Genetic Mutation Screening in an Italian Cohort of Nonsyndromic Pheochromocytoma/Paraganglioma Patients

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ABSTRACT: To assess the prevalence of genetic mutations in nonsyndromic pheochromocytoma/paraganglioma (PHEO/PGL) patients we have performed a systematic search for mutations in the succinate dehydrogenase (SDH) B, C, and D subunits, von Hippel–Lindau (VHL), and RET genes by direct bidirectional sequencing. Patients were selected from the medical records of hypertension centers. After exclusion of syndromic patients, 45 patients with familial (F+, $n = 3$) and sporadic (F−, $n = 42$) cases of isolated PHEO/PGL were considered. They included 35 patients with PHEO, 7 with PGL, and 3 with head/neck PGL (hnPGL). Three patients with PHEO (2F−, 1F+) presented VHL mutations (P86A, G93C, and R167W), six with PGL (4F−, 2F+) were positive for SDH or VHL mutations (SDHB R230G in two patients, SDHB S8F, R46Q, R90Q, and VHL P81L in one subject each), and one with hnPGL carried the SDHD 348–351delGACT mutation. We have also detected missense (SDHB S163P, SDHD H50R and G12S), synonymous (SDHB A6A, SDHD S68S), and intronic mutations that have been considered nonpathological polymorphic variants. No mutation was found in SDHC or RET genes. Our data indicate that germline mutations of VHL and SDH subunits are not infrequent in familial as well as in sporadic cases of nonsyndromic PHEO/PGL (overall, 12 of 45 probands, 22%). Accordingly, screening for such mutations seems to be justified. However, a more precise characterization of the functional relevance of any observed sequence variant and of other genetic and environmental determinants of neoplastic transformation is essential in order to plan appropriate protocols for family screening and follow-up.

KEYWORDS: pheochromocytoma; paraganglioma; genetics

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INTRODUCTION

There is considerable variety in the definition of pheochromocytoma (PHEO) and paraganglioma (PGL). The common denominators are the embryological origin (a subset of neural crest–derived cells) and the histopathological appearance, whereas differential features derive from their anatomical location (adrenal medulla or paraganglia aligned to sympathetic or parasympathetic nervous system) and from the ability of producing catecholamine and various neuropeptides. The definition of PHEO should be reserved for catecholamine-producing tumors originating from the adrenal medulla, whereas the term of PGL is used for the extra-adrenal, usually catecholamine-producing tumors arising from sympathetic paraganglia located in the thorax-abdomen. The definition of head and neck PGL (hnPGL) is reserved for noncatecholamine-producing tumors originating from parasympathetic paraganglia (carotid body, glomus jugulotympanic). The overall incidence of these tumors is about 1 in 300,000.[1]

Clinical records suggest that approximately 10% of cases of PHEO/PGL and up to 50% of hnPGL occur in a familial setting.[2,3] There are four hereditary syndromes that are associated with PHEO/PGL: multiple endocrine neoplasia (MEN) Type 2A and 2B, neurofibromatosis (NF1) and von Hippel–Lindau syndrome (VHL); the genes involved are RET (OMIM 164761), NF1 (OMIM 162200), and VHL (OMIM 193300), respectively.

In addition, recent studies have shown that germline mutations in nuclear genes coding for the subunits B, D, and C of the mitochondrial succinate dehydrogenase complex II (SDH) are often associated with nonsyndromic, familial as well as apparently sporadic, PHEO/PGL. A growing body of data supports that the real prevalence of pathogenic germline mutations in these genes among patients presenting with nonsyndromic PHEO/PGL is probably much larger than supposed earlier on the basis of a positive family history for the disease.

We have therefore performed molecular screening in a sample of Italian patients referring to hypertension clinics for sporadic and familial nonsyndromic PHEO/PGL in order to evaluate the frequency of germline mutations in the genes coding for the VHL, RET, and SDH (B, C, and D) in this particular clinical setting.

