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Germline mutations of the *MEN1* gene in familial multiple endocrine neoplasia type 1 and related states

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Familial multiple endocrine neoplasia type 1 (FMEN1) is an autosomal dominant trait characterized by tumors of the parathyroids, gastro-intestinal endocrine tissue, anterior pituitary and other tissues. We recently cloned the *MEN1* gene and confirmed its identity by finding mutations in FMEN1. We have now extended our mutation analysis to 34 more unrelated FMEN1 probands and to two related states, sporadic MEN1 and familial hyperparathyroidism. There was a high prevalence of heterozygous germline *MEN1* mutations in sporadic MEN1 (8/11 cases) and in FMEN1 (47/50 probands). One case of sporadic MEN1 was proven to be a new *MEN1* mutation. Eight different mutations were observed more than once in FMEN1. Forty different mutations (32 FMEN1 and eight sporadic MEN1) were distributed across the *MEN1* gene. Most predicted loss of function of the encoded menin protein, supporting the prediction that *MEN1* is a tumor suppressor gene. No *MEN1* germline mutation was found in five probands with familial hyperparathyroidism, suggesting that familial hyperparathyroidism often is caused by mutation in another gene or gene(s).

INTRODUCTION

Familial multiple endocrine neoplasia type 1 (FMEN1) is an autosomal dominant disorder characterized by tumors of the parathyroids, gastro-intestinal endocrine tissues and anterior pituitary (1–3). Some of its other associations include foregut carcinoid and lipoma (3). In a recent workshop-based tabulation of 87 families with FMEN1, all showed linkage to 11q13 (4), suggesting mutation in the same gene as the cause of the trait in all or most families.

The prevalence of phenocopies of FMEN1 is unknown. FMEN1 could, in principle, have at least three main types of phenocopy. The first type is the sporadic case with one or more features of FMEN1 in the absence of a family history of MEN1; in particular, MEN1 can be difficult to distinguish from independent occurrence of tumors in two or more FMEN1-related endocrine tissues. A second type of potential phenocopy is a kindred showing incomplete or otherwise atypical features of FMEN1. For example, familial hyperparathyroidism could be an incomplete or early expression of FMEN1 but might also have other causes. Two of six large families, initially reported as familial hyperparathyroidism, later showed features of FMEN1 (5). And one large family with primary hyperparathyroidism but without other features of FMEN1 showed likely linkage to 11q13, with a LOD score of 2.12 for D11S97 at $\theta = 0.05$ (6). A third type of phenocopy is a family meeting the formal definition of FMEN1 with or without certain unusual features; the trait in one family with some added features atypical for FMEN1 was recently shown not to be linked to 11q13 (7).

We recently cloned the *MEN1* gene and proved its identity by finding germline mutations in 15 of 16 probands for FMEN1 (8 and Olufemi, S.-E. *et al.*, in preparation). We have now extended our analyses to a total of 50 kindreds with FMEN1, and we have tested for germline *MEN1* mutations in two related states, sporadic MEN1 and familial hyperparathyroidism.

RESULTS

Germline *MEN1* mutations in FMEN1

Dideoxyfingerprinting (ddF) and direct sequencing were used to characterize *MEN1* mutations in all coding regions and splice junctions of the *MEN1* gene. Data are summarized for 16 FMEN1

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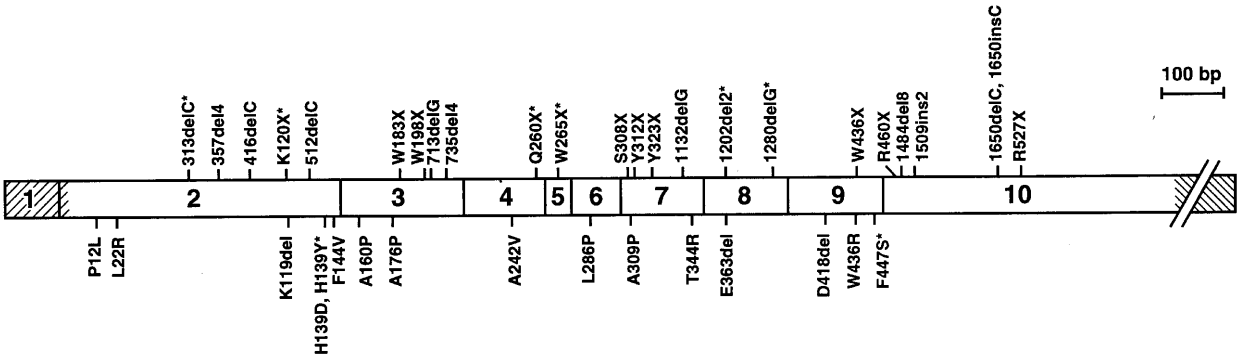

