ONLINE MUTATION REPORT

Mutational and gross deletion study of the *MEN1* gene and correlation with clinical features in Spanish patients

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Multiple endocrine neoplasia type 1 (MEN1, OMIM 131100) is an autosomal dominant disorder characterised by the combination of endocrine tumours, such as parathyroid tumours, enteropancreatic tumours, anterior pituitary tumours, adrenal gland, and neuroendocrine carcinoid tumours, as well as non-endocrine expression, such as lipoma, facial angiofibroma, collagenoma, and ependymoma.[1 2] Primary hyperparathyroidism (HPT) is the first manifestation of MEN1 in approximately 90% of patients, although this percentage differed between studies.[3 4] Gastrinoma is the most frequent enteropancreatic tumour, accounting for approximately 40% of enteropancreatic tumours.[2] It has thus been suggested that MEN1 syndrome should be excluded in patients with gastrinoma.[5] Prolactinomas account for 20% of MEN1 related pituitary lesions,[1 2] while other reported pituitary tumours are relatively uncommon manifestations of MEN1.[1 2]

The *MEN1* gene is located on chromosome 11q13[6 7] and was positionally cloned in 1997.[8 9] It contains 10 exons and encodes menin, a 610 amino acid protein. Menin is known to be a nuclear protein[10] that represses JunD activated transcription[11] and interacts with other proteins, such as Smad3,[12] nm23,[13] and NF-κB,[14] all of which are involved in the regulation of cell proliferation and development.

Inactivating germline mutations found in MEN1 families/patients indicates that the *MEN1* gene is a tumour suppressor gene.[8 9] More than 200 germline and somatic mutations have been identified to date but no hot spots or genotype-phenotype correlations have been observed. Consequently, carriers in a family with MEN1 should be checked periodically for typical and less frequent expressions of the MEN1 syndrome. Taking into account both the absence of hot spots for mutations in the *MEN1* gene and the lack of genotype-phenotype correlations, it is necessary to establish clinical criteria in order to increase the detection rate of *MEN1* germline mutations. It would therefore be more cost effective to perform *MEN1* gene mutation analysis of selected patients, rather than of all patients with apparently MEN1 related tumours. This procedure would also avoid unnecessary anxiety for mutation negative patients.[15]

In this study, 28 MEN1 patients with a family history and 27 MEN1 suspected patients were screened for *MEN1* gene germline mutations such as missense, nonsense, or small deletions or insertions, as well as for gross gene deletions. Subsequently, we measured the association between the presence and absence of germline mutation and the age of diagnosis and clinical characteristics.

Key points

- Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant disorder characterised by a combination of over 20 different endocrine and non-endocrine tumours. No mutational hot spots in the *MEN1* gene have been identified and therefore clinical criteria need to be established to increase the detection rate of *MEN1* germline mutations and to avoid unnecessary anxiety in mutation negative patients.
- In this study, we analysed 28 MEN1 patients with a family history and 27 sporadic MEN1 suspected patients. Sequence analysis showed germline mutations in 89.3% (25/28) of familial cases and 33.3 % (9/27) of sporadic cases, confirming that five out of these nine mutations were de novo, which represents 14% of the total alterations detected in both familial and sporadic groups.
- In all the patients proven negative for mutations by sequencing analysis (three in the familial group and 18 in the sporadic group), we performed Southern blot analysis and detected rearrangements in two out of three families and none in sporadic cases. This suggests that gross deletion analysis is worthwhile for all cases with clear clinical characteristics of MEN1 but negative for mutation.
- Comparing clinical data of cases with and without germline mutation, no patient older than 40 and without familial antecedents had *MEN1* gene mutations. All patients with any type of carcinoid tumour had germline mutation and 87.5% (14/16) of cases with prolactinoma a had germline mutation. No case with growth hormone secreting pituitary adenoma and primary hyperparathyroidism had *MEN1* gene mutations, confirming that this association is a different entity from MEN1 disease.