PATIENTS AND METHODS

Patients

We have examined the medical records of patients referred to the hypertension centers of the University of Brescia and the University of Turin in the last 20 years and then selected those with PHEO/PGL. The diagnosis was supported by clinical, biochemical (plasma and/or urinary catecholamines,

chromogranin A), and imaging data (computed tomography, magnetic resonance, ^{131}I-metaiodbenzylguanidine or In-111 Pentreotide scintigraphy, as needed). After exclusion of syndromic patients with MEN–2, von Hippel–Lindau, or neurofibromatosis features, only familial and sporadic cases of isolated PHEO/PGL were considered. Thus far, we have examined 45 probands, 35 with PHEO, 7 with PGL, and 3 with hnPGL. In this group of subjects there were three probands with a positive family history (1 PHEO, 1 PGL, and 1 hnPGL patient).

After obtaining an informed written consent, germline DNA was extracted from 10 mL of peripheral blood, using the DNA Isolation Kit for Mammalian Blood (Roche Diagnostic, Mannheim, Germany) according to the manufacturer's instructions.

Mutation Screening

DNA (200 ng) was amplified with intronic primers (TABLE 1) flanking the eight exons of SDHB, four exons of the SDHD,[4] six exons of SDHC, three exons of VHL[5] and exons 10, 11, 13, 14, 15, 16 of RET gene. Polymerase chain reaction (PCR) conditions were as follows: 5 min of denaturation at 94°C, then 38 cycles of denaturation (94°C), annealing (see TABLE 1 for temperature conditions), and polymerization (72°C) for 30 sec, respectively. PCR products were purified with Microcon 50 (Millipore, Billerica, MA) and sequenced using BigDye® Terminator v1.1 Sequencing Kit (Applied Biosystems, Foster City, CA). After a purification step performed with CentriSep Spin Columns (Princeton Separations, Adelphia, NJ), the products were analyzed on a 310 DNA Analyzer (Applied Biosystems).

RESULTS

No mutation in the examined RET gene exons was detected in our group of patients.

Each of the three patients (1 PGL, 1 PHEO, and 1 hnPGL) with a positive family history were carriers of genetic variants of potential pathogenic relevance. The patient presenting with familial PGL (Patient 5, see TABLE 2) had the mutation 688 C>G, corresponding to an arginine to glycine substitution (R230G) in exon 7 of the SDHB gene; Patient 8 (PHEO) presented the mutation 277G>T, G93C, in exon 1 of the VHL gene; and Patient 7 (hnPGL) presented the mutation 348–351delGACT in exon 4 of the SDHD gene.

Of the six patients with apparently sporadic PGL four were carriers of germinal sequence variants: one of them (Patient 2, 137G>A, R46Q, in exon 2 of SDHB) has been previously described in association with PHEO/PGL.[6] The remaining three variants (Patient 1, 23C>T, S8F, in exon 1 of SDHB; Patient 6, 688C>G, R230G, in exon 7 of SDHB; and Patient 10, 242C>T, P81L, in exon 1 of VHL) presented novel mutations to our knowledge.

TABLE 1. Set of primers and annealing temperature for PCR amplification of the different gene exons