Figure 1. Mutations identified in probands of 47 MEN1 kindreds and in eight cases of sporadic MEN1. The locations of the 13 frameshift mutations and 11 nonsense mutations are shown above a diagram of the *MEN1* mRNA, with exons numbered; crosshatched areas are untranslated portions of 5′- and 3′-exons. Three in-frame deletions and 13 missense mutations are shown below the diagram. Eight mutations were encountered in FMEN1 more than once: 357del4 (twice), 416delC (five times), K119del (twice), 512delC (six times), 735del4 (twice), E363del (twice), R460X (twice), 1650delC (twice). The eight mutations found in sporadic MEN1 are followed by an asterisk (*). Full details are at http://www.niddk.nih.gov

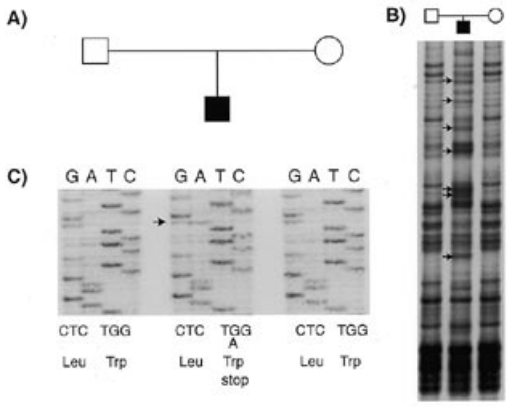

Figure 2. Documentation of a new mutation in the *MEN1* gene in case 5726 with sporadic MEN1. (**A**) Upper panel, family tree with affected subject as solid box, (**B**) dideoxyfingerprinting analysis of exon 5 shows band changes (see arrows) in the son only, (**C**) sequencing gels show heterozygous W265X in the son only. Heterozygous base change is indicated by an arrow.

kindreds reported previously (8 and Olufemi, S.-E. *et al.*, in preparation) plus 34 more kindreds. The kindreds had 1–92 living affected members with a median of 5. Including our recently reported data, heterozygous germline *MEN1* mutations have been found in 47 of 50 probands with FMEN1 (Fig. 1). These consisted of 10 frameshift mutations, eight nonsense mutations, three in-frame deletions and 11 missense mutations. Eight mutations were encountered more than once among probands not previously known to be related (Fig. 1). The 512delC and 416delC mutations were found six and five times respectively. These two clusters included four families and two families respectively that had been grouped together in a prior 11q13 haplotype analysis of 27 of these families (Emmert-Buck, M.R. *et al.*, in preparation), suggesting a common founder that was different for each of these two large clusters.

The prolactinoma variant is the only distinct FMEN1 phenotype reported in more than one family (9–12). Probands were analyzed in all three of these reported families. Two probands showed germline *MEN1* mutations [R460X (11,12; Olufemi, S.-E. *et al.*, in preparation) and Y312X (10)] while the third did not (8,9). The *MEN1* mutation negative kindred showed linkage of FMEN1 to 11q13 with a 2 point LOD score of 3.25 with $\theta = 0.00$ for the marker D11S4909 (Manickam, P. *et al.*, in preparation). Thus the third family has a presumed mutation different from those in the first two and that third mutation is highly likely to be in the *MEN1* gene. There was no unique pattern of the presumed three *MEN1* mutations in the prolactinoma variant of FMEN1 that would readily explain the prolactinoma FMEN1 phenotype.

