4, Table 2) was previously found in PET patients (Bergman *et al.* 2000).

Four common polymorphisms were also found: D418D (rs2071313) in 43 tumors, H433H (rs540012) in 30 tumors, A541T (rs2959656) in 14 tumors, and R171Q (rs607969) in 7 tumors.

The mutational analysis of the three human PET cell lines revealed no genetic abnormalities and identified the presence of two polymorphisms already found in primary neoplasms: R171Q (in CM cell line) and A541T (in BON cell line).

**Table 2** The 27 multiple endocrine neoplasia type 1 (*MEN1*) mutations found in 100 pancreatic endocrine tumors

Mutation ID <sup>a</sup>	Sample ID	Exon	Nucleotide change <sup>b</sup>	Predicted effect <sup>c</sup>
Nonsense				
1	NF23	3	g.3299 G>A	p.W183stop <sup>d</sup>
2	NF1	7	g.4960 G>T	p.E316stop
3	NF38	9	g.6184 G>T	p.E425stop
Missense				
4	NF18	3	g.3286 A>T	p.E179V <sup>d</sup>
5	NF24	3	g.3313 C>T	p.P188L
6	NF27	3	g.3336 G>T	p.V196F <sup>d</sup>
7	NF41	4	g.3685 C>T	p.A242V
8	NF46	6	g.4223 C>T	p.A284V
9	NF7	6	g.4283 G>T	p.R304S <sup>d</sup>
10	F13	9	g.6140 T>C	p.F410S
11	F18	9	g.6233 T>C	p.V441A
Deletions				
12	NF16	2	g.1391delC	fs118stop <sup>d</sup>
13	NF19	2	g.1434_1437delGTCT	fs166stop <sup>d</sup>
14	NF11	2	g.1453delTA	fs115stop
15	NF79	2	g.1552_1566 delGATGT-CATATGGAAC	In-frame deletion
16	F21	3	g.3211_3244del33nt	In-frame deletion
17	NF8	3	g.3261delC	fs185stop
18	NF53	3	g.3384delA	fs223stop
19	NF10	4	g.3683_3684delTG	fs470stop <sup>d</sup>
20	NF13	7	g.4972_4973delCC	fs364stop <sup>d</sup>
21	NF32	9	g.6164delA	fs444stop
22	F3	10	g.6573delG	fs557stop <sup>d</sup>
Insertions				
23	NF78	10	g.6674_6675insC	fs530stop
Splice site				
24	NF67	Intron 3	g.3405delGTA	–
25	NF57	Intron 4	g.4074 A>G	–
26	NF70	Intron 5	g.4118 T>G	–
27	NF63	Intron 9	g.6477 A>T	–

The mutations are listed by mutation type alongside the exons in which they were identified. The nucleotide position of each mutation corresponds to the position of that change on coding strand. NF, non-functioning tumors; F, functioning tumors.

<sup>a</sup>Mutation ID indicates the number reported in Fig. 1.

<sup>b</sup>Mutations are numbered with reference to the *MEN1* genomic sequence U93237 (GenBank); nt, nucleotide; g, genomic sequence; del, deletion; ins, insertion.

<sup>c</sup>p, protein sequence; fs, which denotes frameshift, leads to a premature stop codon at indicated amino acids. The effect of splice site mutations is not determined.

<sup>d</sup>Mutations already found by Moore *et al.* (2001).

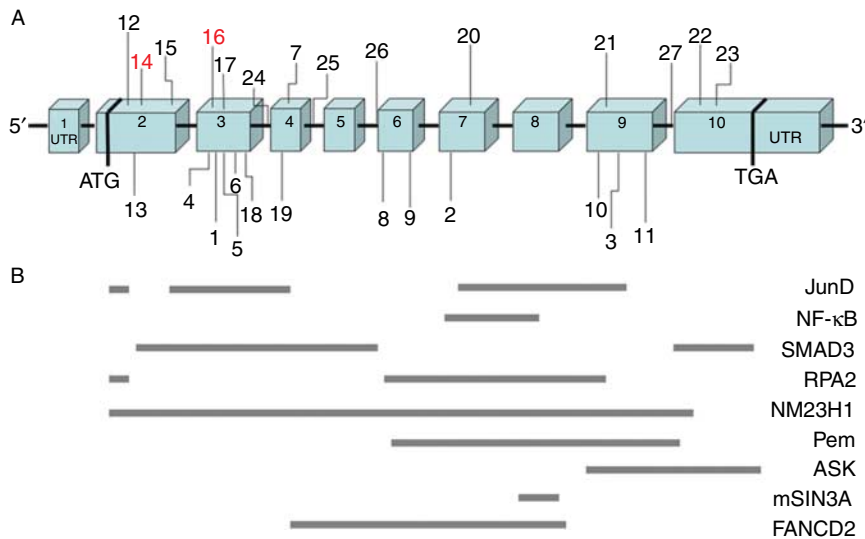
### Menin immunohistochemical expression in 140 PETs