		Temp (°C)
SDHB		
1F GGTCCTCAGTGGATGTAGGC	**1R** TCCTCCATCTCCCTGAGGCCTTG	**61**
2F TGGATATTGAATGCCTGCCT	**2R** GCCTTCCAAGGATGTGAAAA	**63**
3F ATTCCGAAGGTGACCTGAGA	**3R** CTCTATCAGCTTTGGCCAGC	**63**
4F CAGCAAGGAGGATCCAGAAG	**4R** ACAAATCCTGCCCTGAAAAA	**63**
5F CAGTGTCCAAGAAATGGGGT	**5R** TGAACGTTCCTCTCCAGAAT	**63**
6F GCACTGACCCCAAAGGTAAC	**6R** ATGGCAATGAAGGAAACCAG	**63**
7F CCAGAGCTTTGAGTTGAGCC	**7R** TGGTCCCTTTCCTTCTCAAA	**63**
8F AACCCCTATGGTTTTGAGGG	**8R** CTGCGGCAAGTAAAGGAACA	**63**
SDHD		
1F GTTGGTGGATGACCTTGAGC	**1R** TGAGTCCTCACTTCCATCCC	**60**
2F TCAGTCCTGTTAAAGGAGAGGTTC	**2R** CCTGCTAAAGGCATGACCA	**55**
3F AAAGATGTGTGTTTCTCACA	**3R** CAGCAAACAAACTGAGCAGACA	**55**
4F GTATAGTCTTCTAATTTCACT	**4R** CAATTCTTCAAAGTATGAAGT	**55**
SDHC		
1F CGTCACATGACACCCCCAAC	**1R** CTCCCAGTCCCACTGAA	**60**
2F AATCTATCCCTTCACCCCTA	**2R** ATCTCCAGACTTAGAAACTT	**50**
3F TCAAACGGTCATGGTTTTAT	**3R** CTCTGGCTCCAGAATCCTTC	**55**
4F ACTCTCTACTATGGTGTCAT	**4R** TGTGTAAAAACACATATACAT	**50**
5F AGCAGCTGTGACAAGCTACT	**5R** CTCCCTTCACAGAGAAAATG	**55**
6F CTGTTAATGTCCTATTTACT	**6R** CCAAGGAGATCTGAAAATACA	**53**
VHL		
1F TGGTCTGGATCGCGGAGGGA	**1R** GACCGTGCTATCGTCCCTGC	**63**
2F GTGGCTCTTTAACAACCTTTGC	**2R** ACTTACCACAACAACCTTATC	**55**
3F TTCCTTGTACTGAGACCCTAGT	**3R** TCCTGTATCTAGATCAAGACTC	**55**
RET		
10S GGAGGCTGAGTGGGCTACG	**10R** CTGGGAGGTGGTGGTGGTC	**66,5**
11S CTCTGGCGGTGCCAAGCCTC	**11R** GAAGGCAGCTGGGGAGGGCA	**66,5**
13S AACTTGGGCAAGGCGATGCA	**13R** AGAACAGGGCTGTATGGAGC	**65**
14S AAGACCCAAGCTGCCTGAC	**14R** GCTGGGTGCAGAGCCATAT	**66**
15S CCCCCGGCCCAGGTCTCAC	**15R** GCTCCACTAATCTTCGGTATCTTT	**55**
16S AGGGATAGGGCCTGGCCTTC	**16R** TAACCTCCACCCCAAGAGAG	**55**

In the two patients with sporadic hnPGL, genetic screening revealed one previously unreported mutation in Patient 3: a 269G>A transversion, leading to R90Q substitution, in exon 3 of SDHB.

The screening performed on 34 cases of apparently sporadic PHEO showed the presence of sequence variants in eight patients. Two of them were positive for mutations in the VHL gene: Patient 9, with 256C>G, P86A in exon 1 and Patient 11 with 561C>T, R167W in exon 3. Both mutations have been previously described in association with PHEO.[7,8] Patient 4 carried two distinct SDHB variants: a 18A>C transversion, A6A, in exon 1, known as genetic polymorphism[9] and a 487T>C, S163P, in exon 5; such missense mutation has been considered a polymorphic variant in a Spanish population,[10] but we have not found such variation in a series of 70 Italian controls (140 chromosomes).

TABLE 2. Clinical features of patients carrying sequence variants in SDHB, SDHD and VHL genes

