Original Article

Genetic Profile of Indian Pheochromocytoma and Paraganglioma Patients – A Single Institutional Study

Gaurav Agarwal[1,*], Sendhil Rajan[1,2,*], Ramya C. Valiveru[1], Sonam Tulsyan[3], Vinita Agrawal[4], Balraj Mittal[3], Ghazala Zaidi[5], Sabaretnam Mayilvaganan[1], Anjali Mishra[1], Amit Agarwal[1], Saroj Kanta Mishra[1], Eesh Bhatia[5]

Departments of [1]Endocrine and Breast Surgery, [3]Medical Genetics, [4]Pathology and [5]Endocrinology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, Uttar Pradesh, [2]Department of Surgery, St. John's Medical College, Bengaluru, Karnataka, India

*Gaurav Agarwal and Sendhil Rajan have contributed equally to this study, and are joint first authors

Abstract

Background and Aims: Pheochromocytomas (PCCs) and Paragangliomas (PGL) are rare catecholamine producing tumors that may present in sporadic or familial settings. Despite vast strides in understanding of PCC/PGL genetics in the last two decades, there is a dearth of information from India. The aim here is to study the prevalence of genetic mutations in Indian PCC/PGL patients. **Settings and Design:** Tertiary care academic hospital; prospective study. **Methods:** 50 histopathologically diagnosed PCC/PGL patients formed the study group. Clinical, biochemical, pathological attributes and outcomes were documented and the phenotype was compared to the genotype. Succinyl dehydrogenase (SDH), Re-Arranged during Transfection (RET), Von-Hippel-Lindau (VHL) and NeuroFibromatosis-1 (NF1) mutations were studied. Additionally, immunohistochemisty for SDHB was also done, and the results compared to mutational analysis of SDH by MLPA (Multiplex Ligation-dependent Probe Activation). **Statistical Analysis:** Independent samples *t*-test and Fisher's exact test were used as appropriate. *P* values ≤0.05 were considered statistically significant. **Results:** The mean age was 34.3 years. Of the 50 patients, 27 were males and 23 females. 10 patients (20%) in all were detected to have a genetic mutation. 6 patients possessed a *RET* mutation, while two had *VHL* mutations. No patient presented with a NF1 mutation. 2 patients had a SDH mutation, and Immunohistochemistry for SDHB correlated with mutational analysis for these patients. **Conclusions:** The proportion of patients with a familial variant of PCC/PGL is more than what the historic "Rule of Ten" suggests. Our study found that one in five patients have a genetic mutation. PCC/PGL patients with genetic mutations not only require more stringent follow-up, but also screening of family members.

Keywords: Genotype, mutations, NF-1 multiple endocrine neoplasia syndrome-2, paraganglioma, pheochromocytoma, RET mutation, SDH mutations, VHL mutations

Introduction

Pheochromocytomas (PCC) and paragangliomas (PGL) are rare catecholamine secreting tumors arising from chromaffin cells of adrenal medulla.[1] PCC/PGL may present as sporadic or familial disease. The classical "*Rule of 10*" stating that up to 10% of these tumors were associated with genetic mutations has been disproven in recent years. More than 13 susceptibility genes have been implicated in causation of PCC.[2,3] Routine genetic testing is now advocated in all PCC/PGL patients by many.[4] The information on the genetics of PCC/PGL from India is sparse.[5] In this prospective study, we aimed to establish the genetic profile of Indian PCC/PGL patients.

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Methods

In this prospective study conducted at a tertiary care hospital in North India, with approval from IEC, 50 consecutive PCC/PGL patients managed between January 2014 and January 2016, with histopathological confirmation of the disease formed the study cohort. Clinical, biochemical, pathological attributes

Address for correspondence: Prof. Gaurav Agarwal, Department of Endocrine and Breast Surgery, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Rae Bareilly Road, Lucknow - 226 014, Uttar Pradesh, India.
E-mail: gaurav@sgpgi.ac.in, gauravbsi@gmail.com

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and outcomes of all patients were evaluated. Biochemical diagnosis of PCC/PGL was made based on 24-hour urinary fractionated metanephrines estimation in all patients prior to surgery. Patients underwent imaging with ultrasonography and contrast enhanced computed tomography (CECT) in all. Where indicated, additional imaging with MRI and/or Ga^{68} DOTA PET/CT were carried out. Patients underwent evaluation for syndromic/familial forms of PCC/PGL with detailed family history and physical examinations to look for any components of familial PCC/PGL syndromes. Genetic mutation studies were done in a targeted, step-wise fashion as suggested by Manneli *et al*.[3]. Accordingly, not all patients underwent genetic testing for all susceptibility genes. Genetic testing was done for the four most common susceptibility genes, namely- Succinyl DeHydrogenase (*SDH*), Re-Arranged during Transfection (*RET*), von-Hippel-Lindau (*VHL*) and NeuroFibromatosis-1 (*NF1*) genes. All patients underwent curative minimally invasive or conventional open surgery. The diagnoses were established by histopathology on H and E staining and immunohistochemistry (IHC). Additionally, SDHB IHC was performed on all tumors.

