Human Cancer Biology

Clinical Cancer Research

MAX Mutations Cause Hereditary and Sporadic Pheochromocytoma and Paraganglioma

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Abstract

Purpose: Pheochromocytomas (PCC) and paragangliomas (PGL) are genetically heterogeneous neural crest–derived neoplasms. Recently we identified germline mutations in a new tumor suppressor susceptibility gene, *MAX* (MYC-associated factor X), which predisposes carriers to PCC. How *MAX* mutations contribute to PCC/PGL and associated phenotypes remain unclear. This study aimed to examine the prevalence and associated phenotypic features of germline and somatic *MAX* mutations in PCC/PGL.

Design: We sequenced *MAX* in 1,694 patients with PCC or PGL (without mutations in other major susceptibility genes) from 17 independent referral centers. We screened for large deletions/duplications in 1,535 patients using a multiplex PCR-based method. Somatic mutations were searched for in tumors from an additional 245 patients. The frequency and type of *MAX* mutation was assessed overall and by clinical characteristics.

Results: Sixteen *MAX* pathogenic mutations were identified in 23 index patients. All had adrenal tumors, including 13 bilateral or multiple PCCs within the same gland ($P < 0.001$), 15.8% developed additional tumors at thoracoabdominal sites, and 37% had familial antecedents. Age at diagnosis was lower ($P = 0.001$) in *MAX* mutation carriers compared with nonmutated cases. Two patients (10.5%) developed metastatic disease. A mutation affecting *MAX* was found in five tumors, four of them confirmed as somatic (1.65%). MAX tumors were characterized by substantial increases in normetanephrine, associated with normal or minor increases in metanephrine.

Conclusions: Germline mutations in *MAX* are responsible for 1.12% of PCC/PGL in patients without evidence of other known mutations and should be considered in the genetic work-up of these patients. *Clin Cancer Res; 18(10); 2828–37. ©2012 AACR.*

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Translational Relevance

MAX has been recently identified as the tenth susceptibility gene for pheochromocytoma (PCC). However, its clinical relevance was not addressed. This international study, based on an outstanding series of 1,694 unrelated patients with PCC or paraganglioma (PGL), has been able to ascertain the prevalence of *MAX* mutations in PCC patients, extended the spectrum of MAX-related tumors to PGL, uncovered contributions of somatic *MAX* mutations to sporadic disease, and defined an intermediate catecholamine phenotype, which may guide testing of *MAX* gene in patients with PCC/PGLs. This study also confirms a preferential paternal mode of transmission with important consequences for genetic counseling. We establish here that *MAX* germline mutations are responsible for the disease in 1.12% of cases, similarly to the genes recently described, such as *TMEM127*, *SDHAF2*, or *SDHA*, and now *MAX* should be considered in the genetic work-up of affected patients.

Introduction

Pheochromocytoma (PCC) has been referred to as "the 10 percent" tumor due in part to the belief that 10% are hereditary and usually associated with 3 well-known cancer syndromes: von Hippel–Lindau disease, multiple endocrine neoplasia type 2, and neurofibromatosis type 1 due to mutations in *VHL* (1), *RET* (2), and *NF1* (3), respectively. The 10 percent rule was challenged after identification of germline mutations in *SDHD*, *SDHB*, and *SDHC* as important causes of familial paraganglioma (PGL; refs. 4–6) that led Neumann and colleagues to establish that up to a quarter of affected patients carried a PCC/PGL susceptibility gene mutation (7). Since then 3 additional susceptibility genes (*SDHAF2*, *SDHA*, and *TMEM127*) (8–10) have been identified. Thus, the proportion of hereditary PCC/PGLs may exceed estimates of 30% to 40% (11, 12), rendering PCC/PGL one of the most inherited tumor entities in existence.

Findings of other patients with a clinical presentation of PCC/PGL that includes a positive family history, early age of presentation, and bilateral adrenal or multiple tumors, but without known mutations, has suggested the presence of further susceptibility genes. With this observation in mind, we recently identified *MAX* (MYC-associated factor X) as a new PCC tumor suppressor susceptibility gene in 3 independent patients with familial antecedents of the disease (13). Further analysis of 59 patients, selected because they had bilateral PCC and/or an early age of disease presentation, allowed detection of 5 additional cases with *MAX* mutations (13). Preliminary genotype–phenotype associations suggested *MAX* mutations were associated with bilateral PCC and an apparent paternal transmission of the disease (13).