Immunohistochemical expression of menin was evaluated using a series of tissue arrays containing 140 primary PETs, 38 matched metastasis (22 nodal and 16 liver), and 12 normal pancreas. Whenever present, the nuclear and/or cytoplasmic immunopositivity was always uniform in all neoplastic cells.

In normal pancreas, menin expression was detected in all cell types (Fig. 2). Islet cells showed strong nuclear and very faint cytoplasmic immunostaining, while acini and ducts showed a moderately stained cytoplasm. In all normal cell types, nuclei showed a

higher level of expression with respect to cytoplasm, whereas in tumors, variable localization and expression levels were observed (Fig. 2).

In tumors, nuclear immunostaining was found in 111 cases, while 29 (21%) had no detectable nuclear menin. Of the former, 44 (31%) had strong nuclear menin signals, 40 (29%) displayed an intermediate level of expression, and 27 (19%) had weak immunoreactivity. Cytoplasmic staining was observed in 135 cases and was absent in 5 cases (4%). Of the former, 44 cases (31%) had strong menin expression, 70 (50%) were moderately stained, and 21 (15%) had weak staining. The five cases lacking cytoplasmic menin were among



**Figure 1** The 27 *MEN1* gene mutations found in 100 apparently sporadic pancreatic endocrine tumors. Schematic representation of the genomic organization of the *MEN1* gene, illustrating the locations of the identified mutations and the aminoacidic domains that interact with other proteins (see also Table 1). (A) The boxes represent the ten exons of the gene, whereas the lines indicate the introns (not to scale). The start (ATG) and stop (TGA) sites in exons 2 and 10 respectively are indicated. UTR indicates the untranslated regions that comprise the exon 1, the 5' of exon 2, and the 3' of exon 10. *MEN1* mutations are enumerated from 1 to 27, and their positions are shown by vertical black lines. Red numbers refer to germline mutations. (B) Menin domains that have been implicated in the binding to several interacting proteins are represented by gray boxes. These are as follows: JunD (codons 1–40, 139–242, and 323–428); NF- $\kappa$ B (codons 305–381); SMAD3 (codons 40–277 and 477–610); RPA2 (codons 1–40 and 286–448); NM23H1 (codons 1–486); Pem (codons 278–476); ASK (codons 396–610); mSIN3A (codons 371–387); and FANCD2 (codons 219–395).

those that also lacked nuclear staining, while the intensity of cytoplasmic staining was not significantly related to the intensity of nuclear staining. No difference in menin localization or expression level was found between primary and metastatic samples.

