

# Spectrum and Prevalence of *FP/TMEM127* Gene Mutations in Pheochromocytomas and Paragangliomas

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**Context** Pheochromocytomas and paragangliomas are genetically heterogeneous neural crest–derived neoplasms. We recently identified germline mutations of the novel transmembrane-encoding gene *FP/TMEM127* in familial and sporadic pheochromocytomas consistent with a tumor suppressor effect.

**Objectives** To examine the prevalence and spectrum of *FP/TMEM127* mutations in pheochromocytomas and paragangliomas and to test the effect of mutations in vitro.

**Design, Setting, and Participants** We sequenced the *FP/TMEM127* gene in 990 individuals with pheochromocytomas and/or paragangliomas, including 898 previously unreported cases without mutations in other susceptibility genes from 8 independent worldwide referral centers between January 2009 and June 2010. A multiplex polymerase chain reaction–based method was developed to screen for large gene deletions in 545 of these samples. Confocal microscopy of 5 transfected mutant proteins was used to determine their subcellular localization.

**Main Outcome Measures** The frequency and type of *FP/TMEM127* mutation or deletion was assessed and correlated with clinical variables; the subcellular localization of 5 overexpressed mutants was compared with wild-type *FP/TMEM127* protein.

**Results** We identified 19 potentially pathogenic *FP/TMEM127* germline mutations in 20 independent families, but no large deletions were detected. All mutation carriers had adrenal tumors, including 7 bilateral ( $P=2.7 \times 10^{-4}$ ) and/or with familial disease (5 of 20 samples;  $P=.005$ ). The median age at disease onset in the *FP/TMEM127* mutation group was similar to that of patients without a mutation (41.5 vs 45 years, respectively;  $P=.54$ ). The most common presentation was that of a single benign adrenal tumor in patients older than 40 years. Malignancy was seen in 1 mutation carrier (5%). Expression of 5 novel *FP/TMEM127* mutations in cell lines revealed diffuse localization of the mutant proteins in contrast with the discrete multiorganelle distribution of wild-type *TMEM127*.

**Conclusions** Germline mutations of *FP/TMEM127* were associated with pheochromocytoma but not paraganglioma and occurred in an age group frequently excluded from genetic screening algorithms. Disease-associated mutations disrupt intracellular distribution of the *FP/TMEM127* protein.

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**P**HEOCHROMOCYTOMAS AND paragangliomas are chromaffin cell tumors of neural crest origin that arise from the adrenal medulla or extra-adrenal sympathetic paraganglia, respectively, and are frequently catecholamine secreting.<sup>1</sup> These tumors are usually benign and can occur as a single entity or as part of various hereditary tumor syndromes. Genetically, pheochromocytomas and paragangliomas are hetero-

geneous, with at least one-third of cases resulting from germline but not somatic mutations in 1 of several independent genes: *RET*, *VHL*, *NFI*, and succinate dehydrogenase (*SDH*) subunit B, C, and D genes.<sup>2-5</sup> More recently, other candidate susceptibil-

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ity genes have also been reported, including *KIF1B*,<sup>6,7</sup> *Egln1/PHD2*,<sup>8</sup> *SDHAF2*,<sup>9,10</sup> and *SDHA*,<sup>11</sup> although these findings remain restricted to 1 or 2 occurrences of the reported mutations. Despite this broad spectrum of susceptibility genes, the molecular basis for the majority of pheochromocytomas and paragangliomas, including most of the sporadic and rare familial cases, remains unknown. These observations support the existence of additional pheochromocytoma susceptibility genes, which may account for some of the genetically undefined cases.<sup>12,13</sup>

We recently identified *TMEM127*<sup>14</sup> (NM\_017849.3) as the pathogenic target of the familial pheochromocytoma (FP) locus, which we previously mapped to chromosome 2q11.<sup>13</sup> Germline *FP/TMEM127* mutations were found both in familial and sporadic-appearing pheochromocytomas, with loss of the wild-type allele in tumor DNA consistent with its role as a tumor suppressor gene. *FP/TMEM127* encodes a highly conserved 3-spanner transmembrane protein that localizes to multiple intracellular organelles and is linked to regulation of the mTORC1 signaling complex, a critical control node for protein synthesis and cell survival.<sup>14</sup> All but 1 of the 7 initially discovered mutations were truncating and resulted in markedly reduced expression of the *FP/TMEM127* gene in the evaluable tumors, suggesting that the mutations result in loss of *FP/TMEM127* function in the tumor tissue.

In recent years, specific genotype-phenotype associations in pheochromocytomas due to mutations in the various susceptibility genes have spurred the development of guidelines for genetic screening of these patients, with a goal of improving the outcomes of affected and at-risk individuals.<sup>3,5,15,16</sup> To define the prevalence and genotype-phenotype correlations in *FP/TMEM127*-mutated cases, we sequenced the *FP/TMEM127* gene in 990 pheochromocytoma and paraganglioma patients and examined 545 of these samples for germline deletions of the gene. We also deter-

mined the subcellular localization of 5 novel mutations by in vitro confocal microscopy.

## METHODS

### Patients

A total of 990 samples from patients with pheochromocytomas and paragangliomas without mutations in *RET*, *VHL*, *SDHB*, *SDHC*, and *SDHD* and who had no clinical features of neurofibromatosis type 1 were studied. In addition, 319 of these samples were negative for *SDHAF2* and 52 for *KIF1B* gene mutations. A cohort of 898 samples were recruited from a multi-institutional collaborative effort derived from the International Familial Pheochromocytoma Consortium, which encompassed referral centers based in the United States (San Antonio, Texas, and Ann Arbor, Michigan), Italy (Padova, Florence, and Brescia), Spain (Madrid), England (Birmingham and London), Brazil (São Paulo), Belgium (Brussels), and Argentina (Buenos Aires). Written informed consent was obtained from patients in accordance with institutional review board–approved protocols from each center. Eighteen samples were obtained from the Cancer Therapy and Research Center Pathology Tumor Bank at the University of Texas Health Science Center at San Antonio. Diagnosis of pheochromocytoma and/or paraganglioma, including tumors of both sympathetic (thoracic or abdominal) and parasympathetic (head and neck) origin, was established following conventional procedures (including clinical, biochemical, and imaging tests) and the diagnosis was confirmed histologically in every case. Tumors were considered familial when more than 1 affected individual was identified in the family. The study was performed between January 2009 and June 2010.