Although little is known about genetic alterations in sporadic tumors, it has been proposed that mutations in PCC/PGL susceptibility genes are detrimental for neuronal precursor cells, explaining the apparent rarity of somatic mutations in these genes in apparently sporadic PCC/PGL (14, 15). Guided by transcriptome classification and LOH profiles of a large series of 202 PCC/PGL, we nevertheless recently established that 14% of sporadic tumors harbored somatic mutations in *VHL* or *RET* genes (16). Because deregulation of MYC is a prominent hallmark in numerous forms of cancer (17), with activation of *MYC* genes commonly detected in solid human tumors (18), it is plausible that *MAX* somatic mutations may also occur in sporadic PCC/PGL.

Establishing the above associations and the prevalence of *MAX* mutations among patients with PCC/PGL requires analysis of a larger cohort of patients. In a large international collaborative effort, we therefore screened for the presence of germline mutations affecting *MAX* in 1,694 patients without mutations in major PCC/PGL susceptibility genes. Somatic mutations were searched for in tumors from an additional 245 patients.

Materials and Methods

Patients

The study population consisted of 1,694 apparently unrelated index cases with PCC or PGL, from whom blood-leukocyte DNA samples were available and in whom familial antecedents, presence of metastasis, and number of primary tumors are shown in Table 1. Clinical variables collected for this study were the following: gender, number

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Table 1. Clinical features of the entire pheochromocytoma and paraganglioma cohort

Analysis	Clinical features	*n*	Single PCC	Single PGL H&N/TA	mPGL H&N/TA/both	PCC&PGL H&N/TA/both	bPCC/mPCC
	Total	**1,694**[a]	**1,252**	**315**	**29**	**35**	**63**
				110/205	**1/23/5**	**1/34/0**	
	With familial antecedents[b] (%)	37 (2.18%)	24 (1.91%)	5 (1.58%)	0	2 (5.71%)	6 (9.52%)
Index	Malignant[c] (%)	129 (7.61%)	79 (6.30%)	27 (8.57%)	2 (6.89%)	15 (42.85%)	6 (9.52%)
cases	Gender (female/male/unknown)	1,003/682/9					
	Median age first tumor (range)	48 (3–88)					
	Total	**245**[d]	**216**	**29**			
Tumors	Gender (female/male)	150/95					
	Median age (range)	52 (11–83)					

Abbreviations: PCC, adrenal pheochromocytoma; PGL, paraganglioma; mPGL, multiple paragangliomas; bPCC, bilateral adrenal pheochromocytoma; mPCC, multiple pheochromocytomas within the same gland; H&N, Head and Neck; TA, thoracoabdominal; Both – H&N and TA PGL.

[a]Index cases origin (n): France (664), Italy (428), Spain (245), The Netherlands (166), United States (152), Germany (39).

[b]A hereditary cause of PCC/PGL was considered likely when disease affected at least two family members.

[c]Malignancy was defined as the presence of metastases in which chromaffin cells are normally absent.

[d]Tumor origin (*n*): France (106), United States (75), The Netherlands (39), Spain (17), Germany (8).

of PCC/PGLs, tumor location, age of diagnosis for each tumor (in patients with multiple tumors), the biochemical secretion when available, as well as other malignancies developed by probands. These clinical variables were collected electronically into preformatted forms provided to all contributors, and statistically analyzed in a single center. More detailed assessment of clinical data was further obtained for *MAX* mutation carriers. Mutations in *RET*, *VHL*, *SDHB*, *SDHC*, *SDHD*, and *TMEM127* were excluded, and there were no clinical features of neurofibromatosis type 1. Patients were referred from 15 participating centers of the European Network for the Study of Adrenal Tumours (ENS@T) consortium [Madrid, Oviedo, and Seville in Spain (12), Paris, Marseille and Angers in France (19, 20), Leiden, Rotterdam, Nijmegen, and Maastricht in The Netherlands, Padua, Florence, and Brescia in Italy (11), Munich and Dresden in Germany] and 2 centers in the United States (San Antonio and Bethesda). Diagnosis of PCC and/or PGL, 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).

Written informed consent to collect phenotypic and genotypic data was obtained from all participants in accordance with institution review board–approved protocols for each center. DNA from 400 unrelated and unaffected individuals was analyzed as controls.

Tumors

Frozen tumors obtained from a total of 245 apparently unrelated patients without known mutations in the mentioned susceptibility genes were collected through the Spanish National Tumor Bank Network in Madrid (Spain; ref. 21), the Erasmus MC Tissue Bank in Rotterdam (Netherlands), Munich (Germany), the International Familial Pheochromocytoma Consortium of San Antonio and Bethesda (22), Nijmegen Pheochromocytoma Tissue Bank, and the COMETE network in Paris, France (Table 1; refs. 16, 23). From these 245 samples, 106 belonged to patients included in the germline screening. The remaining 139 tumors represented independent cohort. For samples with identified *MAX* mutations, the corresponding mutation was assessed in constitutive DNA when available to classify them as germline or somatic.