The R460X mutation was shared with one more proband, a member of a kindred that could not be classified concerning presence or absence of the prolactinoma phenotype. Haplotype analysis excluded a common founder for these two MEN1 kindreds with identical *MEN1* mutation, since they had different FMEN1-linked alleles (data not shown) at two loci [PYGM (CAGA) and D11S449] within 250 kb of *MEN1* on centromeric and telomeric sides respectively.

Germline *MEN1* mutations in sporadic MEN1

We identified *MEN1* germline mutations in eight of 11 cases with sporadic MEN1. The mutations in sporadic MEN1 were three frameshift mutations, three nonsense mutations and two missense mutations (Fig. 1 and Table 1). The nature and distribution of mutations were similar to those in FMEN1, but no mutation was identical to one found in FMEN1.

Though the family history was negative for MEN1 in the 11 cases, in most instances other family members were not available for examination or for DNA analysis. However, in one case of sporadic MEN1 (ID 5726) we were able to show that the *MEN1* mutation (W265X) (Table 1) was absent in both parents (Fig. 2). Correct assignment of paternity was established by typing of 11 polymorphisms, indicating that only 1 in 500 000 Caucasians would have a genotype compatible with being the father in this case. This documentation of MEN1 arising from a new mutation is an independent confirmation of the recent report (8) that mutation in this gene causes the MEN1 phenotype.

Table 1. Sporadic MEN1 (includes three with MEN1-like state, IDs 37, 601, 5335)

ID	*MEN1* gene mutation	Parathyroid glands	Gastro-intestinal endocrine tissues	Anterior pituitary gland	Other features
6628	313delC	Multiple tumors	Metastatic insulinoma	High prolactin	
487	K120X	Multiple tumors	Zollinger–Ellison, Many islet tumors		
7348	H139Y	Multiple tumors		Prolactin microadenoma	
4266	Q260X	Multiple tumors	Zollinger–Ellison	GH[a]-prolactin macroadenoma	
5726	W265X	Multiple tumors	Insulinoma, Many islet tumors		Lipomas
601	1202del2	Multiple tumors			Lipoma, Thyroid follicular adenoma, Esophageal leiomyoma
6321	1280delG	Multiple tumors	Zollinger–Ellison		Rhabdomyosarcoma
7269	F447S	Multiple tumors	Zollinger–Ellison		Metastatic carcinoid
37	Not found	One tumor?[b]			Lipomas
5335	Not found	One tumor			Bilateral adrenal macronodular Cushings
6721	Not found	Multiple tumors		GH[a] tumor	

Mutation details available at http://www.niddk.nih.gov
[a]GH, growth hormone.
[b]Incomplete information from prior operation(s) at another institution precluded determining if other parathyroid tumors had been removed.

Table 2. Clinical features of pedigrees with familial hyperparathyroidism

ID	*MEN1* gene mutation	Phenotype carriers Total	Avg. age	Parathyroid glands[a]	Renal Ca/Cr clearance ratio[b]	Other features in one carrier
4318	Not found	4	73	Multiple tumors	0.015	
5780	Not found	2	43	Multiple tumors	0.016	
6325	Not found	4	55	Multiple tumors	0.032	
7751	Not found	5	64	Multiple tumors	0.017	Breast cancer
9462	Not found	3	48	Multiple tumors	8.4 mmol per day[b]	Peri-biliary carcinoid, lipomas

[a]Parathyroid gland status available only for proband.
[b]Ca/Cr = calcium to creatinine. Data available only for proband. Expected range is 0.002–0.010 in familial hypocalciuric hypercalcemia and 0.010–0.05 in typical primary hyperparathyroidism (3). For one subject (9462) with a 24 h urine calcium, the equivalent diagnostic separation is at 3.0 mmol per day.

No germline *MEN1* mutation in familial hyperparathyroidism

Five probands with familial hyperparathyroidism, including one (ID 9462) with features resembling FMEN1, were investigated. There are circumstances under which FMEN1 would likely present as familial hyperparathyroidism. A MEN1 family with only young affected cases available might show this. Pancreatic islet tumors are generally expressed at later ages. But the average age of affected cases in these five kindreds with familial hyperparathyroidism was 43–73 (Table 2), and the oldest carrier in each kindred was aged between 58 and 87 years.