Genetic evaluation and mutation testing

For *RET* analysis, venous blood was collected from patients and genomic DNA was extracted using QIAamp DNA blood minikit (*QIAGEN, Hilden Germany*). Susceptible exons of *RET* gene associated with MEN2, namely exons 10, 11, 13, 14, 15 and 16, including their intron-exon boundaries, were amplified by PCR, followed by bi-directional sequencing of amplified PCR fragments. The *VHL* gene was screened for large deletions using a real-time quantitative PCR (RQ-PCR). NF1 mutations were diagnosed on the basis of phenotype alone as generally accepted.[3,6] Careful clinical evaluation for typical NF1 skin and eye lesions accurately correlate with molecular testing, and thus obviate the need for high costs caused by molecular analyses.[7] SDH mutation testing was done by using IHC for SDHB on tumor tissue, as well as mutational analysis by Multiplex Ligation-dependent Probe Activation (MLPA).

SDHB immunohistochemistry

IHC has been shown in previous studies to be a reliable tool in diagnosing SDH-related PCC/PGL syndromes.[8-11] Representative tumor sections were selected by a single pathologist (VA), and the corresponding paraffin blocks were chosen for IHC. Sections were serially cut at 4-micron thickness and then deparaffinised by heating on hot plate and immersing in xylene. Following this, rehydration with 70% ethanol followed by serial distilled water washings were performed. Antigen retrieval was done by heating and treatment with buffer solutions at pH 9. Endogenous peroxidase activity was blocked by washing with 3% H_2O_2 in methanol for 30 minutes at room temperature. SDH-IHC was done using commercially available rabbit polyclonal antibody HPA002868 (*Sigma-Aldrich Corp; St. Louis, MO, USA*). The SDHB primary antibody was used at a 1:500 dilution, and slides incubated for 2 hours, using diaminobenzidine tetrahydrochloride chromogen for 15 min. Sections were counterstained with Harris haematoxylin, rinsed, dehydrated and covered with cover slips. Verification of antigen preservation was performed with Vimentin and Ki-67 immunostaining. Tissue samples from normal adrenal gland and liver were used as positive controls. Dilution buffer instead of the primary antibody, with total absence of staining was used as negative control; sustentacular cells were used as internal control. Results were reported as positive, negative, or weak-diffuse pattern, based on previously published studies by Gill, Castelblanco and Pai.[9-11] A positive SDHB was defined as cytoplasm showing strong granular mitochondrial staining; negative SDHB as cytoplasm showing no staining with positive staining for the internal control; and weak–diffuse pattern as tumours with a cytoplasmic blush lacking definite granularity. The pathologist who reported the results was blinded to the clinical profile and mutational analysis report of the patient.

SDH mutation analysis by MLPA

Five ml of blood drawn in EDTA vials were stored at -80°C. DNA extraction was performed using the DNA isolation kit (*QIAGEN, Hilden Germany*). Quality and quantity of DNA was checked by using the Nanodrop spectrophotometer (*Thermo Fisher Scientific/Nanodrop Products, Wilmington, Delaware, USA*). The extracted DNA was then subjected to MLPA, using the SALSA MLPA probemix P226-C1 SDH (*MRC-Holland, Amsterdam, The Netherlands*).

PCC/PGL clinical presentation and phenotype, including details of various syndromic components, biochemical attributes, surgical procedures performed, histopathology and outcomes were compared between the sporadic and familial groups. All statistical analyses were performed using the SPSS 17 software package (*SPSS Statistics for Windows, Version 17.0, SPSS Inc., Chicago, USA*). Independent samples *t*-test (two-tailed) and Fisher's exact test (two-tailed) were used as appropriate. Any P values ≤ 0.05 were considered statistically significant.

Results

Clinico-pathological attributes of the study group are summarized in Table 1. The mean age was 34.3 years. Of the 50 patients, 27 were male and 23 female. The tumor was right-sided in 28 patients and left-sided in 16, with 6 patients having bilateral disease.

Table 1: Clinico-pathological attributes of patients

Attribute	Value
Age at diagnosis (years): Mean±SD (Range)	34.3±15.3 (8-73)
Tumor size (cm): Mean±SD (Range)	7.2±3.4 (2.3-20)
Tumor weight (gm): Mean±SD (Range)	168.4±255.9 (4-1436)
Male:Female	27: 23
Left: Right: Bilateral	16: 28: 6
Urinary Metanephrines (mcg/24 h): mean (range)	1084 (29-8000)
Urinary Normetanephrines (mcg/24 h): mean (range)	4187 (131-12000)

cm=centimeters, SD=Standard Deviation, mcg=micrograms

The location of the tumors was adrenal gland in 41 (82%), abdominal paragangliomas in 7 (14%), of which 5 were infrarenal and 2 were suprarenal, the remaining 2 (4%) tumors were cervical paraganglioma, both located at the carotid artery bifurcation. Details regarding surgical approach undertaken and histopathology are summarized in Tables 2 and 3, respectively. No peri-operative deaths or major surgical morbidity resulting in prolonged admission or re-admission were observed. After a mean follow-up of 16.7 ± 2.6 months, all symptomatic patients had amelioration of symptoms. All patients had normal 24 hours fractionated metanephrines at 1 to 2 weeks post-operative stage, and in longer-term follow-up.