We previously reported *FP/TMEM127* sequence variations in a cohort of 103 patients with pheochromocytoma and/or paraganglioma.<sup>14</sup> In the present study, 92 of these cases were used for genotype-phenotype associations with the 898 new cases described above. Eleven cases from the original cohort that had germline mu-

tations in other known pheochromocytoma susceptibility genes were excluded.<sup>14</sup> The main features of each series of the entire cohort are shown in TABLE 1.

### DNA Isolation

Of the new series of 898 samples, DNA was isolated from blood in 774 cases and from tumor tissue in 124 cases, following standard procedures.<sup>14</sup>

### Control Population

A control group composed of samples of Europeans, South Americans (heterogeneous population mainly of Latino origin but also including non-Hispanic whites, Asians, African Americans, and indigenous Native Americans), North Americans (including both Hispanic and non-Hispanic whites and African Americans), and Asians totaling 1064 alleles was used as reference samples, as reported.<sup>14</sup> In addition to these, 718 new alleles were screened for exon 3 of the *FP/TMEM127* gene only. This group comprised 318 alleles of white ancestry and 404 alleles of South American origin similar in racial/ethnic composition to those described above.

### Polymerase Chain Reaction and Direct Sequencing

To define the prevalence of *FP/TMEM127* mutation in a large, multi-institutional cohort of pheochromocytomas and paragangliomas, all 4 exons of *FP/TMEM127* were amplified by polymerase chain reaction (PCR) and directly sequenced as previously reported.<sup>14</sup> Primer sequences are listed in eTable 1 (available at <http://www.jama.com>).

The accession numbers for nucleotides (NM\_017849.3) and respective protein variations (NP\_060319.1) detected in this study were deposited in the National Center for Biotechnology Information (NCBI) single-nucleotide polymorphism (SNP) database (<http://www.ncbi.nlm.nih.gov/SNP/transNP/VarBatchSub.cgi>).

### Pathogenic Mutation Call

Variations that included nonsynonymous sequence substitutions, insertions, deletions, duplications, or splice

**Table 1.** Summary of Clinical Features of the Entire Pheochromocytoma and Paraganglioma Cohort by Original Study Sites and Previously Reported Samples

	United States (n = 176)	Spain (n = 168)	Padua, Italy (n = 200)	Florence, Italy (n = 106)	Brescia, Italy (n = 52)	Belgium (n = 67)	Brazil (n = 52)	United Kingdom (n = 77)	Qin et al, <sup>14</sup> 2010 (n = 92)	Total (N = 990)
Age at diagnosis, mean (range), y	46.3 (7-78)	47.0 (11-80)	47.1 (5-79)	44.1 (9-80)	52.1 (20-83)	48 (12-84)	39.3 (7-74)	25.5 (5-65)	41.1 (4-76)	43.3 (4-84)
Sex, No.										
Female	107	94	104	58	27	46	32	51	60	579
Male	63	65	96	48	25	21	20	26	32	396
Unknown	6	9	0	0	0	0	0	0	0	15
Tumor location, No.										
Total adrenal	144	133	190	80	42	37	43	52	76	798
Bilateral adrenal	7	11	15	6	1	1	1	15	8	65
Total extra-adrenal	32	35	10	26	10	30	9	21	16	189
Extra-adrenal head and neck	3	17	0	0	8	25	1	4	2	60
Adrenal and extra-adrenal <sup>a</sup>	2	4	1	1	0	0	0	0	0	8
Unknown	0	0	0	0	0	0	0	4	0	4
Malignancy, No.	12	4	13	3	3	4	5	4	4	52
Familial history, No.	5	4	6	0	1	1	2	0	12	31

<sup>a</sup>Adrenal and extra-adrenal samples are included in the total adrenal samples.

site or nonsense changes and that were absent in the control group were considered potentially pathogenic in the present analysis. Synonymous substitutions, variants affecting intronic regions not immediately adjacent to the intron-exon border, or changes detected in the control group or in the SNP database (dbSNP, NCBI) were not considered of pathogenic relevance for the purposes of this study. We also used 3 prediction software modules to evaluate the pathogenic potential of the identified variants. Two of them, PolyPhen-2 (<http://genetics.bwh.harvard.edu/pph2/>)<sup>17</sup> and SIFT (<http://sift.jcvi.org/>),<sup>18</sup> are algorithms that predict the possible effect of an amino acid substitution on the structure and function of a human protein based on physical/structural considerations (eg, amino acid properties) and sequence homology. The third program, NetStart 1.0, predicts initiation of translation sites in eukaryotes based on a training data set of mRNA derived from genomic sequences with known start sites (<http://www.cbs.dtu.dk/services/NetStart/>).<sup>19</sup>