Molecular genetic analyses

Complete genetic characterization of *MAX* included both point mutation and gross deletion/duplication analyses, the latter done in 1,535 cases with good DNA quality. Primers spanning the 5 exons and intron–exon boundaries of the *MAX* transcript 2 (ENST00000358664, NM_002382.3) were used as previously described (13). To assess for rearrangements, a semiquantitative multiplex-PCR method using labeled primers was designed as previously described for other genes (24). PCR conditions and primers are available upon request. To assess the pathogenicity of variants we used Alamut mutation interpretation software (http://www.interactive-biosoftware.com/software.html).

LOH was estimated by direct sequencing when tumor DNA was available. Uniparental disomy or chromosomal loss was assessed by microsatellite analysis as previously described (13).

Immunohistochemistry

Immunohistochemical staining was done using 3-μm formalin-fixed paraffin-embedded tumor sections from

tumors carrying *MAX* mutations, as previously described (13). Normal adrenal sections and tumors carrying mutations in other PCC susceptibility genes were used as controls. Only cases showing nuclear staining of stromal cells were considered as evaluable.

Biochemical test results and biologic features

Biochemical test results available in patients with *MAX* mutations included urinary fractionated metanephrines in 16 patients measured as part of the routine diagnostic work-up at participating centers, either by liquid chromatography with electrochemical detection (LC-EC) or tandem mass spectrometry. Concentrations of catecholamines (epinephrine, norepinephrine, and dopamine) in tumor tissue available from 7 patients with *MAX* mutations were quantified in frozen specimens by LC-EC as described elsewhere (25). Results were compared with historical data from 57 patients in one group with mutations of *VHL* ($n = 44$), *SDHB* ($n = 10$), and *SDHD* ($n = 3$) and 36 patients in the other group with *RET* ($n = 31$) and *NF1* ($n = 5$) mutations, in all of whom tumor tissue catecholamine results were available (26). Transcriptomic data, involving 2 different microarray platforms (Affymetrix for the French series and Agilent for Spanish series; refs. 27, 28), were further used to determine the expression of mRNA for phenylethanolamine *N*-methyltransferase (*PNMT*).

Statistical analysis

Statistical analyses were carried out using SPSS software package version 17.0 (SPSS, Inc.). The 4 patients carrying variants of unknown significance (VUS) and a subject in whom it was not possible to establish the germline status were not considered for statistical purposes. Thus, only patients with mutations leading to truncated proteins or affecting conserved amino acids were included in the final analysis. Differences between mutation carriers and non-mutation carriers for gender, adrenal multiple tumors, familial history, and malignancy were assessed using a χ^2 test or Fisher exact test, where appropriate. Because age, biochemical, and gene expression data could not be established to be normally distributed, nonparametric analysis by Mann–Whitney and Kruskal–Wallis tests were used to assess statistical significance of differences in these variables among the different groups examined.

Results

Germline and somatic *MAX* variants

Among the 1,694 patients with PCC and/or PGL and no evident germline mutations in *RET*, *VHL*, *SDHB*, *SDHC*, *SDHD*, and *TMEM127* genes (Table 1), we identified 16 different heterozygous variants affecting 23 subjects that spanned all 5 exons of the *MAX* gene (Fig. 1; Table 2). In addition, we analyzed *MAX* in 245 tumors (Table 1) and

