Mutations in the RET proto-oncogene and the von Hippel-Lindau disease tumour suppressor gene in sporadic and syndromic pheochromocytomas

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Abstract

Pheochromocytomas may occur sporadically, or as part of the inherited cancer syndromes multiple endocrine neoplasia (MEN) type 2, von Hippel-Lindau disease (VHL), and, rarely, in type 1 neurofibromatosis. In MEN 2, germline missense mutations have been found in one of eight codons within exons 10, 11, 13, 14, and 16 of the RET proto-oncogene. In VHL, germline mutations within one of the three exons are responsible for the majority of cases. To determine if somatic mutations similar to those seen in the germline in MEN 2 or VHL disease play a role in the pathogenesis of sporadic or familial pheochromocytomas, we analysed 48 sporadic tumours and tumours from 17 MEN 2 and five VHL patients for mutations in RET exons 9, 10, 11, 13, 14, 15, and 16, and the entire coding sequence of VHL. Five of 48 sporadic pheochromocytomas had RET mutations within exons 10, 11, and 16. Of these, one was proven to be germline and two were proven to be somatic mutations. Four of 48 had VHL mutations; these included both the bilateral cases in the series (one was proven to be a germline mutation) and two others, of which one was proven somatic.

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Pheochromocytomas usually occur sporadically but are also a feature of three inherited cancer syndromes with an autosomal dominant pattern of inheritance: von Hippel-Lindau disease (VHL), multiple endocrine neoplasia type 2 (MEN 2), and type 1 neurofibromatosis.¹⁻⁵ The major components of VHL include retinal and cerebellar haemangioblastomas, renal cell carcinoma, pheochromocytoma, and renal, pancreatic, and epididymal cysts.² The frequency of pheochromocytomas in affected subjects within a particular VHL family can range from 0 to >90%, with a mean of 14%.⁶⁻⁸ MEN 2 is divided into three subtypes according to the tissues involved.^{3,4} MEN 2A is characterised by the presence of MTC, pheochromocytoma, and hyperparathyroidism, and MEN 2B by MTC, pheochromocytoma, and developmental abnormalities such as gan-

glioneuromatosis and marfanoid habitus. Familial MTC (FMTC) comprises MTC as the only disease phenotype. In MEN 2, approximately 40% of affected subjects have pheochromocytoma.⁹ Although pheochromocytoma is clearly a feature of type 1 neurofibromatosis, its frequency is only about 1%.¹

The susceptibility genes for VHL and MEN 2 have been identified using positional cloning techniques. Germline deletions and intragenic mutations have been detected along the length of the VHL tumour suppressor gene, which encodes a protein of unknown function, in approximately 70% of VHL kindreds.^{10,11} In the majority of families with the three MEN 2 syndromes, germline missense mutations have been found in one of eight codons of the RET proto-oncogene, which codes for a receptor tyrosine kinase.¹²⁻²⁰ Mutation in one of codons 609, 611, 618, 620 (exon 10), 634 (exon 11), 768 (exon 13), or 804 (exon 14) is responsible for the majority of MEN 2A and FMTC families, while a missense mutation in methionine codon 918 (exon 16) is responsible for >93% of MEN 2B cases.²⁰

To determine if somatic RET and VHL mutations play a role in tumour progression in sporadic or familial pheochromocytomas, mutation analyses of the VHL gene and those exons of the RET proto-oncogene which have been shown previously to be involved in MEN 2 or MTC were performed in pheochromocytomas from sporadic cases and from MEN 2 and VHL patients.

Materials and methods

PHAECHROMOCYTOMAS

A total of 70 pheochromocytomas, 48 sporadic, 17 from MEN 2 cases, and five from VHL cases, were analysed. All pheochromocytomas were obtained as frozen tumours. Results of a more limited analysis of RET in 15 of the 48 sporadic tumours and five of the 17 MEN 2 tumours have previously been reported in two independent studies. The first included 12 sporadic pheochromocytomas and five MEN 2 tumours analysed for somatic mutations in exons 10, 11, and 16 only.¹⁶ The second included a total of three sporadic tumours analysed for exons 10 and 11 only.²¹