No *MEN1* mutation was found in any of these five probands (Table 2). In the absence of *MEN1* mutation, other causes warrant consideration. Hypercalciuria in the proband of each kindred made familial hypocalciuric/benign hypercalcemia unlikely (Table 2) (3).

Benign polymorphisms of the *MEN1* gene

Together with the findings previously reported (8), seven presumably benign polymorphisms have been encountered among 142 normal chromosomes. These were S145S (AGC/AGT) (0.7%), C→T in intron 2 58 nucleotides upstream of exon 3 (0.7%), R171Q (CGG/CAG) (5%), L256L (CTT/CTC) (0.7%), L432L (CTG/CTA) (0.7%), D418D (GAC/GAT) (54% C, 42% T), A541T (GCA/ACA) (96% G, 4% A).

DISCUSSION

Frequent *MEN1* germline mutation in kindreds with FMEN1

This study confirms and considerably extends the finding of a high prevalence of *MEN1* germline mutation in probands with FMEN1 (8 and Olufemi, S.-E. *et al*., in preparation). The parallel implication is that the prevalence of FMEN1 phenocopies caused by mutation in other genes is very low (7). Genetic counseling by testing in the *MEN1* gene will be effective, though there will be many different mutations to be identified.

We have not yet recognized any consistent genotype–phenotype correlations in FMEN1. In particular, we examined three kindreds with one distinct FMEN1 phenotype, $MEN1_{Burin}$ or the prolactinoma variant of FMEN1, and found no characteristic genotype.

Eight mutations occurred more than once. These data, supplemented by a prior haplotype analysis in a subset of these families, revealed that previously tested families in the two largest clusters (512delC and 416delC) shared two identical haplotypes about 11q13. Thus founder effects explain, at least in part, the two most commonly observed *MEN1* mutations (13). In contrast, haplotype analysis excluded a founder effect for another mutation (R460X) shared by two kindreds. Further studies, including added haplotype analyses in these families as well as in families from other geographic and ethnic groups, will be required to explore the prevalence of founder mutations in FMEN1 (13).

It is likely that all or some of the three mutation-negative kindreds with FMEN1 actually do have a mutation in the *MEN1* gene. One mutation-negative family showed convincing linkage of the MEN1 trait to 11q13. The other two families had three and four members with characteristic features, and no such family has yet been encountered in which linkage to 11q13 could be excluded (4). Our mutation screening strategy of ddF and direct sequencing is highly efficient across the entire open reading frame plus 20–40 bases upstream and downstream into introns flanking each exon, but occasional mutations in the open reading frame might be missed (14,15). More importantly, we cannot rule out *MEN1* mutations similar to those that have been identified for many genes in the 5′ and 3′ untranslated regions, introns and promoter regions (16). The methods used here also might not detect a large germline deletion (more than an exon in extent), as only the normal copy of *MEN1* would amplify (16).

Frequent *MEN1* germline mutation in sporadic MEN1

We have begun testing for *MEN1* germline mutation in various sporadic disorders, by analyzing sporadic MEN1, one disorder with particularly high *a priori* likelihood of *MEN1* germline mutation. In eight of 11 cases of sporadic MEN1, *MEN1* germline mutations were found.

Some of the three cases without *MEN1* mutation are quite likely to reflect a phenocopy. Two of these may have had a solitary parathyroid tumor, whereas multiple parathyroid tumors were found in all eight cases with *MEN1* mutation (Table 2). Solitary parathyroid tumor is common in sporadic hyperparathyroidism (17), a likely component of phenocopies, but it is uncommon in FMEN1 (18).

Several features were associated with *MEN1* mutation among these 11 sporadic cases (Table 1). Tumor in multiple parathyroid glands was associated with high likelihood of *MEN1* mutation (eight mutations among nine cases) contrasted with lower likelihood in those with solitary parathyroid (no mutation among two cases). Four of four cases with Zollinger–Ellison syndrome had an *MEN1* mutation, but this clinical feature could not be judged as an independent *MEN1* mutation predictor, as it was always associated with multiple parathyroid tumors. This supports the general experience that primary hyperparathyroidism is usually expressed at an earlier age than Zollinger–Ellison syndrome in FMEN1 (19).