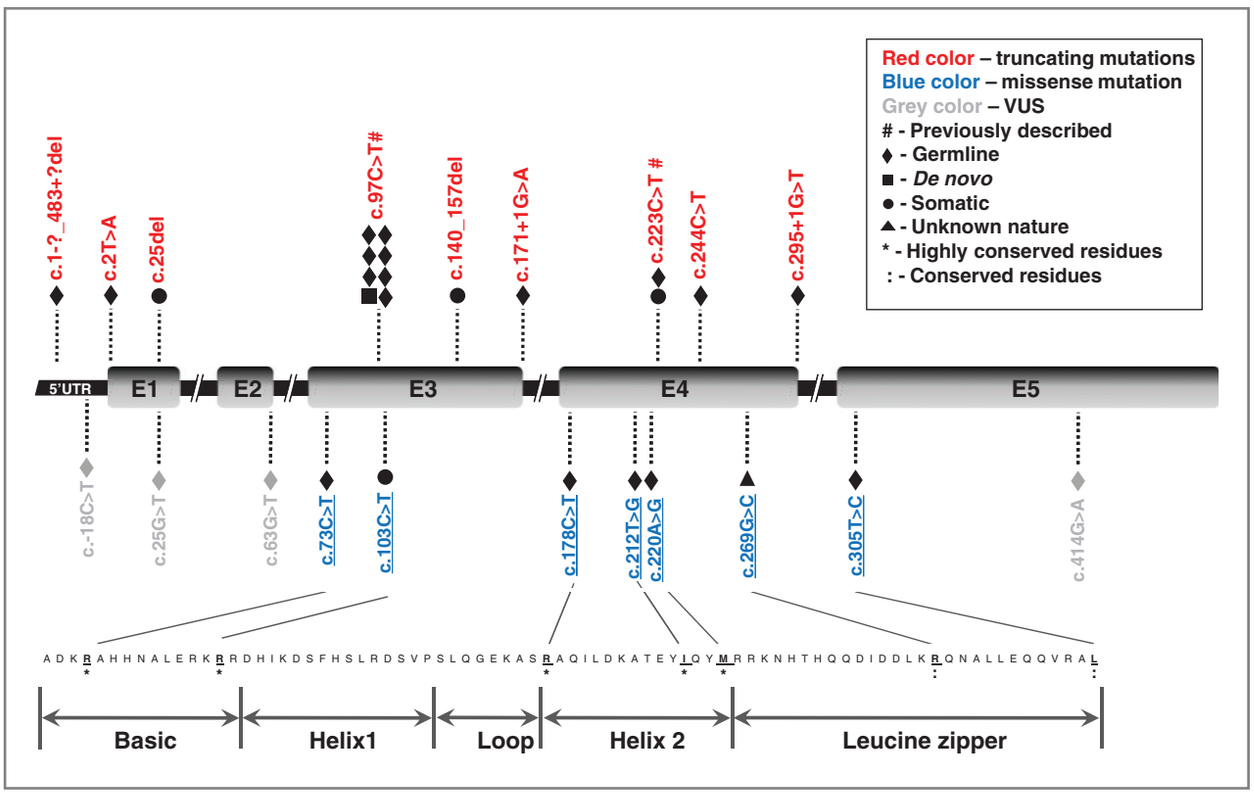

Figure 1. Schematic representation of *MAX* [transcript ENST00000358664] mutations identified in this study. The bottom panel shows the conservation of the amino acids altered by pathogenic missense changes (underlined). Double arrows delimit protein domains between the first and the last missense mutation.

found 4 cases (1.65%) carrying a mutation that was confirmed as somatic by the finding of no mutation in the germline DNA (Table 2). A fifth tumor with a *MAX* mutation (case 24, Table 2) was not considered in further analysis because it was not possible to establish its germline status. None of the variants were found in at least 400 controls, or in public databases (dbSNP132 and 1000 Genomes Project; www.1000genomes.org/).

Table 2. Genetic and clinical features of the 28 *MAX* positive patients[a] and tumors[6]