Table 1 RET mutations in apparently sporadic pheochromocytomas

	This study (n = 48)	Kominoth et al ²⁴ (n = 7)	Lindor et al ²⁵ (n = 29)	Beldjord et al ²⁶ (n = 28)
Exon 9	0	Not analysed	Not analysed	1 del AG
Exon 10	2 C620Y*	0	1 C609Y	0
Exon 11	1 C634G†	0	1 del 632–633	2 D631Y* C634W*
Exon 13	0	Not analysed	Not analysed	Not analysed
Exon 15	0	Not analysed	Not analysed	Not analysed
Exon 16	2 M918T	0	2 M918T D925H*	3 M918T
Total mutations	5 (10.4%)	0 (0%)	3 (10.3%)	6 (21.4%)

* No corresponding germline DNA available.
† Proven germline mutation.
The other cases are proven somatic mutations.

Table 2 VHL mutations in apparently sporadic pheochromocytoma

Patient ID	Clinical	Nucleotide change	Amino acid change	Exon	Origin
AG	Sporadic, bilateral	553 G→A	185 Gly→Ser	1	Probably germline
333	Sporadic, bilateral	569 T→C	190 Phe→Ser	2	Germline
335	Sporadic, unilateral	642 C→G	214 Asp→Glu	2	?
343	Sporadic, unilateral	704 A→G	235 Gln→Arg	3	Somatic

A pheochromocytoma was considered sporadic if there was no history of a first or second degree relative with MTC or pheochromocytoma and there were no other stigmata of MEN 2, VHL, or neurofibromatosis recorded by the clinician providing the material. Among this set of 48 apparently sporadic tumours were 46 unilateral, unifocal tumours (of which one was malignant) and two bilateral tumours. The MEN 2 cases belong to families in which the diagnosis had been made based on pathology of thyroid and adrenal tumours. VHL disease was diagnosed according to standard criteria.² In these MEN 2 and VHL cases, germline mutations in the RET proto-oncogene and the VHL gene have been identified.^{10 14 20}

ISOLATION OF DNA

Genomic DNA and cDNA were prepared from frozen tissues as described previously^{16 22} and from peripheral blood leucocytes using an automated DNA extractor (Applied Biosystems).

PCR AMPLIFICATION AND MUTATION ANALYSIS

Genomic amplicons encompassing exons 7 to 19 of the RET proto-oncogene were created by the polymerase chain reaction (PCR). Primers used in these analyses were as follows: CRT 10G and CRT 10H (exon 7), CRT 10D and 18C (exon 8), CRT 18D and 18E (exon 9), CRT 19S and 2C (exons 10 and 11), CRT 19S and CRT 19E (5'GGA CCT CAG ATG TGC TGT T 3') (exon 10), CRT 19B and CRT 2C (exon 11), CRT 1B and 4C (exon 12), CRT 4E and 4F (exon 13), CRT 4N and 4K (exon 14), CRT 17B and 17G (exon 15), CRT 5G and CRT 5H or f RET 16 and r RET 16 (exon 16), CRT 5J and 6E (exon 17), CRT 6B and 6E (exon 18), and CRT 6J and 14K

(exon 19).^{12 14–16 18 23} Fifty to 100 ng of genomic DNA were amplified using the conditions previously described,^{14 16 23} except for an annealing temperature of 58°C for f RET 16 and r RET 16.¹⁵ Amplicons created from cDNA templates were as previously described.^{12 16} If PCR was carried out using the “Red Hot” *Thermus* *ice-**landicus* DNA polymerase according to the manufacturer’s recommendations (Advanced Biotechnologies, Surrey, UK), commercially supplied buffer with 1.5 mmol/l MgCl₂ was used and, in general, annealing temperatures were raised by 3–5°C.

PCR products were purified by electrophoresis through low melting point agarose and eluted using the Wizard PCR Prep kit (Promega, Southampton, UK) before sequencing. Purified amplification products were sequenced using the appropriate primer with the ΔTaq Version 2.0 cycle sequencing kit (USB, Cleveland, Ohio, USA) or the Cyclist sequencing kit (Stratagene, Cambridge, UK) according to the manufacturers’ recommendations, and resolved on 6% denaturing polyacrylamide gels.