Mutation type	Patient number	Clinical features tumor site	F	Malignancy	Age at first diagnosis	UC	Scintigraphy	Screened relatives/ carriers/ affected	Mutation reference
SDHB 23T>C S8F	1	Left para-adrenal PGL	−	−	28	−	MIBG +	3/2/0	New
SDHB 137G>A, R46Q	2	Right para-adrenal PGL	−	−	45	+	n.a.	5/0	6
SDHB 269G>A, R90Q	3	Bilateral tympanic and carotid hn-PGL	−	−	33	−	n.a.	3/0	New
SDHB 487T>C, S163P 18A>C A6A	4	Right adrenal PHEO	−	−	17	+	MIBG +	−	10, 9
SDHB 822C>G, R230G	5	Left juxta-adrenal interaortocaval multifocal PGL	+	+	9	+	MIBG-OCT +	16/6/1	New
	6	Right para-aortic PGL	−	−	28	+	MIBG +	29/16/2	
SDHD 348–351delGACT	7	Carotid glomous hn-PGL	+	Local invasivity	47	−	OCT +	−	30
VHL 277G>T G93C	8	Adrenal bilateral PHEO	+	−	31	+	n.a.	1/1/1	24
VHL 256C>G, P86A	9	Adrenal bilateral PHEO	−	−	9	+	MIBG +	0	7
VHL 242C>T, P81L	10	Left para-adrenal PGL	−	−	35	+	MIBG +	0	New
VHL 561C>T, R167W	11	Adrenal bilateral PHEO	−	−	28	+	MIBG +	0	8
SDHD 149A>G, H50R	12	Right adrenal PHEO	−	−	65	+	MIBG +	0	11
	13	PHEO	−	−	n.a.	+	n.a.	0	
SDHD 204C>T, S68S 34G>A, G12S	14	Left adrenal PHEO	−	−	54	+	n.a.	0	12
SDHB IVS1–111G>A	15	Right adrenal PHEO	−	−	45	+	MIBG +	0	4
SDHB IVS2 + 35G>A IVS4 + 60T>C	16	PHEO	−	−	40	+	n.a.	0	9

OCT = Octreoscan; UC = urinary catecholamine; n.a. = not available; F = familiarity; − = negative; + = positive; F = familial.

We have found additional known polymorphisms in five other patients with sporadic PHEO: two of them (Patients 12 and 13) presented the nonsynonymous 149A>G substitution, H50R, in exon 3 of SDHD[11] and Patient 14 was a carrier of two polymorphisms,[12] 34G>A, G12S and 204C>T, S68S in SDHD exon 1 and 3, respectively. Three additional intronic polymorphisms of the SDHB gene were found in Patient 15 (IVS1–111 G>A) and Patient 16 (IVS2 + 35 G>A and IVS4 + 60T>C).

We have further investigated the three missense mutations of SDHB gene (S8F, R90Q, and R230G), which have not been described before, in order to define their clinical relevance better. First, we have not found similar gene variants in a cohort of 70 healthy subjects. We have then considered whether the amino acids involved (S8, R90, R230) are conserved among different vertebrate and invertebrate species. As shown in FIGURE 1, positions S8 and S163 are conserved in different mammalian species (*Rattus norvegicus*, *Mus musculus*, *Pan troglodytes*, *Canis familiaris*), while positions R90 and R230 are identical both in mammalian and invertebrate species. We further evaluate loss of heterozygosis (LOH) in the available tumoral tissues; we were able to ex-

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Homo sapiens          MAAVVALSLRRRLPATTLG--GACLQASRGAQTAAATAPRIKKFAIYRWDPDKAGDKPHM 58
Rattus norvegicus     MAAVVGVSLKRGFSATALGRVGLQFQACREAQTAAAAAPRIKTFAIYRWDPDKAGDKPRM
Mus Musculus          MAATVGVSLKRGFPAAVLGRVGLQFQACRGAQTAAAAAPRIKKFAIYRWDPDKTGDKPRM
Pan troglodytes       MAAVVALSLRRRLPATTLG--GACLQASRGAQTAAATAPRIKKFAIYRWDPDKAGDKPHM
Canis familiaris      MAAVVGVSLKRRFPAAALG--GACLQACRGAQTAAATAPRIKKFAIYRWDPDKTGDKPHM
Drosophila m.         LSRVGSLVARNQMRAISNG--TA--QLEQQAQPKEAQEPQIKKFEIYRWNPDNAGEKPYM
S.pombe                                                   LKTFEIYRWNPEKPEVKPKL
M.grisea                          PSAALR--KPAMVSVRSMASVGESESKMKTFQIYRWDPSQPSEKPRM
A.thaliana                      RLIPARWTSTGA—EAETKASSGGGRGSNLKTF  QIYRWNPDNPG-KPEL