ID	Gender/ age	Fam	PCC	PGL	Mets	Other disease[d]	cDNA mutation[b]/ Protein alteration[b]	Predicted Pathogenicity[c]	LOH	IHC
1	M/27	No	PCC	No	No	No	c.1-?_483+?del/p.?	n.a.	Yes	Neg
2	F/46	No	bPCC	1 TA	No	No	c.2T>A/p.?	n.a.	Yes[UPD]	Neg
3	F/43	No	bPCC	4 TA	No	BrC, RO	c.73C>T/p.(Arg25Trp)	Y	—	—
4	M/23	Yes	bPCC	No	No	No	c.97C>T/p.(Arg33*)	n.a.	—	—
5	M/27	Yes[p]	bPCC & mPCC	No	No	No	c.97C>T/p.(Arg33*)	n.a.	Yes	—
6	M/34	No[dn]	bPCC	No	No	No	c.97C>T/p.(Arg33*)	n.a.	Yes	Neg
7	F/58	Yes	mPCC	No	No	No	c.97C>T/p.(Arg33*)	n.a.	Yes	Neg
8	F/26	Yes[p]	PCC	No	No	No	c.97C>T/p.(Arg33*)	n.a.	Yes	Neg
9	M/38	No	PCC	No	No	SCCT	c.97C>T/p.(Arg33*)	n.a.	—	—
10	M/24	Yes	bPCC	No	No	CCH	c.97C>T/p.(Arg33*)	n.a.	—	—
11	F/43	No	PCC	1TA	No	No	c.97C>T/p.(Arg33*)	n.a.	Yes	—
12	F/18	No	bPCC	No	No	No	c.171+1G>A/p.?	n.a.	Yes	—
13	F/55	No	bPCC	No	No	No	c.178C>T/p.(Arg60Trp)	Y	—	—
14	F/34	No	bPCC	No	No	No	c.212T>G/p.(Ile71Ser)	Y	Yes[UPD]	Pos
15	M/57	No	mPCC	No	No	PA	c.220A>G/p.(Met74Val)	Y	Yes	Pos
16	M/18	No	bPCC	No	No	1ºHPT	c.223C>T/p.(Arg75*)	n.a.	—	—
17	F/18	Yes	PCC	No	Yes	No	c.244C>T/p.(Gln82*)	n.a.	—	—
18	F/40	No	bPCC	1 TA	Yes	No	c.295+1G>T/p.?	n.a.	Yes	Neg
19	M/13	Yes[p]	PCC	No	No	No	c.305T>C/p.(Leu102Pro)	Y	—	—
20[e]	F/48	No	—	1 H&N	No	No	c.-18C>T/p.(=)	N	—	—
21[e]	M/13	No	PCC	1 TA	No	No	c.25G>T/p.(Val9Leu)	N	No	Pos
22[e]	M/22	No	PCC	No	No	No	c.63G>T/p.(=)	N	No	—
23[e]	F/80	No	PCC	No	No	No	c.414G>A/p.(=)	N	—	—
24[6g]	F/29	No	PCC	No	No	No	c.269G>C/p.(Arg90Pro)	Y	Yes	—
25[6f]	M/39	No	PCC	No	No	No	c.223C>T/p.(Arg75*)	n.a.	Yes	Neg
26[6f]	F/57	No	PCC	No	No	RC	c.103C>T/p.(Arg35Cys)	Y	Yes	Pos
27[6f]	M/24	No	PCC	No	No	No	c.140_157del/p.(Arg47_Ser52del)	n.a.	Yes	Neg
28[6f]	F/56	No	PCC	No	No	No	c.25del/p.(Val9Trpfs*56)	n.a.	Yes[UPD]	Neg

Abbreviations. Gender: F, female; M, male. Fam: familial antecedents; PCC, adrenal pheochromocytoma; bPCC, bilateral adrenal pheochromocytoma; mPCC, multiple pheochromocytomas within the same gland; PGL, paraganglioma; H&N, Head and Neck; TA, thoracoabdominal; Mets, presence of metastases in which chromaffin cells are normally absent; Other disease: BrC, breast Cancer; RO, renal oncocytoma; SCCT, squamous cell carcinoma of the tongue; CCH, C-cell hyperplasia; PA, pituitary adenoma; 1ºHPT, primary hyperparathyroidism; RC, renal carcinoma; n.a., not applicable;—, not available; UPD, uniparental disomy; IHC, immunohistochemistry: Pos, positive; Neg, negative.

[a]Only data from probands are shown in the table.

[b]All cDNA and protein nomenclature is based on reference sequence ENST00000358664. All MAX variants were named following Human Genome Variation Society and checked using Mutalyzer Name Checker (www.mutalyzer.nl).

[c]Pathogenicity potential of missense variants was examined by Alamut mutation interpretation software (version 2.5), which provides variant interpretation according to several prediction methods (AlignGVGD, Polyphen, SIFT, ESEfinder, GeneSplicer, RESCUE-ESE).

[d]Other tumors in the proband.

[p]Paternal familial antecedents.

[dn]*de novo* case.

[e]VUS, not considered for examination of phenotypic associations.

[f]Somatic mutation.

[g]This tumor was not considered in further analysis because it was not possible to establish its germline status.

Overall, taking into account the germline and the somatic findings, we identified 18 novel variants affecting *MAX* and 2 previously reported mutations, c.97C>T and c.223C>T (13). Seven mutations disrupted the MAX protein because they affected the initial methionine (c.2T>A), created a premature stop codon (c.25del, c.97C>T, c.223C>T, and c.244C>T) or affected a donor/acceptor splice site (c.171 + 1G>A and c.295 + 1G>T; Fig. 1). In addition, 2 deletions were identified: the first caused an in-frame loss of 6 highly conserved amino acids within the first helix of the protein (c.140_157del), and the second, detected by multiplex-PCR (Supplementary Fig. S1), spanned the whole gene (c.1-?_483+?del). Immunohistochemical detection of MAX in tumor-embedded paraffin slides showed complete loss of the protein in all analyzed tumors that carried truncating mutations (Supplementary Fig. S2).