#### Quantitative Multiplex PCR of Short Fluorescent Fragments

To assess larger deletions that might disrupt the *FP/TMEM127* gene and that would not be detected by direct sequencing, a quantitative multiplex PCR

of short fluorescent fragments method was designed. The assay comprised 6 sets of primers spanning the 5' UTR, exons 1 through 4, and 3' UTR of the *FP/TMEM127* gene (eTable 1). Each forward primer was labeled with a 6-FAM-5' fluorescent tag, and fragments were amplified in a multiplex PCR as previously described.<sup>20</sup> In addition, amplicons spanning distinct chromosomal regions (chromosomes 1, 11, or 17) were included in the multiplex reaction as internal controls. Two to 3 normal DNA samples were run within each batch to serve as references for normal, 2-copy gene pattern. A total of 545 germline samples with high-quality, high-molecular-weight DNA were successfully processed through this assay. Results were analyzed using Peak Scanner software, version 1.0 (Applied Biosystems, Foster City, California) as previously reported.<sup>20</sup> Reliability of the assay was validated by the detection of monoallelic *FP/TMEM127* loss in tumor DNA from previously reported mutant samples, which were known to carry loss of the wild-type *TMEM127* allele<sup>14</sup> and, thus, represented models of single-copy *TMEM127* loss (eFigure 1).

#### Loss of Heterozygosity Analysis

Loss of heterozygosity (LOH) analysis is a classic method to test whether a gene involved in tumor susceptibility

has features of a tumor suppressor gene. In classic tumor suppressor genes, LOH consists of loss of the wild-type allele in tumor DNA, or the "second hit" (the first hit being the germline mutation in hereditary tumors), which results in loss of functional copies of the putative tumor suppressor gene in the tumor tissue. To identify the second hit of *FP/TMEM127* inactivation in samples carrying a mutation, LOH analysis was performed in 4 cases in which tumor DNA was available. Loss of heterozygosity was identified when the wild-type-mutant allele ratio was less than 0.5 based on sequence data from germline and tumor DNA, as described.<sup>14</sup> Loss of heterozygosity data were available from 6 previously reported samples.<sup>14</sup>

#### RNA and Reverse Transcription-PCR Analysis

To demonstrate the effect of the c.409 + 1G>T mutation on splicing of the *TMEM127* transcript, RNA was obtained from peripheral lymphocytes of the patient carrying this mutation. Complementary DNA was prepared by reverse transcription using standard procedures<sup>14</sup> and used for a PCR spanning nucleotides 180 to 677 of the *FP/TMEM127* open reading frame (primer sequences listed in eTable 1), followed by direct sequencing of the products.

**Constructs, Transfections, and Confocal Microscopy**

To begin to determine the effects of FP/TMEM127 mutations in vitro, we generated clones carrying 5 novel substitution mutations identified in the present series. Four of the mutations (c.280C>T, p.Arg94Trp; c.208G>A, p.Asp70Asn; c.419G>A, p.Cys140Tyr; and c.418T>C, p.Cys140Arg) were obtained using site-directed mutagenesis (Phusion, New England Biolabs, Ipswich, Massachusetts) from wild-type FP/TMEM127

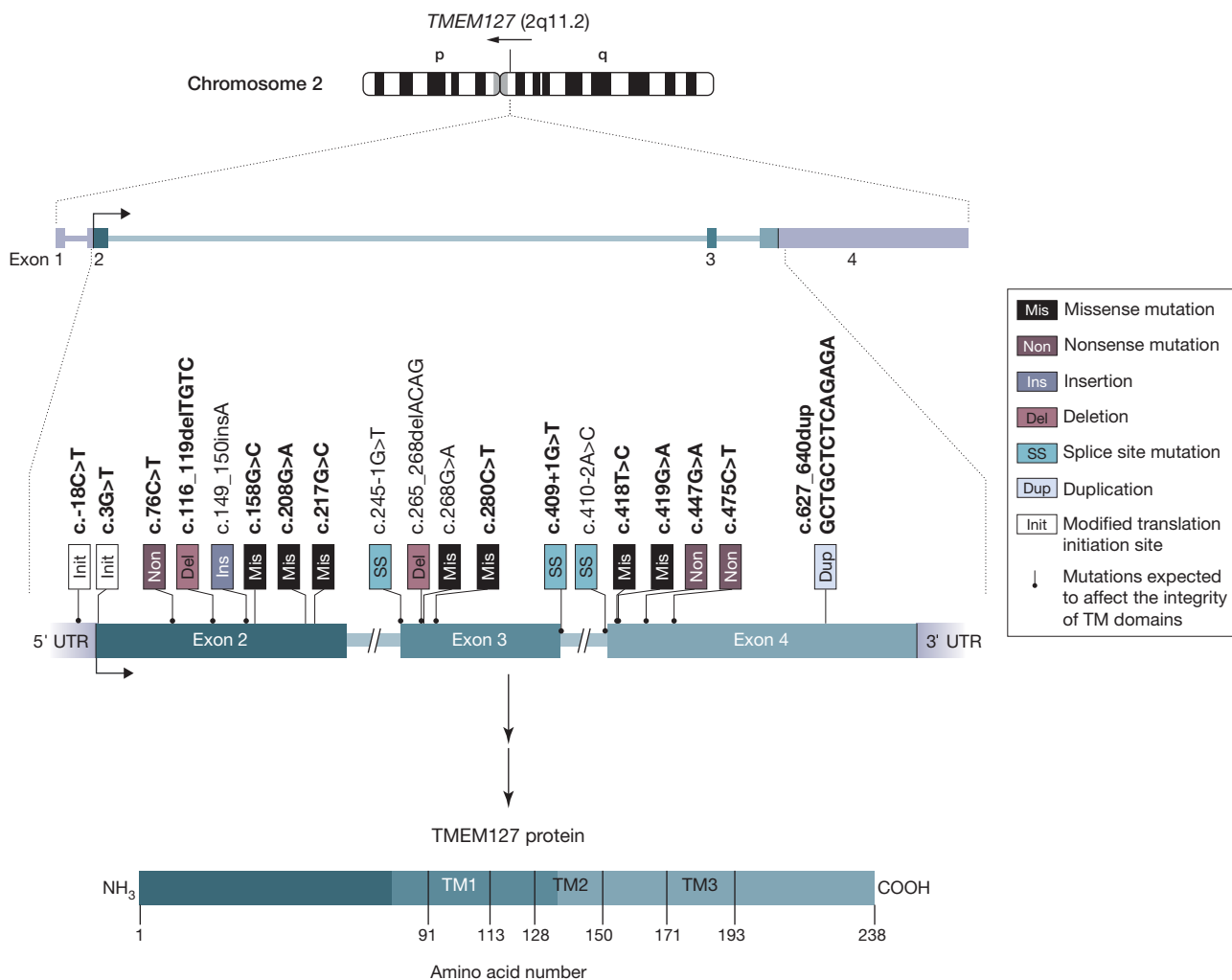
sequence cloned into *HindIII/EcoRI* sites of the pEGFP-C2 vector (Clontech, Mountain View, California), as previously described.<sup>14</sup> Additionally, the c.3G>T (p.Met1?) mutation was generated by PCR, using as the start codon an in-frame methionine at position 85 and cloned as above. All constructs were verified by sequencing. The remaining constructs that led to truncating products had previously shown to be unstable for protein detection.<sup>14</sup> Each construct, as well as the wild-type version, was