Homo sapiens          QTYKVDLNKCGPMVLDALIKIKNEVDSTLTFRRSCREGICGSCAMNINGGNTLACTRRID 118
Rattus norvegicus     QTYKVDLNKCGPMVLDALIKIKNEIDSTLTFRRSCREGICGSCAMNINGGNTLACTRRID
Mus Musculus          QTYEVDLNKCGPMVLDALIKIKNEVDSTLTFRRSCREGICGSCAMNINGGNTLACTRRID
Pan troglodytes       QTYEVDLNKCGPMVLDALIKIKNEVDSTLTFRRSCREGICGSCAMNINGGNTLACTRRID
Canis familiaris      QTYEIDLNKCGPMVLDALIKIKNEIDSTLTFRRSCREGICGSCAMNINGGNTLACTRRID
Drosophila m.         QTYEVDLRECGPMVLDALIKIKNEMDPTLTFRRSCREGICGSCAMNIGGTNTLACISKID
S.pombe               QKYTVDLTKCGPMVLDALIKIKNEQDPTLTFRRSCREGICGSCAMNINGSNTLACICNIK
M.grisea              QTYTLDLNKTGPMVLDALVRIKNELDPTLTFRRSCREGICGSCAMNINGTNTLACLCRIP
A.thaliana            QNYQIDLKDCGPMVLDALIKIKNEMDPSLTFRRSCREGICGSCAMNIDGCNGLACLTKIQ

Homo sapiens          TNLNKVSKIYPLPHMYVIKDLVPDLSNFYAQYKSIEPYLKKKDESQE--GKQQYLQSIEERE 178
Rattus norvegicus     TDLGKVSKIYPLPHMYVIKDLVPDLSNFYAQYKSIEPYLKKKDESQE--GKQQYLQSIEDRE
Mus Musculus          TDLSKVSKIYPLPHMYVIKDLVPDLSNFYAQYKSIEPYLKKKDESQE--GKQQYLQSIEDRE
Pan troglodytes       TNLNKVS------------------SNFYAQYKSIEPYLKKKDESQE--GKQQYLQSIEERE
Canis familiaris      TNLSKVSKIYPLPHMYVIKDLVPDLSNFYAQYKSIEPYLKKKDESQE--GKQQYLQSIEDRE
Drosophila m.         INTSKSLKVYPLPHMYVVRDLVPDMNNFYEQYRNIQPWLQRKNEAGEKKGKAQYLQSVEDRS
S.pombe               KD-NKPTKIYPLPHCFIVKDLVPDLTYFYKQYKSIEPWLQNDNIPKD----KEFYQSRADRA
M.grisea              TEASSEVKIYPLPHTYVVKDLVPDLTHFYKQYRSIKPYLQRDEPAPDG---KEYRQSKEDRR
A.thaliana            DEASETT-ITPLPHMFVIKDLVVDMTNFYNQYKSIEPWLKRKTPASV--PAKEILQSKKDRA

Homo sapiens          KLDGLYECILCACCSTSCPSYWWNGDKYLGPAVLMQAYRWMIDSRDDFTEERLAKLQDPF 238
Rattus norvegicus     KLDGLYECILCACCSTSCPSYWWNGDKYLGPAVLMQAYRWMIDSRDEFTEERLAKLQDPF
Mus Musculus          KLDGLYECILCACCSTSCPSYWWNGDKYLGPAVLMQAYRWMIDSRDDFTEERLAKLQDPF
Pan troglodytes       KLDGLYECILCACCSTSCPSYWWNGDKYLGPAVLMQAYRWMIDSRDDFTEERLAKLQDPF
Canis familiaris      KLDGLYECILCACCSTSCPSYWWNGDKYLGPAVLMQAYRWMIDSRDDFTEERLAKLQDPF
Drosophila m.         KLDGLYECILCACCSTSCPSYWWNAEKYLGPAVLMQAYRWIIDSRDENSAERLNKLKDPF
S.pombe               KLDGLYECILCACCSTSCPSYWWNSEEYLGPAVLMQAYRWIIDSRDQATAKRLDVMQNSM
M.grisea              KLDGLYECILCACCSTSCPSYWWNSEEYLGPAILLQSYRWLADSRDQRKVERRLALDNSM
A.thaliana            KLDGMYECILCACCSTSCPSYWWNPESYLGPAALLHANRWISDSRDEYTKPRLEAIDDEF
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FIGURE 1. Conservation of amino acid position S8, R90, S163, and R230 of SDHB protein among different species.

tract DNA from an abdominal, catecholamine-producing PGL from Patient 6 (R230G mutation) and amplify them with a set of primers surrounding microsatellites D1S199, D1S2644, D1S2697, and D1S507: the results (data not shown) confirmed somatic LOH. Finally we considered PHEO/PGL penetrance among relatives of probands carrying the same mutation. In particular, we have been able to examine two large kindreds from Patient 5 (familial PGL) and Patient 6 (sporadic PGL), both carriers of the R230G mutation in SDHB gene: among 22 carriers of such mutation found in the two kindreds, three more subjects were affected by PHEO/PGL.