Among the 11 nontruncating variants, 7 mutations (c.73C>T, c.103C>T, c.178C>T, c.212T>G, c.220A>G, c.269G>C, and c.305T>C) changed conserved or highly conserved amino acids located within the basic helix-loop-helix leucine zipper (bHLH-Zip) domain of the MAX protein (Fig. 1) and were classified as deleterious by the Alamut software. The remaining 4 nontruncating variants (c.25G>T, c.63G>T, c.414G>A, and c.-18C>T) were classified as VUS because these were predicted as benign through bioinformatic tools; it was also not possible to show their pathogenicity with further analyses (Table 2). Positive immunohistochemistry staining was observed in all nontruncating variants assessed (Supplementary Fig. S2).

LOH of the *MAX* wild-type allele was found in 16 of 18 tumors analyzed (Table 2). *MAX* wild-type allele was present in 2 tumor associated with the variants c.25G > T/p.(Val9Leu), and c.63G>T/p.(=), both considered as VUS.

In summary, we found pathogenic germline *MAX* variants in the 1.12% of the 1,694 index cases included in this analysis. All mutations, except one gross deletion, consisted of a single nucleotide substitution.

Clinical presentation of *MAX* carriers

Only those 19 patients who harbored a germline variant defined as pathogenic were considered for examination of phenotypic associations (Table 2). The presence of familial antecedents of disease was found in 7 of the 19 patients (37%) and appeared in the paternal branch in the 3 pedigrees with more than one generation of affected members (Supplementary Fig. S3). These 19 patients developed at least one PCC, with 13 (68.4%) showing either bilateral PCC or multiple PCCs within the same gland, a 48-fold higher rate ($P < 0.001$) than in *MAX*-negative cases (4.28%). Age at diagnosis was lower ($P < 0.001$) in mutation carriers than in cases without mutations (median 34, range 13–58 years vs. 48 range 3–88 years). Three of the 19 patients (15.8%) developed additional tumors at thoracoabdominal sites at a median age of 48 years (range 44–64 years). Importantly, these tumors presented as PGLs, distinct from recurrences of the earlier adrenal tumors. Two patients (10.5%) developed metastatic disease.

Among 4 sporadic cases with *MAX* somatic mutations, the median age at diagnosis was 47.5 years (range 24–57 years), which was not significantly different from those with *MAX*-negative sporadic tumors (median 52, range 11–83 years; Table 1).

Biochemical test results and biologic features

All patients with *MAX* mutations showed increased urinary outputs of normetanephrine that did not differ from patients in the group with *VHL* and *SDHB/D* mutations or the other with *RET* and *NF1* mutations (Fig. 2A). In contrast, urinary outputs of metanephrine were either normal or moderately increased in patients with *MAX* mutations and showed an intermediate distribution, significantly ($P < 0.001$) higher than in the VHL/SDH group, but lower than in the RET/NF1 group (Fig. 2B). Similarly, tumor tissue concentrations of epinephrine also showed an intermediate distribution, representing 8.4% of total catecholamine contents in *MAX* tumors, a proportion 5.6-fold higher ($P < 0.001$) than in VHL/SDH tumors, but a sixth ($P = 0.003$) that in *RET/NF1* tumors (Fig. 2C). Furthermore, levels of PNMT expression in *MAX* tumors were 14-fold higher ($P < 0.001$) than in *VHL/SDH* tumors and a little under a half ($P = 0.05$) than in *RET/NF1/TMEM127* tumors (Fig. 2D).

Discussion

It is widely accepted that MYC deregulation is not restricted to translocations and amplifications at the MYC locus, which suggests that the impact of its deregulation on human cancer incidence is higher than previously thought (18). We recently found *MAX* germline mutations in patients with PCC (13), suggesting that alterations in this most important regulator of the MYC/MAX/MXD1 network promote hereditary susceptibility to neoplasias. This study followed up on these observations, taking advantage of a large international collaborative network to determine the prevalence and the genotype–phenotype correlations of *MAX* mutations in 1,694 PCC/PGL patients previously negative for 6 major PCC/PGL susceptibility genes. We establish here that *MAX* germline mutations are responsible for the disease in 1.12% of cases, a similar contribution to that of the recently reported *TMEM127* mutation (29). Furthermore, our findings reveal the presence of *MAX* somatic mutations in sporadic tumors, extend the spectrum of *MAX*-related tumors to PGLs, ascertain that *MAX* tumors are not particularly prone to malignancy, and show that *MAX* tumors produce predominantly norepinephrine, but with some capacity to also produce epinephrine.