All tumour samples were analysed by direct sequence analysis or by appropriate restriction enzyme digestions for known mutations in RET exons 9, 10, 11, 13, 14, 15, and 16.^{14 16 18 19} Thus, the 12 sporadic and five MEN 2 pheochromocytomas previously analysed at exons 10, 11, and 16¹⁶ were further analysed for mutations in exons 9, 13, 14, and 15 in this study; the three sporadic tumours previously analysed for exons 10 and 11²¹ were also further analysed for mutations in exons 9, 13, 15, and 16. In addition, DNA from 12 sporadic tumours was analysed by sequencing exons 7–19, which code the entire cysteine rich and tyrosine kinase domains of RET.

Mutations analysis for the VHL gene was performed using six primer pairs. Each PCR fragment was analysed by single strand conformational polymorphism (SSCP) and heteroduplex analysis as previously described.¹⁰ Samples showing aberrant band patterns were sequenced directly using nested primers.

Results

SPORADIC PHAEOCHROMOCYTOMAS

Of 48 sporadic pheochromocytomas, five had mutations in the RET proto-oncogene and four others in the VHL gene (tables 1 and 2). The only malignant tumour in this series did not have mutations. Among the tumours with RET mutations, two had somatic codon 918 mutations altering ATG to ACG (M918T), which were not present in germline (peripheral blood) DNA. One of these two M918T mutations has been reported previously.¹⁶ One tumour had a codon 634 TGC to GGC mutation (C634G); when germline DNA was examined, the mutation was also detected in the germline. This patient had no other stigmata of MEN 2, and did not have a family history of MEN 2, MTC, or pheochromocytoma elicited by the clinician at the time of operation. Once this unexpected germline mutation was detected in the proband, her first degree relatives were examined

for the presence of the germline mutation. Her father and two of three of her sibs carried this mutation. Subsequent history showed that the grandfather "died of a goitre" and examination of the proband's father showed a thyroid mass and bilateral phaeochromocytomas. Two other tumours had codon 620 TGC to TAC mutations (C620Y).¹⁶ Unfortunately, germline DNA was not available from these two cases with mutation at codon 620. Of the four tumours which carried VHL mutations, two had exon 2 missense mutations (F190S, D214E), one had an exon 1 missense mutation (G185S), and one had an exon 3 missense mutation (Q235R) (table 2). The Q235R mutation occurred in a patient with unilateral disease and was proven to be somatic. The F190S mutation occurred in a patient with bilateral phaeochromocytoma diagnosed at the age of 11 years and this mutation was detected in both tumours and the germline. The patient, whose tumour contained the G185S mutation, also had bilateral tumours and the same mutation was detected in both tumours, suggesting that this mutation was also germline in origin. The D214E mutation occurred in a patient with unilateral disease but no germline DNA was available. Of these four patients with VHL mutations, none had other clinical signs of VHL disease nor family histories of VHL or phaeochromocytomas.

A more extensive search for mutations, encompassing RET exons 7–19, coding for the cysteine rich domain and the tyrosine kinase domain, was carried out in 12 sporadic phaeochromocytomas. No further mutations were detected. In addition, two sporadic phaeochromocytomas, previously postulated not to express the region of RET exons 10 or 11 because of failure to amplify by RT-PCR with primers encompassing those exons,²¹ have subsequently been shown to amplify the region of RET exons 10 and 11 by RT-PCR using different sets of primer pairs. No mutations were detected.

PHAEOCHROMOCYTOMAS FROM MEN 2 CASES

Neither somatic RET mutations in exons 9, 10, 11, 13, 14, 15, or 16 nor VHL mutations were detected in tumours from MEN 2A and MEN 2B patients. Germline RET mutations have been previously reported in each of these patients.^{12 14 16}

PHAEOCHROMOCYTOMAS FROM VHL CASES

Apart from the germline VHL mutations detected in tumour tissue, no somatic RET and VHL mutations were detected. The germline mutations were detected in the heterozygous state in each of the tumours. Loss of heterozygosity (LOH) studies using 3p markers flanking the VHL gene were performed on three of these tumours (and compared to the allele pattern obtained in germline DNA). In two, all markers were uninformative (that is, either homozygous or hemizygous) and the third showed retention of heterozygous markers (data not shown).