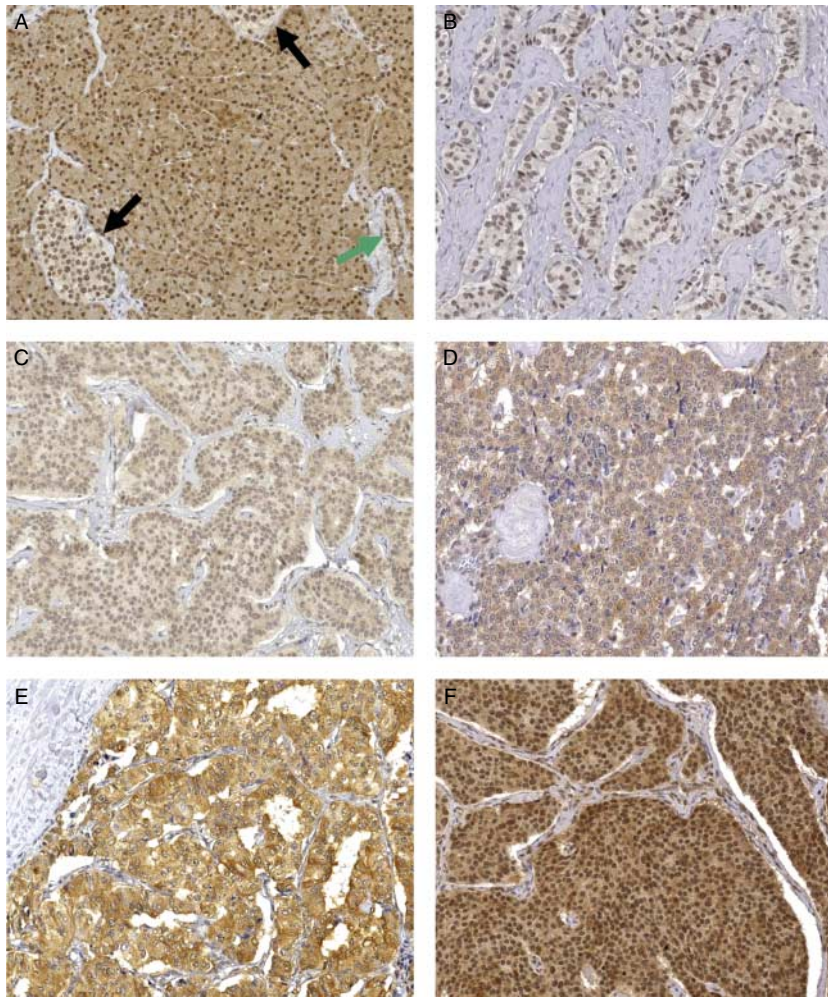
### Menin expression in human PET cell line

IF was performed on confluent and subconfluent human PET cell lines (BON, QGP-1, and CM). The results of the IF analyses were consistent with the immunohistochemical staining observed for the majority of PET tissues. Figure 3 shows the results of the IF analyses of a subconfluent BON cell culture revealing that menin is expressed in both the nucleus and the cytoplasm of the cells (Fig. 3C, overlay image). Interestingly, this cell line is not affected by *MEN1* gene mutations and only presents the A541T polymorphism in a heterozygous state.

### Western blot analysis

To confirm the presence of menin suggested by the immunohistochemical positivity in cases harboring different *MEN1* gene mutations, we performed western blot analysis of five primary PETs selected on the basis of their immunohistochemical and mutational profile

(Table 3). Two different polyclonal anti-menin antibodies were used: the A300-105A from Bethyl Laboratories that is affinity purified for an epitope located at the C-terminus of the protein; and the H-300 from Santa Cruz Biotechnology that is raised against the N-terminus of menin. The specificity of antibodies was tested analyzing protein lysates of three PET cell lines (Supplementary Figures 1 and 2, see section on supplementary data given at the end of this article). For the A300-105A antibody, which is the very same antibody used to obtain menin immunostaining of PET tissues as well as menin IF of cell lines, the specificity was also assessed preadsorbing the antibody with the blocking peptide in a saturating concentration (Supplementary Figure 3, see section on supplementary data given at the end of this article). The western blot analysis of primary tumors shown in Fig. 4 is representative of three independent experiments that gave identical results. Menin was present in all samples except for case F8, the tumor that lacked menin immunohistochemical staining. A normal size protein was detected in wild-type tumor NF61 and tumor F18 carrying a missense mutation. A band of lower molecular mass (~50 kDa) was observed in case NF38 harboring a nonsense mutation and tumor NF32 that had a frameshift-stop mutation. Noteworthy, these



**Figure 2** Menin immunohistochemical staining in normal pancreatic tissue and pancreatic endocrine tumors. Immunohistochemistry with anti-menin antibody. Original magnification,  $\times 20$ . (A) Normal pancreatic tissue, islets, and ducts are indicated by black and green arrows respectively; (B) PET with moderate nuclear and absent cytoplasmic staining similar to that seen in the normal islet shown in (A) (NF46, wild-type sequence); (C) PET with weak nuclear intensity and faint cytoplasmic staining (case NF41, missense mutation); (D) PET with absent nuclear and moderate cytoplasmic expression (case NF32, truncating mutation); (E) PET with absent nuclear and intense cytoplasmic staining (NF6, wild-type sequence); (F) PET with strong nuclear and cytoplasmic protein expression (NF40, wild-type sequence).