transfected into HEK293T, HeLa, or MPC9/30 (mouse pheochromocytoma cell line<sup>21</sup>) cells. After 24 hours, cells were fixed and imaged by confocal microscopy as previously reported.<sup>14</sup> Green fluorescent protein (GFP) fluorescence of transfected cells was examined for sub-cellular localization as punctate (wild-type) or diffuse patterns.

**Statistical Analysis**

Statistical analyses were carried out using SPSS software, version 17.0 (SPSS

**Figure 1.** Pathogenic FP/TMEM127 Gene Mutations Identified in Pheochromocytomas



Variants considered to be pathogenic by in silico and/or in vitro predictions are shown by their location along the coding gene and corresponding protein structure. The type of mutation is shown in the key. Mutations newly presented are indicated in boldface. An identical splice site mutation at C.410-2A>C was detected in 2 unrelated families. TM indicates transmembrane domain.

Inc, Chicago, Illinois). Because *FP/TMEM127* mutations were detected exclusively in patients with pheochromocytoma, only this group was used for statistical studies. Differences between pheochromocytoma mutation carriers and non-mutation carriers

were assessed for sex, bilaterality, familial history, and malignancy using a  $\chi^2$  test or the Fisher exact test when appropriate. The Mann-Whitney test was applied for testing age differences. Nominal 2-sided  $P < .05$  was considered statistically significant. A modified

Bonferroni-corrected nominal threshold of  $P = .05/N^*$  was used to correct for multiple hypothesis testing where  $N^*$  is the number of independent comparisons. We further evaluated age at disease onset of the current series in comparison with 2 previ-

**Table 2.** Genetic and Clinical Features of the 20 Proband With *FP/TMEM127* Mutations Considered Likely to be Pathogenic<sup>a</sup>

Mutation <sup>b</sup>	Sex/ Age, y <sup>c</sup>	Tumor Location	Malignancy Status	Family History	Other Disease	LOH	Predicted Truncation or Aberrantly Sized Protein <sup>d</sup>	Predicted Pathogenicity <sup>e</sup>	Total Samples/ Total Adrenal Tumors <sup>f</sup>	No. of Mutation Samples	% of Total Samples With Mutations/ Adrenal % of Tumor Samples With Mutations <sup>g</sup>
<b>United States</b>									176/144	3	1.70/2.08
c.280C>T, p.Arg94Trp	F/43	A	B	N		ND	N	Y			
c.208G>A, p.Asp70Asn	F/50	A	B	N		Y	N	Y			
c.3G>T, p.Met1?	F/61	A	B	N	Acrocyanosis	Y	Y	Y			
<b>Padua, Italy</b>									200/190	5	2.50/2.63
c.217G>C, p.Gly73Arg	M/44	A	B	N		ND	N	Y			
c.76C>T, p.Gln26X	F/37	A	B	N		ND	Y	ND			
c.447G>A, p.Trp149X	F/40	A(Bi)	B	N		ND	Y	ND			
c.158G>C, p.Trp53Ser	M/21	A	B	N	Bone marrow failure	Y	N	Y			
c.419G>A, p.Cys140Tyr	F/59	A(Bi)	M	N	Diffuse metastases	ND	N	Y			
<b>Spain</b>									168/133	1	0.60/0.75
c.409 + 1G>T, r.245_409del (p.Asp82_Thr136del)	F/38	A	B	Y	Breast cancer	ND	Y	ND			
<b>Florence, Italy</b>									106/80	1	0.94/1.25
c.627_640dupGCTGCTCTCAGAGA, p.Met214Serfs98X	F/26	A(Bi)	B	N	Papillary thyroid carcinoma, medullary thyroid hyperplasia	ND	Y	ND			
<b>United Kingdom</b>									77/52	1	1.30/1.92
c.-18C>T, p.?	M/44	A	B	N		ND	Y	Y			
<b>Brazil</b>									52/43	1	1.92/2.32
c.116_119delTGTC, p.Ile41ArgfsX39	F/34	A	B	N	Macrovascular disease	ND	Y	ND			
<b>Belgium</b>									67/37	1	1.49/2.70
c.418T>C, p.Cys140Arg	F/47	A	B	N		ND	N	Y			
<b>Qin et al,<sup>14</sup> 2010</b>									92/76	7	7.61/9.21
c.410-2A>C, r.410_417del (p.Leu138CysfsX12)	F/34	A(Bi)	B	Y		Y	Y	ND			
c.149_150insA, p.Pro51ThrfsX57	F/25	A	B	U		Y	Y	ND			
c.475C>T, p.Gln159X	F/72	A(Bi)	B	Y		Y	Y	ND			
c.265_268delACAG, p.Thr89fsX35	M/46	A(Bi)	B	N		Y	Y	ND			
c.410-2A>C, r.410_417del (p.Leu138CysfsX12)	F/37	A	B	Y		Y	Y	ND			
c.245-1G>T, r.245_264del (p.Phe83SerfsX18)	F/66	A(Bi)	B	Y		Y	Y	ND			
c.268G>A, p.Val90Met	F/32	A	B	N		ND	N	Y			

Abbreviations: A, adrenal; B, benign; Bi, bilateral; LOH, loss of heterozygosity; M, malignant; ND, not done; U, unknown (adopted); Uni, unilateral.