Though it has been reported that somatic mutations in the known PCC susceptibility genes constitute an extremely rare event, we recently found 14% of sporadic PCC/PGL carrying somatic mutations in *VHL* or *RET* (16). The presence of somatic *MAX* mutations in 1.65% of sporadic tumors described here is in agreement with this latter

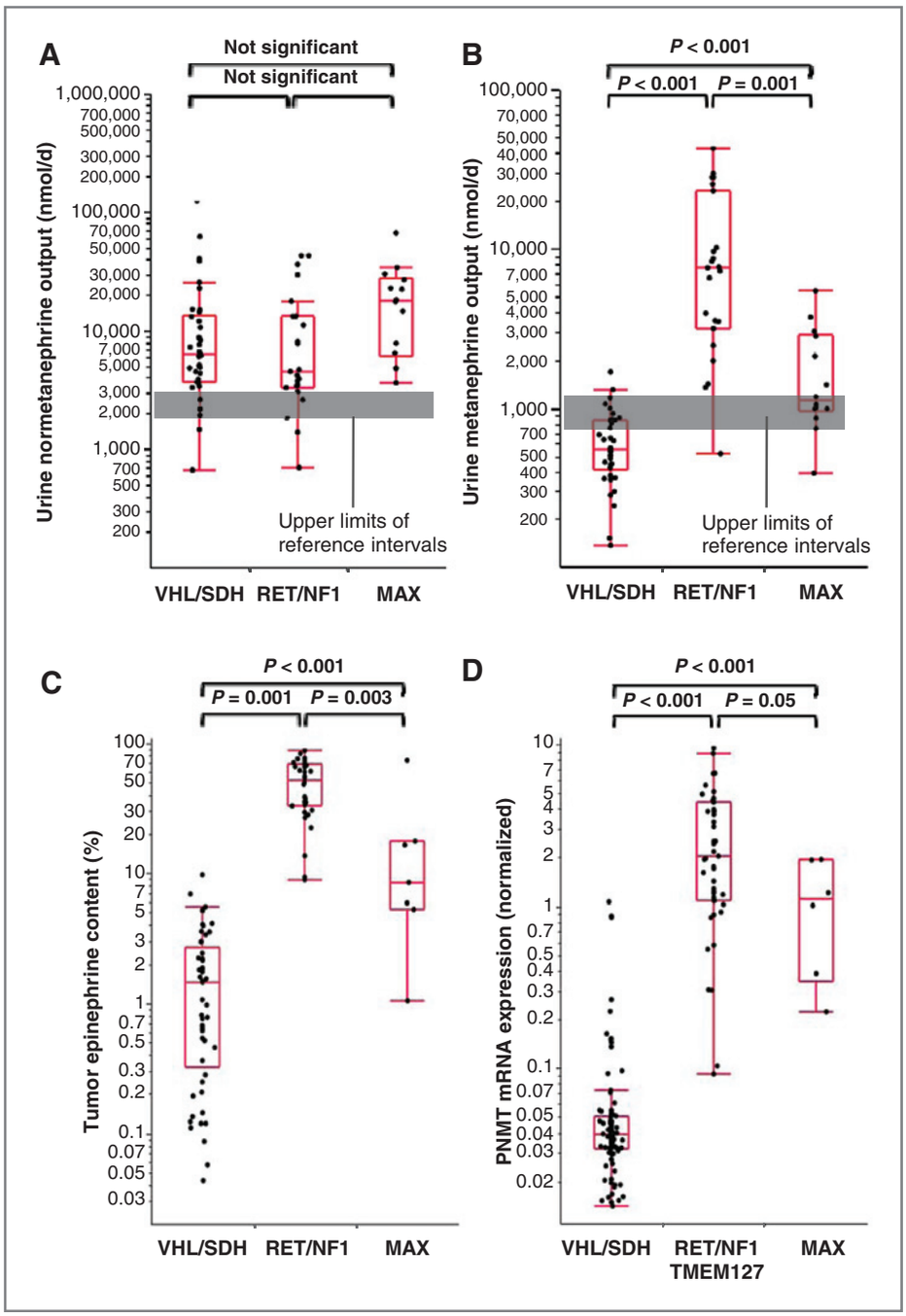

Figure 2. Dot-box plots illustrating urinary outputs of normetanephrine (A) and metanephrine (B), tumor tissue contents of epinephrine (C), and expression of PNMT mRNA (D) for patients with *MAX* mutations compared with those with *VHL* and *SDHB/D* mutations or *RET*, *NF1*, and *TMEM127* mutations.

finding and highlights the importance of the MYC/MAX/MXD1 network in the development of neural crest tumors. It is well known that somatic amplification and overexpression of MYCN is a genetic hallmark in neuroblastoma (30), so ablation of *MAX* transcriptional repression of MYC in PCC could lead to the same oncogenic MYC dysregulation that occurs in neuroblastoma. Nevertheless, no meaningful trend for a contribution of *MAX* mutations to other neoplasms, including neuroblastoma, was found in the current series.