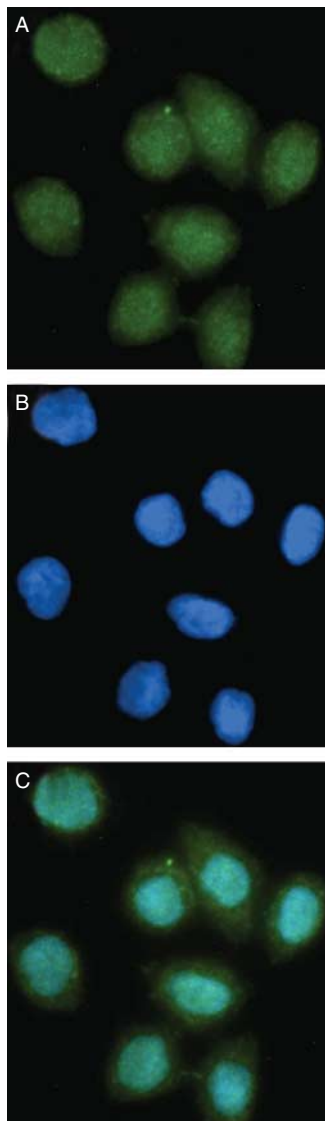
two mutations are likely to give a shortened protein, whose predicted size is about 50 kDa (Table 3). The absence of a band corresponding to the wild-type protein in these two cases is due to the loss of the normal allele, as assessed by the analysis of loss of heterozygosity (LOH) using PCR amplification of microsatellite markers (LOH, Table 3).

Furthermore, as the shortened proteins may miss the C-terminal epitope eventually recognized by the A300-105A antibody, tissue lysates (cases NF32, NF38, NF61, and F18) were independently re-probed with the menin (H-300) antibody directed against the N-terminus of the protein thus ensuring that the bands observed were not artifacts (Fig. 5). The results

of this western blot were in line with those previously obtained. Indeed, a band of about 50 kDa was still detected in tumors harboring truncating mutations (Fig. 5, lanes 2 and 4) and therefore displaying a shortened protein.

### Menin expression and *MEN1* mutations

Immunohistochemical data were available for 71 samples screened for *MEN1* mutations (Table 4). A significant association was found between the lack of menin nuclear expression and the presence of truncating mutations (Fisher's test, odds ratio (OR) 5.1, 95% CI 1.0–25.9;  $P=0.025$ ). A significant correlation



**Figure 3** Menin expression in human PET cell line. Immunofluorescence of cell lines (BON) was carried out using the anti-menin antibody A300-105A (Bethyl Laboratories) and FITC-conjugated anti-rabbit antibody (Jackson Immuno-Research Laboratories, Inc.) to detect menin (green signal, A), and DAPI (blue signal, B) to identify nuclei. The overlay image is shown in (C). Original magnification,  $\times 63$ .

was also observed between the intensity of cytoplasmic positivity and the presence of mutation regardless of the mutation type (Fisher's test,  $P=0.006$ ). In fact, all the mutated cases had moderate to strong cytoplasmic staining (Table 3). We also sought to investigate the combined effect of nuclear and cytoplasmic immunohistochemical staining in relation to the mutational status of MEN1. Interestingly, in a multivariate logistic model, we estimated that an increase of one unit of cytoplasmic score had the

effect to double the probability of carrying a mutation (OR 2.2; 95% CI 1.0–4.9;  $P=0.033$ ), whereas the opposite effect was observed for nuclear score (OR 0.5; 95% CI 0.5–0.9;  $P=0.021$ ).

### Correlation of MEN1 gene status and protein expression with clinical–pathological information

There was no significant correlation between MEN1 mutations or polymorphisms and clinical–pathological parameters, including WHO classification, proliferation index, and progression-free and overall survival. Nuclear immunostaining had no significant correlation with any clinical–pathological parameter.