SUBJECTS AND METHODS

Patients and families

Informed consent was obtained from all subjects before their participation in this study. Fifty-six unrelated probands with MEN1 were studied (31 females and 24 males), 28 of whom had a family history of the disease. A total of 152 family members, including not only familial but also sporadic MEN1 cases, were included in the study; 49 were affected, and 103 were unaffected at the time of the study. Tables 1 and 2 show the clinical features of 28 familial MEN1 cases and 27 sporadic MEN1 cases.

Classification of patients and clinical criteria

The diagnosis of sporadic MEN1 patients was based on the presence of at least two of the three main MEN1 related endocrine tumours (HPT, enteropancreatic endocrine tumours, and pituitary tumour) without a clear family history of the disease,[2] obtained from the proband and from available relatives. In these cases, the presence of MEN1 related lesion(s) was excluded by biochemical testing.

Table 1 Clinical features of familial MEN1 cases and detected germline mutations

Case	Age at diagnosis*	Sex*	Parathyroid lesions†	Anterior pituitary lesions	Enteropancreatic lesions	Other lesions	Germline *MEN1* mutation	Location	Family examination: Non-carriers	Family examination: Mutant carriers
F1**	33	M	4	PRL (2)	NFT (1)		1619del25	Exon 10	4	3
F2**	19	M	12	PRL (1), ACTH (1)	I (1), NFT (2)		1650del1	Exon 10	1	5
F3**	50	F	8	ACTH (1), PRL (1)	NFT (1)		379del2	Exon 2	4	5
F8**	16	F	8	PRL (1)			359del4	Exon 2	1	
F11**	29	F	6	PRL (2)	G (2)		239del3	Exon 2	1	2
F13**	33	F	2	PRL (1)		Thymic carcinoid	Q209X	Exon 3	4	3
F20**	50	F	4	PRL (1), NFT (1)	I (1)		W341X	Exon 7	5	4
F24	48	M	4	PRL (1), NFT (1)	Carcinoid (1)	Lung carcinoid	1489dup10‡	Exon 10	6	3
F27§	30	M	1			Thymic carcinoid	W341X	Exon 7	–	–
F28	63	F	4	PRL (1), NFT (1)	NFT (1)	Adrenal adenoma	Gross deletion		1	3
F29	33	M	5	Mixed (1), PRL (1)	G (1)	Gastric carcinoid	1071ins1‡	Exon 7	4	8
F31	40	M	2	NFT (1)	G (1)	Adrenal adenoma	1498del5‡	Exon 10	2	1
F38	32	M	2		G (1)	Lung carcinoid	H139P‡	Exon 2	1	1
F48	16	F	3	PRL (1)			738del4‡	Exon 3	4	8
F49	26	M	9		G (5)		1505del48‡	Exon 10	2	1
F50	25	M	2	PRL (1), GH (2)	I (1)		1780del3	Exon 10	2	2
F52	18	F	3	PRL (2)	G (1)		E45K	Exon 2	4	3
F59	33	F	6		I (3)		360ins2	Exon 2	1	3
F64	42	M	1	PRL (1)	G (1)		A49F‡	Exon 2	5	1
F66¶	35	M	4				Gross deletion		1	4
F70	37	F	5	PRL (1)			666del1‡	Exon 3	4	4
F75	46	F	3		G (1)		A337D	Exon 7	6	5
F82	22	F	4	PRL (1)			345del1‡	Exon 2	–	4
F83§	34	M	1	NFT (1)			806del 4bp‡	Exon 4	–	–
F84	29	F	1	NFT (1)	NFT (1)		464del 3bp	Exon 2	–	–
F88	17	F	1	NFT (1)	G (2)		738del 4bp	Exon 3	–	–
F89	39	F	1	PRL (1)	I (1)		R108X	Exon 2	–	–
F22	30	M	4		G (2)		Linkage to 11q13		1	4