Some of these observations will be immediately applicable to genetic counseling. The high prevalence of *MEN1* mutations in sporadic MEN1 excludes the possibility that many of these subjects have a phenocopy. Sporadic MEN1 cases with particularly high likelihood of *MEN1* germline mutation can be readily identified as those with multiple, not solitary, parathyroid tumor. *MEN1* mutation analysis can be a useful diagnostic tool for any patient with sporadic MEN1.

Muscle tumors may represent a component of the MEN1 spectrum. Two cases of sporadic MEN1 with associated muscle tumor (rhabdomyosarcoma and esophageal leiomyoma) had germline frameshift mutations in exon 8 (Table 1). Prior tumor studies of a mother and son in a large MEN1 kindred (20) revealed no 11q13 loss of heterozygosity in an esophageal leiomyoma or in a renal angiomyolipoma (20). The *MEN1* mutation in this family is K119del in exon 2. There is insufficient evidence that any of these muscle-containing tumors is somehow caused by a germline *MEN1* mutation.

Further work must be done to establish the prevalence of *MEN1* mutations among patients with other sporadic disorders. The most obvious sporadic disorders to investigate for *MEN1* germline mutation are Zollinger–Ellison syndrome, primary hyperparathyroidism (with multiple parathyroid tumors), pro-

lactinoma, and foregut carcinoid. But many other disorders will also be of interest.

No *MEN1* germline mutation in familial hyperparathyroidism

Surprisingly, *MEN1* germline mutations were not found in any of the five probands with familial hyperparathyroidism, although FMEN1 can clearly present in this manner (5). Some small FMEN1 kindreds with mainly young members are likely to present as isolated primary hyperparathyroidism, since hyperparathyroidism has the highest penetrance in FMEN1 and also an early age of onset (85% by age 35) in FMEN1 (19). The relatively advanced ages in our kindreds with familial hyperparathyroidism (Table 2) may have biased our series against FMEN1. One or more of these families might have a distinct phenotype of FMEN1 with a class of mutation not recognized by our analyses (see above). However, it seems more likely that most or all of these families represent several distinct etiologies of hyperparathyroidism with few if any mapping to *MEN1*. Such etiologies could include the syndrome of recurrent parathyroid adenoma, parathyroid cancer, jaw tumors, and renal tumors, mapped to 1q (21) and also familial hypocalciuric or benign hypercalcemia (5) mapping to the calcium-sensing receptor at 3q (22) and to unknown genes at other loci (23). The latter was partially excluded by the occurrence of hypercalciuria in all the probands with familial hyperparathyroidism (Table 2). It will be important to test these pedigrees with polymorphisms from 11q13 to see if linkage can be excluded.

Molecular interpretations of *MEN1* germline mutations

A broad spectrum of germline mutations was identified in the *MEN1* gene. None of these were seen in ddF analyses of 142 normal chromosomes. The common feature of most of these mutations was likely loss of function of the encoded menin protein. This further supported predictions that *MEN1* is a tumor suppressor gene (8,24).

The prevalence of frameshifts and nonsense mutations (23/40 among ascertained mutations and 35/55 among ascertained probands) indicates that a protein truncation type of mutation screening assay for MEN1 could be feasible if RNA from the mutant allele is expressed (25), but the frequency of in-frame deletions and missense mutations would still cause a false negative yield in the range of 30%.

The *MEN1* mutations were distributed throughout the open reading frame with no apparent hotspots for mutation. In prior reports, only two missense mutations were found among 13 different mutations in FMEN1 (8 and Olufemi, S.-E. *et al*., in preparation); however among 27 additional mutations, there were nine more missense mutations in FMEN1 and two in sporadic MEN1. Each of these 13 missense mutations in FMEN1 and sporadic MEN1 was not found in ddF analysis of 142 normal chromosomes. In the absence of information about function and three dimensional structure of menin (8), we cannot measure the consequences of the 13 missense mutations and the three in-frame deletions. Most predict changes in the menin protein likely to be significant in terms of structure or function: eight charge changes, two changes of hydrophobicity, one loss of bulky aromatic residue, and five gain or loss of proline. Chou-Fasman prediction of secondary structure indicates that four of the five proline changes are in regions of likely alpha helix. Complexity based segmentation analysis reveals regions of high and low complexity in menin (26). All of the missense mutations are in regions of high complexity. These mutations provide the first indication that widespread domains of menin may be critical to its still unknown functions. This is supported by extensive genomic sequence conservation throughout the open reading frames between human and mouse menin (Guru, S.C. *et al*., unpublished). For the above reasons, all or most of these mutations are not likely to be benign polymorphisms. But this can only be definitively analyzed after development of assays of gene/protein function.