### Discussion

The results of our combined genetic and immunohistochemical study of MEN1 gene and protein status in 169 apparently sporadic PETs may be summarized as follows: i) MEN1 gene mutations were found in 27/100 cases, including 23/75 (30%) non-functioning PETs and 4/25 (16%) functioning PETs; two of the cases revealed to harbor germline mutations in the lack of any familial cancer history; ii) mutations were distributed along the gene and most were of the loss of function type; iii) menin IHC showed that normal islet cells had intense nuclear and very faint

**Table 3** The five pancreatic endocrine tumors selected for menin western blot analysis shown in Fig. 4

Sample ID	Mutation type <sup>a</sup>	IHC score <sup>b</sup> (n/c)	Predicted protein size (kDa) <sup>c</sup>	LOH <sup>d</sup>
NF38	Nonsense	1/3	47	Yes
F8	Wild-type	0/0	67	Yes
NF32	fs-stop	0/2	49	Yes
F18	Missense	3/2	67	NI
NF61	Wild-type	3/3	67	No

NF, non-functioning tumors; F, functioning tumors; NI, not informative.

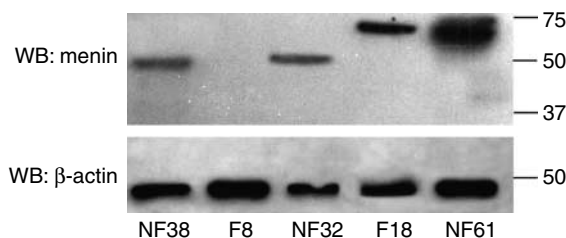
<sup>a</sup>Wild-type, tumor with no mutation of MEN1 genomic sequence; fs, which denotes frameshift, leads to a premature stop codon; see Table 2 for detailed information about the predicted effects of the mutations found in tumors NF38, NF32, and F18.

<sup>b</sup>IHC score, immunohistochemical score; separate scores were assigned for nucleus (n) and cytoplasm (c); see Materials and methods section for details.

<sup>c</sup>The predicted size of the mutant proteins was estimated assuming 110 kDa as the molecular weight of each amino acid.

<sup>d</sup>LOH, loss of heterozygosity. Matched normal and tumor DNAs were analyzed for loss of heterozygosity on chromosome 11q using the microsatellite markers D11S898 and D11S908. Analysis was performed on an ABI 310 DNA sequencer as described in Moore et al. (2001).





**Figure 4** Western blot analysis of menin in five pancreatic endocrine tumors with known mutational and immunohistochemical status reported in Table 3. Western blot analysis of five primary pancreatic endocrine tumors, whose *MEN1* mutational and immunohistochemical expression status is reported in Table 3. Protein extracts (30 μg/lane) from paraffin-embedded tissues probed with anti-menin antibody. Reprobing of the blot with anti-β-actin antibody served for normalization. Case numbers are indicated below; the molecular weight standards (kDa) are reported on the right. An expected normally sized protein of ~70 kDa is detected in immunohistochemical positive tumors NF61 having a wild-type *MEN1* gene and F18 carrying a missense mutation. Immunohistochemical positive tumors NF38 and NF32 display bands of lower molecular mass corresponding to the shortest protein of ~50 kDa predicted by the truncating mutations of their *MEN1* gene. The absence of a band corresponding to the wild-type protein in these two PET cases is due to the loss of the normal allele. The lack of the menin band in tumor F8 parallels the negative immunohistochemical staining of this case. LOH analysis of this tumor revealed the loss of one allele, and suggest that the lack of the protein is due to the inactivation of the remaining *MEN1* allele, having a germline sequence, by epigenetic mechanisms, e.g. promoter methylation.

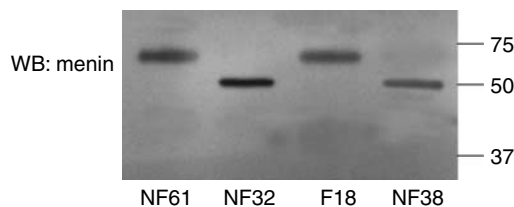
cytoplasmic staining, while 56/140 (40%) PETs lacked or had weak nuclear immunostaining and 114/140 (80%) had cytoplasmic intensity higher than normal; iv) about one-third of the cases showing an altered localization and intensity of menin coincided with those harboring a *MEN1* gene mutation.

This study involved the largest panel of PETs that has been ever screened for mutations of *MEN1*, and concluded that about 25% of sporadic PETs harbor a somatic *MEN1* mutation. Of the 27 mutations, 2 revealed to be germline, suggesting that a small proportion of cases may indeed have a *MEN1* syndrome acquired at the time of zygote formation or inherited from an asymptomatic carrier parent.