*Age at diagnosis and sex of probands (F, female; M, male). †Number of subjects with HPT in the family (independently of whether they were hyperplasia or adenomas). All probands had parathyroid lesions at the time of the study; (n) number of tumours in the family. NFT, non-functioning tumour; PRL, prolactinoma; ACTH, ACTH secreting tumour; GH, GH secreting tumour; mixed, prolactin and ACTH secreting tumour; I, insulinoma; G, gastrinoma. ‡Mutation not previously described. §Familial antecedents compatible with the disease reported by patients. ¶Family with apparently FHTP. **Results partially described by Cebrián *et al*.[19]

Familial MEN1 was considered when a MEN1 patient had at least one first degree relative with one of these three tumours.[2 15]

A family apparently affected with familial hyperparathyroidism (F66), and another two cases (F27 and 83) with familial neoplasia antecedents compatible with the disease and the existence of members of the family who had died at an early age reported by the index cases were also included.

The diagnosis of primary hyperparathyroidism was established by the simultaneous presence of raised ionised or albumin adjusted serum calcium concentrations and intact parathyroid hormone levels at least twice. Histological study after surgery defined adenoma or hyperplasia. Pituitary adenomas were diagnosed by the presence of a mass lesion on high resolution CT scanning or MR imaging of the sella turcica. Baseline hormonal concentrations and dynamic test of pituitary reserve were used to diagnose hormone secreting tumours. Histopathology and immunohistochemistry analysis after surgical explorations of the pituitary fossa confirmed the presence of an adenoma or hyperplasia. Pancreatic islet cell tumours were suspected based upon the evidence of clinical manifestations and raised serum concentrations of pancreatic hormones (gastrin, insulin, glucagon). The presence of endocrine pancreatic lesions was confirmed by abdominal CT or MR imaging. Histological analysis confirmed the presence of hyperplasia or neoplasia. Pituitary or enteropancreatic tumours without an apparent rise of basal hormone levels were designated non-functioning tumours (NFTs).[8] Other MEN1 associated lesions were diagnosed by means of imaging techniques, appropriate hormonal determinations, and histological confirmation after surgery.

Molecular analysis of the *MEN1* gene

Standard methods[16] were used to extract genomic DNA from peripheral blood of the patients and their relatives.

Mutation screening was performed by CSGE analysis, and variants were confirmed by sequencing analysis. Cases without variant shifts were sequenced to confirm the absence of mutations. The electrophoretic and amplification conditions used for the CSGE analysis have been previously described.[17] Mutations were named according to standard nomenclature.[18]

Nucleotide sequencing of PCR products.

The PCR products were purified using columns (EZNA cycle pure kit, OMEGA Bioteck) and bi-directionally sequenced with a Big Dye terminator cycle sequencing Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions.

Haplotype analysis

To perform the haplotype analysis in familial cases without germline mutation in the *MEN1* gene, we used four previously published extragenic polymorphic DNA markers: D11S1883, PYGM (CA) (GA), D11S913, and D11S1889.

PCR amplification was performed in 20 µl of a mixture containing 100 ng of peripheral blood DNA. The PCR conditions and the primer sequences were obtained from the Genome Data Base (http://www.gdb.org). The PCR product was mixed