Discussion

We found RET mutations in approximately 10% of sporadic phaeochromocytomas. This is consistent with other reports (summarised in table 1).^{25 26} All MEN 2 germline mutations²⁰ and MTC somatic mutations described to date lie within the region of the gene covered in this analysis.^{15 16 18–20 27–29} However, the results of this study and others^{25 26} suggest that a wider spectrum of RET mutations can occur in sporadic phaeochromocytomas than in sporadic MTC, including some mutations, such as the exon 9 splice site mutation²⁶ and the two codon deletion in exon 11,²⁵ not so far found in the MEN 2 syndromes. This raises the possibility that other mutations may be present elsewhere in RET in these tumours. We sequenced exons 7–19 and found no mutations in a sample of 12 tumours, but it is possible that such mutations could rarely be present in the 5' region of the gene or the introns. Alternatively, other genes may be involved.

Phaeochromocytomas are a component of VHL.² Five of nine sporadic phaeochromocytomas and five of nine MEN 2 phaeochromocytomas were previously shown to have LOH at 3p markers,³⁰ suggesting the putative involvement of the VHL gene, which lies on chromosome arm 3p, in tumour progression. Unexpectedly, we found VHL mutations in only 8% of apparently sporadic tumours: one was proven somatic, two were probably germline, and one was undetermined (summarised in table 2). Two of the four VHL gene missense mutations are new.^{10 31–34} No somatic VHL mutations were identified in nine sporadic phaeochromocytomas studied by Gnarr *et al.*³² Together with our series, only one proven somatic VHL mutation has been detected in 57 sporadic phaeochromocytomas. The rarity of somatic VHL mutations in sporadic phaeochromocytomas contrasts with their frequent presence in sporadic clear cell renal cell carcinoma, a tumour which is a component of VHL.^{31–33} The discrepancy between the paucity of VHL mutations in sporadic phaeochromocytomas and the frequent finding of LOH on 3p raises the possibility that the 3p LOH may reflect mutation in a gene which lies in the 3p region but is distinct from VHL, analogous to the findings in sporadic Wilms' tumours.³⁵

We detected no somatic VHL mutations in the second allele in five phaeochromocytomas from VHL cases. The VHL gene is believed to be a tumour suppressor gene.³⁶ Consistent with this, germline mutations in the VHL gene are predicted to be inactivating.^{4 10} Possibly, the second allele in the VHL phaeochromocytomas has been inactivated by loss of all or part of that allele (for example, the two VHL tumours that were uninformative for 3p markers may be hemizygous), or by methylation or an inactivating mutation within the VHL promoter in the remaining allele, which we would not have detected.

The rarity of somatic RET and VHL mutations in sporadic phaeochromocytomas contrasts with the finding that 23 to 40% of sporadic MTC, in series of 10 or more tumours,

have a somatic RET codon 918 (MEN 2B type) mutation.^{15 16 28 29} There are probably several different pathways which can lead to the genesis of pheochromocytomas, and any of several genes in these pathways could be the target for somatic mutation and not just RET or VHL. For example, in a series of 20 sporadic MEN 2 and VHL pheochromocytomas combined, seven were found to have reduced expression of the NF1 gene.³⁷ Another possible reason for the low frequency of somatic VHL mutation in sporadic pheochromocytomas comes from the analogy with the RET 634 mutation, which is the most common germline mutation in MEN 2A²⁰ yet very infrequent as a somatic mutation in sporadic MTC.²⁹ Thus, as has been suggested for RET 634 mutations,²⁹ VHL mutations may only promote tumorigenesis if present at a critical period of adrenal chromaffin cell development or cell cycling.

From a clinical standpoint, it may be prudent to consider screening patients with apparently sporadic pheochromocytomas for inherited mutations in the RET and VHL gene. This is especially true in a case with an apparently sporadic bilateral tumour, where the possibility of heritable disease is high. In unilateral cases, the yield of such mutation analyses is likely to be low, based on the objective evidence (this study; see tables 1 and 2). However, it can be argued that even a few percent chance of detection of an occult or de novo case of MEN 2A would be worthwhile in view of the important implications and possible benefits for that person and his family.

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