*MEN1* mutations were scattered throughout the coding sequence and splice sites. Although the majority of mutations were localized in the 5' half of the gene, no obvious hot spot was identified. The alterations affected predominantly the evolutionary conserved regions of menin, and are likely to result in the alteration of protein function. In fact, 12 of the 25 somatic mutations are predicted to give a truncated menin (numbers 1–3, 12–13, 17–19, and 20–23, Table 2) that would lack at least one of the NLS sites due to premature stop codon, thus preventing the

protein to enter the nucleus and exert its function. Six missense mutations (numbers 4–7 and 9–10) involve JunD-binding domains, and are therefore predicted to prevent menin-repressive action on JunD-mediated transcription (Agarwal *et al.* 1999). The effects of the remaining two missense mutations, one in frame deletion, and four splice site alterations (numbers 8 and 11, 15, and 24–27 respectively) are difficult to predict. However, it has been previously demonstrated by *in vitro* assays that missense mutations could lead to a loss of function due to an enhanced proteolytic degradation of menin (Yaguchi *et al.* 2004), whereas the in-frame alteration could delete crucial amino acid residues. Those mutated proteins were also hypothesized to affect the interaction with other known menin interactors (Fig. 1B; Ohkura *et al.* 2001, Jin *et al.* 2003, Sukhodolets *et al.* 2003). Finally, it is likely that the pathogenic effects of *MEN1* mutations are shown through disruption of multiple and cross-linked interaction pathways.

We also found four common polymorphisms that are often classified as benign. However, several pieces of evidence suggest that the non-synonymous A541T and R171Q polymorphisms that were found in 14 and 7 cases respectively might be considered as pathogenic missense mutations (Shan *et al.* 1998), which may alter protein function and exhibit higher clinical expression in some genetic context (Bazzi *et al.* 2008, De Carlo *et al.* 2008). The frequencies of these non-synonymous polymorphisms in our cohort cases are 14 and 7% for A541T and R171Q respectively. Based on the data reported in the HapMap Project (<http://hapmap.ncbi.nlm.nih.gov/>), the frequencies of these polymorphisms in the European population are 1 and 2% for A541T



**Figure 5** Menin expression in four pancreatic endocrine tumors with the H-300 antibody. Western blot analysis of four primary pancreatic endocrine tumors, whose mutational and immunohistochemical expression status is reported in Table 3. Protein extracts (30 μg/lane) from paraffin-embedded tissues probed with anti-menin raised against the N-terminus of the protein (H-300, 1:400, Santa Cruz Biotechnology). Case numbers are indicated below; the molecular weight standards (kDa) are reported on the right. The results of this western blot analysis confirmed those obtained probing tissue lysates with the anti-menin (A300-105A, Bethyl Laboratories) that is affinity purified for an epitope located in the C-terminus of the protein (see Fig. 3).

**Table 4** Frequency of multiple endocrine neoplasia type 1 (*MEN1*) mutations by immunohistochemical score and mutation type

IHC score <sup>b</sup>	Mutation type <sup>a</sup>			Total mutated (n=21)	Wild-type (n=50)
	Missense	Splicing	Truncating		
Nuclear					
0	1	0	5	6	6
1	2	0	3	5	13
2	2	3	4	9	18
3	1	0	0	1	13
Cytoplasmic					
0	0	0	0	0	2
1	0	1	0	1	11
2	4	1	8	13	25
3	2	1	4	7	12

<sup>a</sup>Splicing, mutation occurring in splice sites; truncating, both nonsense and frameshift mutations that lead to a premature stop codon.

<sup>b</sup>IHC score refers to the staining intensity that was classified into four grades: 0 (absent), 1 (weak), 2 (moderate), and 3 (strong).

and R171Q respectively; therefore, we can speculate that the expression of these genetic changes has eventually a clinical significance.

All the gene alterations identified did not correlate with clinical–pathological features including tumor stage, suggesting that *MEN1* alteration is an early and common event in PET pathogenesis. The fact that a large proportion of PETs (70%) do not harbor *MEN1* mutations suggest that other genes or other mechanisms of *MEN1* inactivation that have been only marginally investigated might be involved in PET tumorigenesis (Arnold *et al.* 2007).