Table 2 Clinical features of sporadic MEN1 cases and detected germline mutations

Case	Age at diagnosis*	Sex*	Parathyroid lesions	Anterior pituitary lesions	Enteropancreatic lesions	Other lesions	Germline *MEN1* mutation	Location	Family examination, non-carriers
C6‡	26	F	Yes	PRL			848del4/ins9	Exon 4	–
C10‡	28	M	Yes	PRL	I	Colagenomas	355ins1	Exon 2	Fa, Mo
C21	26	M	Yes	ACTH			R98X	Exon 2	Fa, Mo
C23	21	M	Yes	Mixed			IVS6+1G>A	Intron 6	Fa, Mo, S
C33	35	M	Yes	PRL	NFT		357del4	Exon 2	Fa, Mo
C41	34	M	Yes		Gl	Skin alterations	Q261X	Exon 4	Fa, Mo
C45	30	M	Yes		I		W423R†	Exon 9	–
C54	35	F	Yes	NFT	I, Gl		357del4	Exon 2	–
C4	15	F	Yes	PRL			–	–	–
C5‡	52	F	Yes	GH		Thyroid adenoma	–	–	–
C9	60	F	Yes	GH			–	–	–
C12	45	M	Yes		G		–	–	–
C15	50	F	Yes	GH			–	–	–
C25	57	F	Yes		G		–	–	–
C34	54	M	No	NFT	NFT	Mesenchymal tumour	–	–	–
C35	66	F	Yes		G			–	–
C39	81	F	Yes		G		–	–	–
C42	38	M	Yes	GH			–	–	–
C51	55	F	Yes	GH			–	–	–
C53	41	M	Yes	NFT	G		316ins5	Exon 2	–
C57	44	F	Yes	GH			–	–	–
C62	74	F	Yes	PRL			–	–	–
C65	55	F	Yes	GH			–	–	–
C72	39	F	Yes	GH			–	–	–
C77	53	F	Yes	NFT			–	–	–
C78	62	M	Yes	ACTH			–	–	–
C80	65	F	Yes		I	Adrenal adenoma	–	–	–

*Age at diagnosis and sex of probandus (F, female; M, male); NFT, non-functioning tumour; PRL, prolactinoma; ACTH, ACTH secreting tumour; GH, GH secreting tumour; Mixed tumour, prolactin and ACTH secreting tumour; I, insulinoma; G, gastrinoma; Gl, glucagonoma. †Mutation not previously described. Fa, father; Mo, mother; S, son. ‡Results partially described by Cebrián *et al*.[19]

with loading buffer (95% formamide, 0.05% xylene cyanol blue, 0.05% bromophenol blue, 10 mmol/l NaOH), denatured at 95ºC for five minutes, and loaded on denaturing 8% acrylamide/7 mol/l urea gels. The gels were run at a fixed power of 75 W for 4.5 hours. After electrophoresis, the gel was silver stained and dried for documentation.

Southern blot analysis

A total of 7 µg of genomic DNA from each proband were digested overnight with 30 U of selected, infrequently cutting restriction enzymes (*Eco*RI, *Bam*HI, *Sac*I) (MBI Fermentas, Germany). Samples were electrophoresed through 0.8% agarose gels and transferred to Zeta-Probe GT Genomic Tested Blotting membranes (Bio-Rad, Hercules, CA, USA) using standard protocols.[16] Following transfer, membranes were rinsed in 2 × SSC, dried, and UV cross linked. For hybridisation, filters were incubated at 60°C in 10 ml hybridisation solution (0.5 mol/l Na_2PO_4H, 7% SDS) for four hours before addition of probe, then hybridised overnight in 10 ml of the same hybridisation solution. Two low (2 × SSC/0.1% SDS) and two high (0.1 × SSC/0.1% SDS) stringency washes were performed at 60°C, and filters were exposed to Kodak autoradiographic film at –80°C for 48 hours. A probe was generated by restriction digestion with *Xho*I from a *MEN1* cDNA containing the entire coding region of the gene (clone IMAGE 3138471). Probes were radiolabelled with [α-^{32}P]dCTP using a Megaprime™ DNA labelling systems kit (Amersham Pharmacia Biotek, UK).

Statistical analysis

The association between presence or absence of mutations and other dichotomous variables was determined using Fisher's exact test. Average ages of diagnosis of patients with and without mutation were compared using the *t* test.