<sup>a</sup>Only data from probands are shown in the table.

<sup>b</sup>Nucleotide and protein nomenclature according to Human Genome Variation Society.

<sup>c</sup>Summary mean age, 42.8 years.

<sup>d</sup>Predicted changes that affect protein size or frame are considered truncating mutations; others are missense variants.

<sup>e</sup>Pathogenicity potential of missense variants was examined by PolyPhen2, SIFT, and NetStart 1.0 software.

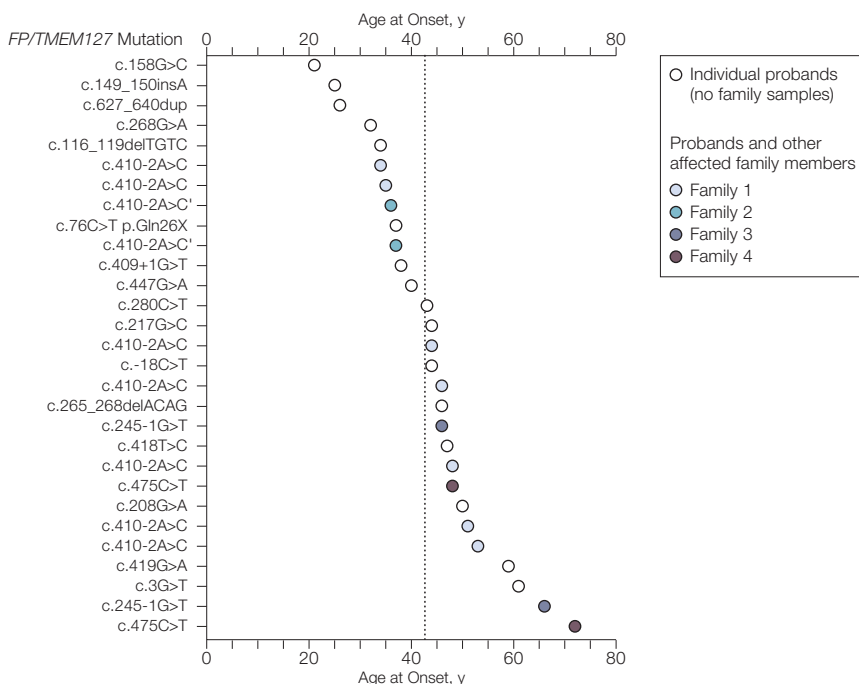
<sup>f</sup>Only study groups in which a mutation was found are shown; the total number of tested samples is 990.

<sup>g</sup>Total frequency of *FP/TMEM127* mutations does not include the 92 samples previously reported because they are biased by the presence of familial pheochromocytoma-linked samples.<sup>14</sup> Summary mean frequency of mutations is 1.89%.

**Table 3.** Relative Frequency of *FP/TMEM127* Mutation in Patients With Single Adrenal Tumor Presentation Without Family History (Truly Sporadic Samples) vs Other Pheochromocytoma Susceptibility Genes, by Age Group

Mutated Gene	No. of Mutations/Total Cases (%) <sup>a</sup>	No. of Mutations/Cases Aged >40 y (%)	No. of Mutations/Cases Aged >45 y (%)
Current series <i>TMEM127</i>	11/547 (2.01)	6/335 (1.8)	3/269 (1.1)
Spanish series <sup>4</sup>			
<i>VHL</i>	2/95 (2.1)	0	0
<i>RET</i>	0	0	0
<i>SDHB</i>	2/95 (2.1)	1/66 (1.52)	1/51 (1.96)
<i>SDHC</i>	0	0	0
<i>SDHD</i>	0	0	0
Italian series <sup>5</sup>			
<i>VHL</i>	6/233 (2.57)	0	0
<i>RET</i>	2/233 (0.86)	0	0
<i>SDHB</i>	3/233 (1.29)	0	0
<i>SDHC</i>	0	0	0
<i>SDHD</i>	3/233 (1.29)	1/157 (0.6)	1/141 (0.71)
Combined series <sup>4,5</sup>			
<i>VHL</i>	8/328 (2.43)	0	0
<i>RET</i>	2/328 (0.60)	0	0
<i>SDHB</i>	5/328 (1.52)	1/223 (0.44)	1/192 (0.52)
<i>SDHC</i>	0	0	0
<i>SDHD</i>	3/328 (0.91)	1/223 (0.44)	1/192 (0.52)

<sup>a</sup>Numbers of mutations and total cases included only unilateral adrenal tumors without family history for the 3 cohorts examined.

**Figure 2.** Age-Related Distribution of Patients and Affected Relatives Carrying *FP/TMEM127* Mutations

Dotted line represents mean age at onset (42.8 years). Mutations from families with a single affected individual are indicated by open circles. Families with more than 1 affected individual (c.245-1G>T, c.475C>T, c.410-2A>C, and c.410-2A>C') are color-coded. The latter 2 are unrelated families with an identical *FP/TMEM127* mutation.

ously published pheochromocytoma/paraganglioma cohorts (the Spanish series<sup>4</sup> and the Italian series<sup>5</sup>). A proportions-difference test for 2 independent samples was applied to determine the degree of heterogeneity between these series. This test showed that the cohorts were not heterogeneous and, thus, could be combined for the comparative analysis.