MATERIALS AND METHODS

Subjects

All probands were evaluated at NIH. Relatives were tested at NIH or through local physicians. All participation was with informed consent, approved by the NIDDK Institutional Review Board. MEN1 was defined as endocrine tumor in two of the three principal MEN1-related tissues; familial MEN1 was defined as MEN1 plus at least one first degree relative with an MEN1-related endocrinopathy. Study cases included 50 FMEN1 probands, five familial hyperparathyroidism probands and 11 cases of sporadic MEN1. Data were included from the 16 probands of MEN1 kindreds reported previously (8 and Olufemi, S.E. *et al*., in preparation). The cluster of four Newfoundland families with $MEN1_{Burin}$ were analyzed as one extended family, based on a known founder effect from haplotype analysis and mutation analysis (11 and Olufemi *et al*., in preparation). The prolactinoma phenotype can be best recognized in a large MEN1 kindred; among members expressing the trait, there is high penetrance (~95%) for primary hyperparathyroidism, relatively high (~30%) for prolactinoma and low (~10%) considering MEN1 for Zollinger–Ellison syndrome. The comparable penetrance frequencies in typical FMEN1 are 95, 15 and 40% respectively (3). The sporadic MEN1 group included three patients with an MEN1-like state consisting of primary hyperparathyroidism and either (a) lipomas, (b) lipoma and thyroid follicular adenoma, or (c) hypercortisolism from bilateral ACTH independent macronodular adrenal hyperplasia. Familial hyperparathyroidism was defined as primary hyperparathyroidism in two or more family members without MEN1; one family had one member with lipomas, suggestive but not diagnostic of MEN1. The control DNAs from 71 normals have been reported (8).

Two point LOD scores were calculated with FASTLINK (26).

Analyses of DNA

Genomic DNA was isolated from blood using the Qiagen Kit (Chatsworth, CA).

Haplotype analysis was as recently described (28), using a proximal (PYGM-CAGA) and a distal (D11S449) polymorphic marker, each within 250 kb of the *MEN1* gene (8, Manickam, P. *et al*., in preparation).

The Amplitype-PM PCR amplification and typing kit (Perkin-Elmer) was used to test for paternity. DNA typing was according to the manufacturer's protocol for six polymorphic loci. This was supplemented by haplotype analysis as above, using D1S243 (29), D6S310 (29), D22S351 (30), D11S480 (31), and D11S4908 (GDB accession ID: 3811807).

MEN1 exons 2–10 were amplified individually or in groups using primers designed from intron sequences (8) with minor modifications available at http://www.niddk.nih.gov. The products were used for ddF reactions. PCR conditions for amplification of exons and ddF procedures have been reported earlier (8). ddF reactions were electrophoresed on a nondenaturing gel [0.75× MDE (FMC Bioproducts, Rockland, ME) in 0.5× TBE] on a sequencing apparatus as described (8).

Samples showing changes from normal in band patterns were subjected to cycle sequencing using the same PCR product and the same end-labeled primer as was used in the ddF reaction.

For insertion or deletion type changes in which the actual bases involved could not be ascertained from the sequence of the heterozygous patient sample, the primary PCR product was cloned in the TA cloning vector pCRII (In Vitrogen, San Diego, CA) and then sequenced.

For the 11 cases in which a mutation was not detected by ddF, the PCR products containing individual exons amplified from genomic DNA (exons 2–10) were sequenced on both strands.

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ABBREVIATIONS

FMEN1, familial multiple endocrine neoplasia type 1; MEN1, multiple endocrine neoplasia type 1; ddF, dideoxyfingerprinting.

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