RESULTS

Germline *MEN1* mutations in familial MEN1

We used sequence analysis of the coding region of the gene to establish that 89.3% of patients with a family history (25/28) had germline mutations scattered throughout the whole coding region. Each family showed a different alteration and 10 out of 25 mutations had not been previously described (40%) (table 1). Some of these results have been partly described by Cebrián *et al*.[19] Three of them generated a change of amino acid: H139P (F38), E45K (F52), and A49F (F64). In order to rule out that the missense mutations not previously described (H139P and A49F) were benign polymorphisms, we performed the study in 200 independent alleles of the control population by means of CSGE and these variants were not present, suggesting the pathogenic character of the change. Moreover, in two of them (E45K and A49F), we were able to perform a segregation analysis to confirm that these variants segregated with the disease.

We identified 77 mutant gene carriers, of which 30 were asymptomatic, and 64 were non-carriers among first degree relatives of the patients with *MEN1* mutation (table 1).

Moreover, we identified five previously described polymorphisms; 2265-16 C→G (6%), S145S (AGC→AGT, 2%), R171Q (CGG→CAG, 7%), 6025-3 C→G (2%), and D418D (GAC→GAT, 44%) whose frequencies were similar to those reported in the white population.[20–22]

In the three families in which CSGE detected no variants, the absence of mutations was confirmed by sequence analysis, and it was possible to perform a haplotype analysis in two of them. Both displayed segregation between the disease and the 11q13 region. In the third family (F66), the haplotype analysis could not be performed because an insufficient number of relatives were available.

We carried out a Southern blot analysis in these three cases to determine if one copy of the *MEN1* gene was being inactivated by a method that could not be detected by genomic DNA sequence analysis. Using three restriction enzymes (*Eco*RI, *Bam*HI, and *Sac*I), we found different rearrangements or gross deletions in two out of three MEN1 families (F28 and F66) (fig 1).

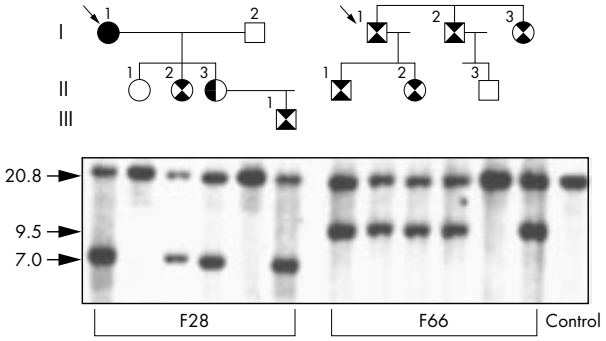

Figure 1 *Eco*RI Southern blot analysis of DNA from MEN1 families (F28 and F66) carrying large germline deletions. The *Eco*RI restriction analysis of I.1 (index patient) of F28 showed a rearrangement that segregated with the disease in this family (II.2, II.3, and III.1). The mutant pattern presented an additional band of 7 kb that was not observed either in the unaffected husband (I.2) or in the unaffected daughter (II.1). In F66, the index case (I.1) showed an additional band of 9.5 kb that segregated with the disease in this family (I.2, I.3, II.1, and II.2). Unaffected subjects are represented by unfilled symbols; patient affected by a parathyroid tumour, pituitary NFT, pancreatic NFT, and adrenal adenoma by a filled black circle; patients with a parathyroid tumour and prolactinoma by a half filled circle; and those with a parathyroid tumour by symbols filled with two black triangles.

Germline *MEN1* mutations in sporadic MEN1

We analysed 27 sporadic MEN1 cases and identified heterozygous germline mutations located throughout the entire coding region of *MEN1* gene in nine cases (33%) (table 2). Some of these results have been partially described by Cebrián *et al*.[19] The parents of five of these cases could be analysed, thereby confirming that the mutations were de novo (table 2). In the 18 cases without germline mutation, we performed a Southern blot analysis but did not detect any rearrangements.