## RESULTS

A total of 44 distinct *FP/TMEM127* variants were detected in 990 samples from pheochromocytoma or paraganglioma patients (FIGURE 1, TABLE 2, and eTable 2). Of these, 19 mutations found in 20 patients were considered of potential pathogenic significance (2.02%) (Table 2). Thirteen of these variants were novel changes, while the remainder had been previously reported.<sup>14</sup> Approximately half of the mutations (10/19) comprised small deletions, duplications, or nonsense or splice site substitutions that led to truncation or extension (1 mutation) of the predicted *FP/TMEM127* product. The other 9 variants were nonsynonymous missense mutations affecting conserved codons of the *FP/TMEM127* sequence (Table 2). None of these 19 sequence variants was detected in an ethnically matched control group comprising 1064 alleles (or, in the case of exon 3, 718 additional alleles). The constitutive (germline) nature of the mutation could be determined in all but 1 case in which only somatic tissue was available for the analysis (c.208G>A, p.Asp70Asn) (eFigure 1A). Loss of heterozygosity of the wild-type allele was detected in 4 new cases in which tumor DNA was available (Table 2 and eFigure 1A). None of the 545 germline samples analyzed for larger *FP/TMEM127* deletions showed evidence of partial or complete deletion of the gene.

The mutations spanned all 3 coding exons of *FP/TMEM127* (Figure 1), and 13 of them affected 1 or more transmembrane domains of the predicted protein (Figure 1, Table 2, and eFigure 2), highlighting the relevance of these domains for *FP/TMEM127* func-

tion. All of the missense mutations were considered to be potentially pathogenic by at least 1 of the 3 prediction programs used to preliminarily evaluate the likelihood of pathogenicity of the novel missense variants (Table 2 and eFigure 2) and/or by in vitro assays, as described herein.

Next, we examined the association of *FP/TMEM127* mutations with various clinical variables, including age at onset, tumor location, and familial history of pheochromocytoma. The mean age at development of *FP/TMEM127*-mutated tumors was 42.8 years (95% confidence interval [CI], 36.44-49.25 years) and the median was 41.5 years. This age at onset is similar to the mean age of non-mutated cases in this series, 43.2 years (95% CI, 41.88-44.52 years) (median, 45 years) and to the reported average diagnostic age for sporadic pheochromocytomas (47.01 years; 95% CI, 45.42-48.6 years).<sup>4,5</sup> This is in contrast with patients with hereditary pheochromocytomas due to mutations in other susceptibility genes, in whom the disease has an earlier manifestation (eTable 3). The single exception are individuals with *RET* mutations (eTable 3), who can usually be distinguished by their unique clinical presentation.<sup>2,4,5</sup>

Furthermore, *FP/TMEM127* mutation frequency in patients presenting with unilateral pheochromocytomas without a family history or other syndromic features (11/547 cases [2%])—ie, truly sporadic-appearing cases—is similar to that reported for mutations of the *VHL* and *SDHB* genes and higher than that described for other pheochromocytoma susceptibility genes (TABLE 3).<sup>4,5</sup> In the group diagnosed after age 45 years, *FP/TMEM127* showed the highest frequency of mutations (1.1%) compared with all other susceptibility genes (Table 3). When all 20 probands and other affected members from 4 different families were combined (n=29), the age at diagnosis of pheochromocytoma was older than 40 years in two-thirds of these individuals and older than 50 years in almost one-third of the cases (FIGURE 2). In a single family in which multiple af-

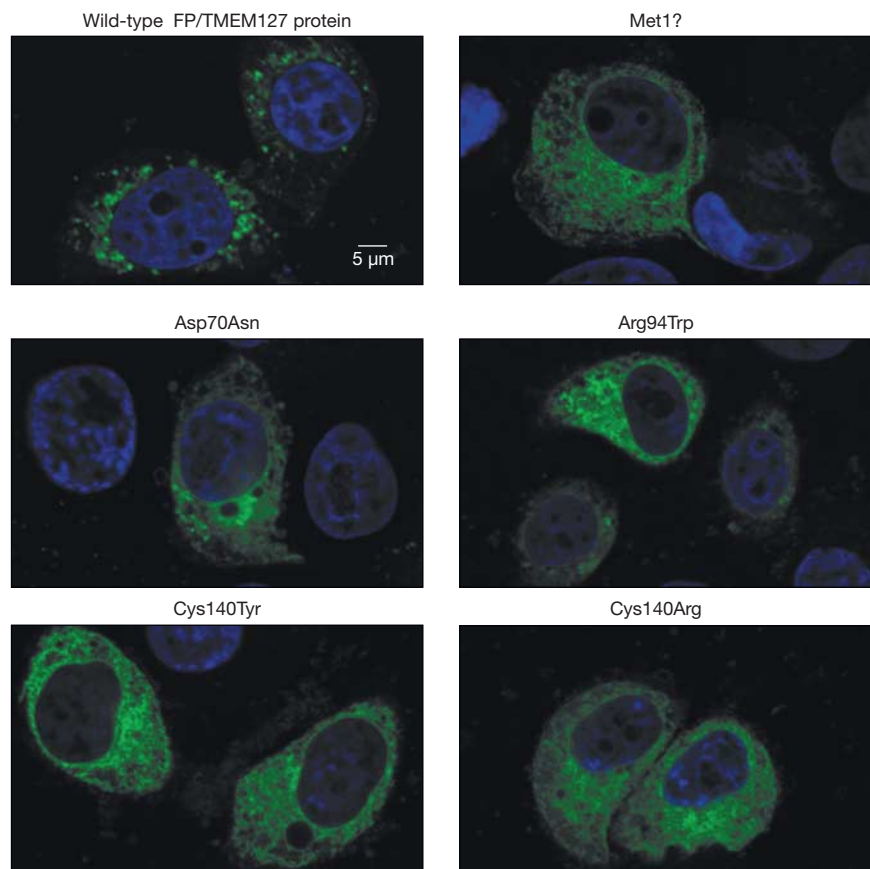
ected individuals were available for analysis, 7 of 11 individuals carrying the c.410-2A>C mutation had clinical disease by age 55 years, while 1 mutation carrier remained free of disease at age 60 years.<sup>14</sup>