DISCUSSION

In the present screening of an Italian sample of PHEO/PGL patients representative of the specialized hypertensive clinical setting, we have been able to find 17 sequence variants in genes potentially involved in PHEO/PGL pathogenesis. Of these, five (SDHB R46Q, SDHD 348-351 delGACT, VHL G93C, P86A, and R167W) have been previously described in association with PHEO/PGL, while three (SDHB S8F, R90Q, and R230G and VHL P81L) are novel mutations that presumably play a causative role. In addition, we have found eight known genetic polymorphisms. Accordingly, the overall prevalence of clinically significant mutations is 10 of 45 patients (22%), with relative contribution of 5 of 45 (11%), 4 of 45 (9%), and 1 of 45 (2%) for SDHB, VHL, and SDHD genes, respectively. If we limit our consideration to PHEO/PGLs presenting in a familial setting, the prevalence of pathogenically relevant mutations is 100% (3 of 3), but in sporadic forms the frequency of mutation is also very high, in substantial agreement with the literature, showing similar data in a large collection of patients with sporadic PHEO/PGL (TABLE 3).

In our sample no patient presented mutations of RET or SDHC genes. As a matter of fact, we preliminarily excluded PHEO/PGL occurring in syndromic form, and PHEO is almost never the first clinical manifestation of a MEN2 syndrome; on the other hand, only eight cases of SDHC-associated PGL have been reported thus far.

TABLE 3. Occult germline mutations in sporadic nonfamilial phaeochromocytomas

Gene	No. of patients	Subjects with mutations	Percent	Reference	Present data (%)
RET	712	22	3.1 (0–21.4)	16–26	0
VHL	586	41	7.0 (0–20)	18, 20, 23, 24–27	7.9
SDHB	536	39	7.3 (4.9–14.3)	25, 26, 28, 29	12.5
SDHD	561	27	4.8 (0–8)	25, 26, 28–30	0

It should also be noted that the prevalence of SDHD mutations is lower than previously reported, but this may be at least in part due to the small number of hnPGLs included in our samples, which are more frequently associated with SDHD mutations.

From these data and previous evidence from the literature, we may argue that the most cost-effective mutation screening strategy in the setting of hypertensive patients with nonsyndromic PHEO is probably the sequential analysis of VHL, SDHB, and SDHD, whereas in PGL the sequence should be SDHB, SDHD, VHL, and SDHC.

We have been able to perform an extended pedigree analysis only in two kindreds, both carrying the novel SDHB R230G mutation. Notably, only 5 of 22 mutation carriers had a diagnosis of PHE/PGL (two subjects in proband 6 family and three subjects in proband 5 family). None of the remaining 17 carriers, clinically asymptomatic, showed evidence of the disease on thorough biochemical and imaging investigation.

Although our mutation screening by direct bidirectional sequencing is very sensitive, we cannot exclude the occurrence of other mutations in intronic or, regulatory regions, large deletions of the entire gene,[13] or exon skipping promoted by Alu intronic sequence, as recently reported by various authors.[14,15] Southern blot or RNA analysis (RT-PCR or Northern blot) could give us more complete results.

In conclusion, the present findings in an Italian cohort of PHEO/PGL patients recruited through hypertension clinic facilities suggest that genetic screening for mutations of candidate genes (at least SDHB, VHL, and SDHD) is indicated not only in familial, but also in sporadic forms, given the relatively high prevalence of pathogenically relevant mutations. However, a more precise characterization of the functional relevance of any observed sequence variant and of other genetic and environmental determinants of neoplastic transformation is essential in order to plan appropriate protocols for family screening and follow-up.

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