Aside from the five polymorphisms previously mentioned, we identified another variant, A541T (GCA→ACA, 2%), whose frequency was similar to that described in the white population.[20–22]

Genotype-phenotype correlations

We defined two groups to compare their clinical features. The first included all familial and sporadic cases with germline mutations or with confirmed segregation with 11q13 (n=37). The second group comprised all cases without germline mutation (n=18). The analysis was not biased given that table 1 shows clinical data and the age of development of the disease from the index patients of the families. Fig 2 shows the mean age at which the first lesion was diagnosed in both groups: 33 years (50th centile =32) in cases with germline mutations and 53.6 years (50th centile=54.5) in sporadic cases without mutation. This difference was statistically significant ($p<0.0001$). Fig 3 shows the clinical characteristics of both groups. Worthy of note is that nearly 100% of all patients developed HPT, 37.8% (14/37) of cases with mutation developed prolactinoma, whereas only two cases without alteration developed it. Furthermore, only two cases with mutation had GH secreting pituitary tumours, whereas eight out of 18 cases without mutation (44.4%) had developed it in conjunction with HPT, this difference being statistically significant ($p=0.001$).

DISCUSSION

To date, in MEN1 families there is no specific correlation between the kind of tumours and the *MEN1* germline mutation. Its clinical presentation, age of onset, and natural history of the disease vary even in members of the same family, probably owing to modifying genes which can influence

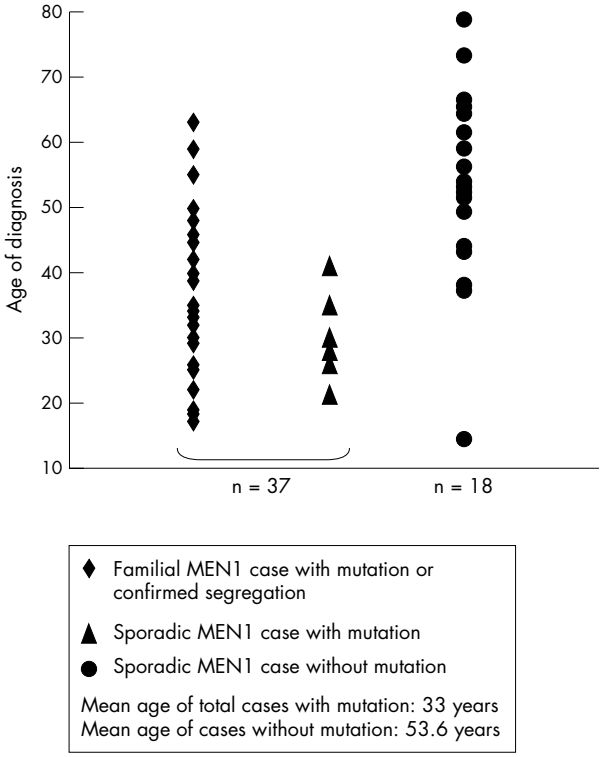

Figure 2 Representation of age of diagnosis of 28 familial MEN1 cases and 27 sporadic MEN1 cases. The age difference between cases with and without mutation was statistically significant.

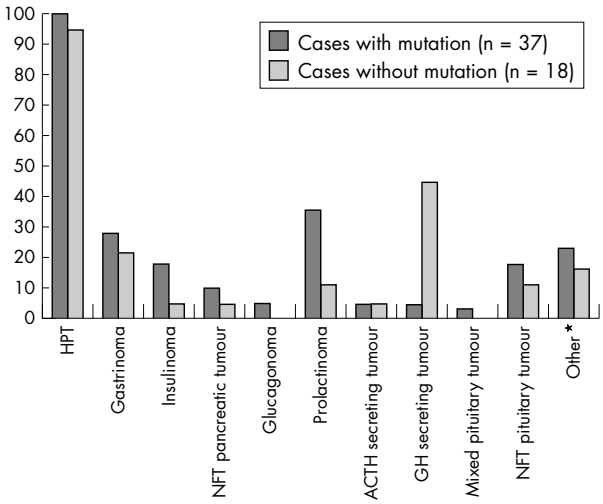

Figure 3 A graph comparing clinical characteristics from MEN1 cases with and without mutation. Each bar represents the percentage of cases that developed each type of tumour.