The identified variants were distributed along the gene but were especially frequent in exons 3 and 4, matching some of the most important residues within the conserved bHLH-Zip domain of MAX. The majority of mutations lead to truncated proteins, and the expected LOH affecting the remaining wild-type allele of the *MAX* tumor suppressor gene was further supported by the absence of the protein by immunohistochemistry.

The most frequently found mutation was the previously described c.97C > T variant (13) discovered in 8 unrelated

patients from 5 nations (Italy, Spain, United States, France, and The Netherlands). This recurrent mutation affects a CpG dinucleotide located contiguous to Glu32, the crucial residue for DNA binding, and represents the first hotspot mutation affecting *MAX*. In agreement with this, one of the c.97C > T mutation carriers was a *de novo* case, further suggesting the high mutability of this dinucleotide. The 6 missense variants that altered conserved MAX residues were predicted as deleterious by the Alamut software (Table 2) and have been reported as critical for dimerization and DNA binding of HLH proteins or for interactions within the protein structure (31, 32). Mutations affecting highly conserved amino acids within the bHLH-Zip domain of MAX, involved in protein–protein interactions and DNA binding, can be expected to destroy the ability of MAX to antagonize MYC-dependent cell transformation leading to tumor development.

The absence of familial antecedents in more than 65% of individuals, as well as the paternal transmission identified in 3 pedigrees, further supports previous suggestions of a paternal mode of transmission (13). This mode of inheritance, with its consequence of generation skipping, complicates identification of candidate mutation carriers. In general, the phenotypic characteristics of *MAX* mutant patients overlap with clinical features observed for other PCC/PGL-related hereditary disorders. For example, the presence of a significant proportion of bilateral/multiple PCC cases among *MAX* germline mutation carriers, representing 21% (13 of 63) of patients included in this cohort (Table 1), is in agreement with the high percentage (35%–60%) of bilateral tumors found in patients with mutations in *VHL*, *RET*, or *TMEM127* (7, 29, 33, 34). The age at diagnosis of PCCs in *MAX* mutation carriers (34 years) was clearly lower than in negative cases (48 years), also lower than the reported on average in patients with *TMEM127*, *RET*, and *NF1* mutations (38–42 years), but higher to that for *VHL* and *SDH* mutation carriers (27–32 years; refs. 19, 20, 29, 33–35).

Extraadrenal thoracoabdominal PGL are relatively common compared with adrenal tumors in patients with *SDHB* and *SDHD* mutations, less common in *TMEM127* and *VHL* mutation carriers, and rarely associated with *RET* and *NF1* germline mutations (12, 33, 36). Interestingly, in all *MAX* patients with PGL, the extraadrenal tumor was diagnosed after the adrenal tumor. This contrasts with VHL patients with head and neck PGLs, 50% of whom showed no adrenal tumors (34). In this study, only 2 patients developed metastasis, suggesting that unlike *SDHB* mutations, mutations of *MAX* are not associated with a high risk of malignancy.

The catecholamine-related information available from patients with *MAX* mutations indicated a biochemical phenotype intermediate between the established phenotypes of epinephrine producing tumors due to *NF1* and *RET* mutations and the predominantly norepinephrine producing tumors due to *VHL* or *SDHB/D* mutations (26). This intermediate diagnostic phenotype, manifested by at least 3-fold larger tumor-associated increases in urinary outputs of normetanephrine than of metanephrine, was explained by a significant but limited capacity to produce epinephrine. This latter finding is supported by the intermediate tissue concentrations of epinephrine and expression of mRNA for PNMT, the enzyme responsible for conversion of norepinephrine to epinephrine. The intermediate biochemical phenotype associated with *MAX* mutations, together with lack of MAX immunohistochemical staining of tumor tissue, may prove useful for guiding testing of the *MAX* gene in patients with PCC/PGLs.

In summary, this study involving an unprecedented international effort to genotype and phenotype a substantial number of patients with PCC/PGLs reveals the importance of the MYC/MAX/MXD1 network in the development of both hereditary and sporadic forms of these tumors. *MAX* is the tenth PCC/PGL susceptibility gene described to date, which now should be considered in the genetic work-up of affected patients.

Disclosure of Potential Conflicts of Interest

The authors have no potential conflicts of interest to declare.

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