All mutated tumors arose from the adrenal medulla. No mutations were detected among the 189 extra-adrenal tumors, including 60 head and neck paragangliomas. Overall, one-third of patients with a mutation had bilateral tumors (7/20;  $P = 2.7 \times 10^{-4}$ ). *FP/TMEM127* mutations were detected in 11% (7/65) of all bilateral pheochromocytomas without other genetic cause. None of the 9 bilateral cases diagnosed

in the pediatric group (younger than 20 years) had an *FP/TMEM127* mutation. A clear family history of pheochromocytoma was present in only a quarter of patients carrying a mutation (5/20;  $P = .005$ ).

Most tumors with an *FP/TMEM17* mutation were benign. However, 1 patient carrying a missense mutation (c.419G>A, p.Cys140Tyr) had multiple vertebral metastases. In another patient (c.116\_119delTGTC, p.Ile41ArgfsX39), features of a more aggressive histological profile (capsular and vascular tumor invasion) were reported; however, stringent criteria for malignancy were not met.

**Figure 3.** Confocal Microscopy of HeLa Cells Transfected With c.280C>T, p.Arg94Trp; c.208G>A, p.Asp70Asn; c.419G>A, p.Cys140Tyr; c.418T>C, p.Cys140Arg; and c.3G>T, p.Met1? Mutant or Wild-Type *FP/TMEM127* Constructs



Each construct expresses green fluorescent protein fused to the *FP/TMEM127* sequence at the N terminus. Cells were fixed and stained with 4',6-diamidino-2-phenylindole (nuclei) 24 hours after transfection. Green fluorescent protein fluorescence was measured at 488 nm, as previously reported.<sup>14</sup> Scale bar is indicated. The 5 mutant constructs have a diffuse cytoplasmic location in contrast with the wild-type protein, which is distributed in the plasma membrane and in discrete organelles within the cytoplasm.

All tumors with *FP/TMEM127* mutations were catecholamine secreting, with no preferential production of either norepinephrine or epinephrine. Moreover, no recurrent clinical manifestations other than pheochromocytoma were detected in the *FP/TMEM127*-mutated cases, although other neoplasias were reported in 3 of these patients (Table 2).

Enforced expression of GFP fusion constructs of 5 novel mutations in HeLa cells revealed that these variants had a subcellular distribution distinct from that of wild-type *FP/TMEM127*, which shows a typical plasma membrane and punctate cytoplasmic localization pattern corresponding to endomembrane organelles (endosome, lysosome, and Golgi body) (FIGURE 3 and eFigure 3). The mutant proteins were localized diffusely within the cytoplasm (Figure 3). Similar results were obtained with 2 independent cell lines, HEK293T and MPC9/30 (eFigure 3), indicating that these effects were not cell-specific.

## COMMENT

In this study, we assessed the prevalence of mutations of *FP/TMEM127*, a recently identified pheochromocytoma susceptibility gene, in a large, multi-institutional, ethnically diverse cohort of 990 individuals with pheochromocytomas and paragangliomas (Table 1). Overall, 19 mutations detected in 20 independent families were considered potentially pathogenic (2%) based on a combination of sequence conservation data, prediction algorithms, LOH, clinical segregation of the mutation in families, and in vitro studies.

The main phenotypic associations that could be drawn from our analysis suggest the following pattern in association with *FP/TMEM127*: (1) mutations were detected only in patients with tumors of adrenal localization (pheochromocytomas) but not with paragangliomas; (2) age at onset of tumors in patients with mutations was similar to that of patients with sporadic disease; (3) while combined bilateral and familial cases accounted for almost half

of the mutant cases, more than one-third of the mutation carriers presented with a sporadic-appearing, benign pheochromocytoma after age 40 years; (4) malignancy was rarely found among *FP/TMEM127* mutation carriers; and (5) no recurrent pattern of manifestations other than pheochromocytoma was detected among the affected individuals or families, although the presence of other neoplastic manifestations in a few patients, including medullary carcinoma of the thyroid, supports the idea that clinical disease associated with *FP/TMEM127* mutations may mimic other pheochromocytoma-associated syndromes (eg, multiple endocrine neoplasia type 2A).

A clear familial history of pheochromocytoma was observed in only a quarter of cases, suggesting low penetrance of *FP/TMEM127* mutant alleles. However, a comprehensive assessment of age-related penetrance of *FP/TMEM127*-associated disease was not available in the current families and awaits further analyses. The preliminary genotype-phenotype associations reported in the present study can be used to further refine genetic testing priorities in patients with pheochromocytomas and suggest that *FP/TMEM127* mutation screening may be recommended for patients presenting at an older age with adrenal tumors, especially but not exclusively those with bilateral disease.