the penetrance of disease phenotypes.[1] Thus, the *MEN1* carriers in a family with either typical or atypical expression of MEN1 should be monitored similarly for expression of MEN1 tumours.[2] It is essential to establish useful clinical criteria to perform a *MEN1* mutation test, not only in MEN1 families but also in sporadic MEN1 cases, in which the identification of *MEN1* germline mutation has clear consequences in the follow up.

In our series of Spanish patients, we tried to identify which tumour combination would most probably show a *MEN1* germline mutation in patients without a clear familial history

of the disease, and to discover if all typical MEN1 families could be explained by alterations in the *MEN1* gene.

Germline *MEN1* mutations in MEN1 families and sporadic cases

A total of 89.3% (25/28) of familial MEN1 cases showed germline mutations in the *MEN1* gene. Each case had an individual alteration and all but seven of them generated a truncated protein. The type of mutation was characteristic of the tumour suppressor gene, and the frequency of these mutations was similar to that reported by other authors.[23 24]

No germline mutation was detected in three families analysed. A haplotype study was conducted in those two families with a sufficient number of relatives and we were able to confirm that the 11q13 region segregated with the disease in both families. As gross deletions have been previously described to be a *MEN1* inactivation mechanism,[25–27] we investigated the presence of this type of alteration in cases without germline mutation. Rearrangements were detected in two out of three analysed families, implying that this type of analysis should become a standard procedure in mutation screening of the *MEN1* gene. Following the strategy described in this study, we were able to define the molecular alteration of the *MEN1* gene to explain more than 96% of Spanish families. Regarding the family in which 11q13 segregated but had no germline mutation (F22, table 1), the existence of alterations in other regions of the gene or the presence of one or more additional genes in this region requires further investigation. There is controversy over the existence of genetic heterogeneity in this syndrome, as genetic linkage studies have suggested that the familial MEN1 trait always arises from the same gene,[7] while, on the other hand, this idea has already been opposed by other authors.[20 28]

To date, the 27 sporadic cases constitute the largest series of patients with sporadic MEN1 to be reported. We detected germline mutations in nine of them (33.3%). This incidence of *MEN1* gene mutations in sporadic cases was similar to that described by Hai *et al*[29] (40%) but was lower than that reported by other authors,[21 30] probably because of the different selection criteria used in these studies.

In five out of nine patients with germline mutations (13.8% of all detected mutations, including gross deletions), we were able to confirm by analysis from parental DNA that these mutations were de novo. In the rest of the patients, it was not possible to obtain the collaboration of their relatives to carry out the analysis. Although none of the relatives had symptoms related to the disease, we cannot establish whether these cases involved incomplete penetrance or were indeed de novo cases. The confirmation of a de novo mutation in near to 14% of our series is of great clinical importance both for the probands and for the genetic counselling of the rest of family, for whom carrier status may be determined and a suitable clinical and biochemical follow up instigated for the early detection of the disease.

Genotype-phenotype correlations

Recently, some menin domains have been defined as being important for menin function and essential for its interaction with other proteins, such as JunD or nm23.[11 13] We have tried to find some associations between mutations located in these domains and more aggressive phenotypes than those produced by mutations located outside these regions. Our study and others[24 31] have all found the phenotype to be independent of the location of the mutation. As carriers of germline *MEN1* mutations without a family history evidently showed the same clinical behaviour as MEN1 patients with antecedents of the disease, we established two different groups (cases with germline mutation or confirmed segregation *v* cases without germline mutation) to formulate clinical criteria to identify disease gene carriers among patients with apparently sporadic MEN1 in the Spanish population. The mean age of diagnosis in the two groups was significantly different: 33 years in patients with germline mutation and 53.6 years in cases without alteration ($p<0.0001$) (fig 2). Among sporadic cases only, all patients younger than 40 years of age had a germline mutation except two women, 15 and 39 years old, and a 38 year old man, who will be discussed in greater detail below. These results support those of other authors,[15] who identified novel MEN1 families from MEN1 suspected patients younger than 35 years and/or multiple MEN1 related lesions in a single organ or two distinct organs affected.