Despite numerous data have been accumulated about the mutational status of *MEN1* in PET cases, only a limited number of studies deal with the expression pattern of menin in human pancreas and PET. Indeed, Cavallari *et al.* (2003) provided the first description of the expression pattern of menin in human pancreas using an in-house characterized monoclonal antibody (C126) to perform an indirect IF/laser-scanning microscopy protocol on frozen tissues. They described that the protein is mainly located in the nucleus of all pancreatic cell types. They also analyzed seven pancreaticoduodenal endocrine tumors showing variable expression levels of menin compared to normal tissue.

In a recent study, the same protocol was applied to examine menin expression pattern in normal exocrine pancreas and in pancreatic ductal adenocarcinoma (Cavallari *et al.* 2009) using interchangeably the mouse monoclonal antibody and the A300-105A polyclonal antibody from Bethyl Laboratories. Their analysis of normal exocrine pancreas confirmed that the protein mainly localizes in the nucleus of both acinar and duct cells, but it is also detected in the cytoplasm.

The A300-105A was the very same antibody we used for our immunohistochemical analysis of PET tissues. This antibody gave, in our hands, the most reproducible results compared with other commercially available antibodies tested. Indeed, the specificity of this antibody in immunostaining as well as in western blot experiments was addressed in competitive assays using the commercially available blocking peptide from Bethyl Laboratories.

In the present study, immunohistochemical expression of menin was seen in all cell types of normal pancreas, with the protein mainly localized into the nucleus. Cytoplasmic signal was very faint to absent in islet cells and moderate in acini and ducts. Most tumors expressed menin with a broad spectrum of immunoreactive intensity, which varied from a level comparable to that detected in normal tissue to an almost undetectable signal, and further, with variable levels of protein distribution between nucleus and cytoplasm.

Indeed, immunohistochemical data suggested that a localization defect of menin exists in most cases, including those lacking or with very faint nuclear staining and those with cytoplasmic immunostaining more intense than the faint signal seen in normal islets. Half of the cases lacking nuclear menin and one-third of cases showing either weak nuclear or moderate to intense cytoplasmic protein carried *MEN1* mutations. To further confirm the menin expression pattern observed in tumors, we performed an IF analysis of human PET cell lines screened for gene mutations. This analysis revealed that menin signal is still detected in the cytoplasm of the cells despite the absence of evident genetic abnormalities.

Interestingly, the absence of nuclear protein expression correlates with the presence of truncating mutations, while positive cytoplasmic protein intensity is associated with the presence of mutation regardless of its predicted effect on the protein functionality. These results suggest that some of the truncating mutations cause the premature block of transcription generating an incomplete protein that accumulates into the cytoplasm due to the lack of at least one of the NLSs. Non-truncating mutations could partially affect nuclear transport as well, possibly causing an incomplete protein processing or preventing menin interaction with other proteins. This can explain the abundance of cytoplasmic protein observed in mutated cases.

Western blot data, demonstrating protein expression in tumors carrying different types of *MEN1* gene mutations, are in line with this hypothesis. Indeed, a band of the expected normal molecular size was detected in both a wild-type tumor (i.e. displaying no sequence abnormalities) and in a tumor carrying a missense mutation, whereas bands of lower molecular mass were observed in tumors affected by truncating mutations and these were of the size predicted by the truncating mutations observed.

At partial support to our hypothesis concerning a localization defect of menin in PETs, there is a recent paper by *Cao et al. (2009)*, which demonstrated *in vitro* that menin is able to shuttle between nucleus and cytoplasm through newly identified nuclear export signals (NES). More interestingly, menin was shown to directly interact with  $\beta$ -catenin and, when over-expressed, to prevent the nuclear accumulation of  $\beta$ -catenin carrying the protein outside the nucleus. Finally, they demonstrated that mutations within these domains impair the nuclear export function of menin. Although none of the mutations we identified in our study affects the NES domain, the results of this very interesting paper suggest that abnormal interaction involving menin might alter the cellular localization of its physical interactor (e.g.  $\beta$ -catenin). These data allow us to speculate that an inverse situation, which is the mislocation of menin, might occur in the event of alterations affecting one of its physical interactors.

In conclusion, our data suggest that gene mutations affect the subcellular localization of menin causing its accumulation in the cytoplasm. However, the majority of PETs show abnormal menin distribution in the lack of *MEN1* gene mutations, suggesting that other genes, either independently or more probably as physical and/or functional partners of *MEN1*, are altered and are responsible for the impairment of menin functionality.

## Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1677/ERC-10-0028>.

## Declaration of interest

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

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