Loss of the wild-type *FP/TMEM127* allele was detected in all informative cases, suggesting a classic mechanism of tumor suppressor gene inactivation similar to other pheochromocytoma susceptibility genes. However, in contrast with a minority of patients with *SDHB*, *SDHC*, *SDHD*, or *VHL* gene deletions,<sup>4,5,22</sup> our investigation of alternative mechanisms of primary gene disruption via germline deletions of the entire gene or specific exons did not reveal any abnormalities in more than 500 samples tested. These results suggest that small mutations are the main mechanism of *FP/TMEM127* disruption, although it cannot be excluded that methylation of the gene pro-

motor, which has not yet been characterized, can also occur in some cases.

Finally, the aberrant subcellular distribution of newly identified *FP/TMEM127* mutations strongly suggests that intracellular distribution within the endomembrane system is relevant for *FP/TMEM127* function. Future studies should determine quantitative intracellular effects of individual variants. We previously reported an effect of *FP/TMEM127* on mTOR (mammalian target of rapamycin) signals.<sup>14</sup> The mislocalization of mutated proteins raises the possibility that *FP/TMEM127* might affect mTOR accessibility to its regulators and offers a useful platform to test functions of *FP/TMEM127* in protein trafficking and mTOR signaling in vitro.

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## REFERENCES

- Manger WM. An overview of pheochromocytoma: history, current concepts, vagaries, and diagnostic challenges. *Ann N Y Acad Sci.* 2006;1073:1-20.
- Neumann HP, Bausch B, McWhinney SR, et al; Freiburg-Warsaw-Columbus Pheochromocytoma Study Group. Germ-line mutations in nonsyndromic pheochromocytoma. *N Engl J Med.* 2002;346(19):1459-1466.
- Gimenez-Roqueplo AP, Lehnert H, Mannelli M, et al; European Network for the Study of Adrenal Tumours (ENS@T) Pheochromocytoma Working Group. Pheochromocytoma, new genes and screening strategies. *Clin Endocrinol (Oxf).* 2006;65(6):699-705.
- Cascon A, Pita G, Burnichon N, et al. Genetics of pheochromocytoma and paraganglioma in Spanish patients. *J Clin Endocrinol Metab.* 2009;94(5):1701-1705.
- Mannelli M, Castellano M, Schiavi F, et al; Italian Pheochromocytoma/Paraganglioma Network. Clinically guided genetic screening in a large cohort of Italian patients with pheochromocytomas and/or functional or nonfunctional paragangliomas. *J Clin Endocrinol Metab.* 2009;94(5):1541-1547.
- Schlisio S, Kenchappa RS, Vredeveld LC, et al. The kinesin KIF1B $\beta$  acts downstream from Egr1 to induce apoptosis and is a potential 1p36 tumor suppressor. *Genes Dev.* 2008;22(7):884-893.
- Yeh IT, Lenci RE, Qin Y, et al. A germline mutation of the KIF1B $\beta$  gene on 1p36 in a family with neural and nonneural tumors. *Hum Genet.* 2008;124(3):279-285.
- Ladroue C, Carcenac R, Leporrier M, et al. *PHD2* mutation and congenital erythrocytosis with paraganglioma. *N Engl J Med.* 2008;359(25):2685-2692.
- Yao G, Xu L, Wu X, Xu L, Yang J, Chen H. Preventive effects of salivarianic acid B on transforming growth factor- $\beta$ 1-induced epithelial-to-mesenchymal transition of human kidney cells. *Biol Pharm Bull.* 2009;32(5):882-886.
- Bayley JP, Kunst HP, Cascon A, et al. *SDHAF2* mutations in familial and sporadic paraganglioma and pheochromocytoma. *Lancet Oncol.* 2010;11(4):366-372.
- Burnichon N, Brière JJ, Libé R, et al. *SDHA* is a tumor suppressor gene causing paraganglioma. *Hum Mol Genet.* 2010;19(15):3011-3020.
- Opocher G, Schiavi F, Conton P, Scaroni C, Mantero F. Clinical and genetic aspects of pheochromocytoma. *Horm Res.* 2003;59(suppl 1):56-61.
- Dahia PL, Hao K, Rogus J, et al; Familial Pheochromocytoma Consortium. Novel pheochromocytoma susceptibility loci identified by integrative genomics. *Cancer Res.* 2005;65(21):9651-9658.
- Qin Y, Yao L, King EE, et al. Germline mutations in *TMEM127* confer susceptibility to pheochromocytoma. *Nat Genet.* 2010;42(3):229-233.
- Cascón A, López-Jiménez E, Landa I, et al. Rationalization of genetic testing in patients with apparently sporadic pheochromocytoma/paraganglioma. *Horm Metab Res.* 2009;41(9):672-675.
- Neumann HP, Eng C. The approach to the patient with paraganglioma. *J Clin Endocrinol Metab.* 2009;94(8):2677-2683.
- Adzhubei IA, Schmidt S, Peshkin L, et al. A method and server for predicting damaging missense mutations. *Nat Methods.* 2010;7(4):248-249.
- Ng PC, Henikoff S. Predicting deleterious amino acid substitutions. *Genome Res.* 2001;11(5):863-874.
- Pedersen AG, Nielsen H. Neural network prediction of translation initiation sites in eukaryotes: perspectives for EST and genome analysis. *Proc Int Conf Intell Syst Mol Biol.* 1997;5:226-233.
- Cascón A, Landa I, López-Jiménez E, et al. Molecular characterization of a common *SDHB* deletion in paraganglioma patients. *J Med Genet.* 2008;45(4):233-238.
- Powers JF, Evinger MJ, Tsokas P, et al. Pheochromocytoma cell lines from heterozygous neurofibromatosis knockout mice. *Cell Tissue Res.* 2000;302(3):309-320.
- Burnichon N, Rohmer V, Amar L, et al; PGL.NET Network. The succinate dehydrogenase genetic testing in a large prospective series of patients with paragangliomas. *J Clin Endocrinol Metab.* 2009;94(8):2817-2827.