Regarding the clinical aspects (fig 3), we observed no differences between the development of HPT or gastrinoma and the presence or absence of mutations in the *MEN1* gene. An explanation could be the prevalence of HPT in the population older than 40 years, or that pancreatic islet tumour can typically cause symptoms from hormone excess after this age,[2] and then gene carriers probably will develop enteropancreatic tumours later. Five patients developed carcinoid tumours and all of them had germline mutations. These data support the suggestion of Chanson *et al*[32] that *MEN1* gene analysis should be obligatory in patients with thymic or bronchial carcinoid. One striking result was that 37.8% (14/37) of cases with germline mutation developed prolactinoma, whereas only 11.1% (2/18) of patients without mutation had it.

Significantly, one of these cases was a 15 year old girl (C4, table 2) who, owing to her age at diagnosis, was considered a de novo case, with no mutation detected either by sequence analysis or by Southern blotting, pending confirmation or ruling out the existence of mutations in other regions of the *MEN1* gene, such as the promoter. This difference between cases with and without mutation in the development of prolactinoma was not great enough to be statistically significant ($p=0.106$) for the small sample size currently available. That said, our results suggest that there is a need to study the *MEN1* gene in all patients younger than 40 years of age who present the combination of HPT and prolactinoma, because 14/16 cases with prolactinoma had germline mutation. Moreover, whereas only one case with germline mutation developed GH secreting pituitary tumours, 8/18 (44.4%) patients without mutation had this tumour in combination with HPT. It is worth highlighting the coincidence in clinical manifestations in our patients without mutation and those provided by Hai *et al*.[29] The patients in these studies had developed HPT and GH secreting pituitary tumour. These data support the view that the association of HPT and GH secreting pituitary tumours should be considered a distinct entity from MEN1, a hypothesis supported more firmly because two of these patients were the subjects aged 38 and 39 years mentioned before. This would explain why these patients do not have a mutation in the *MEN1* gene. This hypothesis was suggested before the *MEN1* gene was cloned.[33] When the gene was isolated, GH secreting tumours were found to be more frequent in cases suspected of having sporadic MEN1 but without germline mutation.[24 29 34 35] In our series, there was a strong association between the presence of GH secreting tumour and the absence of mutation in the *MEN1* gene ($p=0.001$), indicating that these patients represent a different entity with a distinct aetiology to MEN1 syndrome.

To summarise, we have found germline mutations (including Southern blot analysis) in 96.4% of MEN1 families and 33.3% of sporadic cases. We have not observed any association between the location of mutations and the clinical characteristics of the patients. Our results suggest the following.

- Patients with only two of the three main clinical manifestations of the disease, without a family history and older than 40 years of age, should not be included in the study of *MEN1*. Hence, they are probably not MEN1 cases, but instead phenocopies generated by mutations in other genes or by random development of HPT and other MEN1 related tumour owing to the high frequency of some of these pathologies in the population older than 40 years of age.

- Patients with any type of carcinoid tumour should be studied in order to rule out the possibility of MEN1 syndrome.
- Analysis of the *MEN1* gene should be obligatory for patients with HPT and prolactinoma, because 87.5% (14/16) of cases had germline mutation.
- The association of GH secreting tumours and HPT seems to be a different entity to MEN1 disease, and the gene responsible for this condition remains to be localised.
- We recommend the analysis of gross deletions in the *MEN1* gene in families without germline mutation as this type of alteration was detected in 2/3 of such cases.

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