Genetic evaluation and mutation testing identified 10 (20%) patients with one or the other mutations. Six patients possessed the *RET* mutation, while two had *VHL* mutations. No patient presented with a NF1 mutation. Two patients had a SDH mutation, and IHC for SDHB correlated with mutational analysis for these patients.

Of the 6 (12%) patients with *RET* mutations, 5 had a mutation involving codon 634. These included a 50-year-old female (C634Y), a 35-year-old female (C634G), a 30-year-old female (C634Y), a 22-year-old male (C634R) and a 29-year-old male (C634Y). The first three patients had bilateral disease at initial presentation, while the last two had unilateral disease, and have not developed metachronous bilateral PCC up to 2 years in follow-up. All patients of codon 634 RET mutation had the MEN2A phenotype and had medullary thyroid carcinoma (MTC), for which they underwent a second operation, 1-3 weeks following the PCC surgery. The sixth *RET* mutated patient had a Codon 804 (V804M) mutation, a rare cause of MEN2-associated PCC, reported by us earlier.[5] The two patients of VHL mutation included a 10-year-old boy and a 32-year-old male, both exhibiting bilateral disease at the time of diagnosis. The latter also had renal cell carcinoma at the time of initial presentation. Two patients had a SDH mutation- both males, aged 39 years and 19 years, respectively. Both had unilateral retroperitoneal benign PGL. IHC studies on tumors of these two patients showed absence of SDHB immuno-staining, signifying mutation in SDH sub-units. These results correlated with mutational analysis by MLPA for these patients. Comparisons of clinico-pathological attributes between patients detected to have a genetic mutation and those without, are summarized in Tables 4-6. Owing to small number of patients with specific mutations, a genotype-phenotype correlation was not performed.

Table 2: Surgical approach

Approach	*n*	%
Minimally invasive approach		
Laparoscopic	16	32%
Retroperitoneoscopic	3	6%
Conventional (open) surgical approach		
Open Transperitoneal (inclusive of conversion from laparoscopic to open approach in 4 patients)	26	52%
Open Retroperitoneal	3	6%
Cervical Paraganglioma Excision	2	4%

Table 3: Histopathology

Pathology	*n*	%
Benign Pheochromocytoma	40	80%
Malignant Pheochromocytoma	1	2%
Benign Paraganglioma	8	16%
Malignant Paraganglioma	1	2%

Discussion

The historic "rule of 10" attributed to PCC/PGL, according to which 10% of patients were thought to suffer from a genetic form of disease, has now been discarded. Prior to 2000, the only hereditary variants of PCC/PGL known were *VHL/RET/NF-1*. In the year 2000, *SDHD* gene mutations were shown to cause head and neck paragangliomas,[12] and *SDHB* mutations were shown to cause familial PCC/PGL syndrome in 2001.[13] Other genetic variants of genetic disease have since been described, which led to PCC/PGL being called a "10 gene tumor", as 10 susceptibility genes were implicated in the causation, which too has since evolved. Currently, more than 13 susceptibility genes have been described and many more are being evaluated.[2]

The majority of data regarding genetics of PCC/PGL comes from American and European literature;[3,4,14] and recent Japanese and Korean studies.[15,16] Literature from India is sparse and is limited to case reports and series, barring a single study on a database of 50 PCC/PGL patients, which reported 4 (8%) patients exhibiting RET mutations, VHL and SDH mutations accounting for 6 (12%) patients each, with total prevalence of genetic disease being 32%.[17] Our study has shown that 12% of PCC/PGL patients possess the RET mutation, and 4% each having mutations either in VHL or

Table 4: Comparison of clinico-pathological attributes between patient groups with and without RET mutation

Attribute	RET mutation present (*n*=6)	RET mutation absent (*n*=40)	*P*
Age (Years, Mean±SD)	32.7±9.3	35.3±16.1	0.694
Size (cm, Mean±SD)	9.1±6.5	7.0±2.8	0.180
Weight (g, Mean±SEM)	331.0±232.3	156.5±29.5	0.133
Urine Metanephrines (mcg/24 h, Mean±SEM)	3310±1430	803±210	0.002*
Urine Normetanephrines (mcg/24 h, Mean±SEM)	4945±2144	4080±378	0.494

cm=centimeters, g=grams, SD=Standard Deviation, SEM=Standard Error of Mean, mcg=micrograms

Table 5: Comparison of clinico-pathological attributes between patient groups with and without VHL mutation

Attribute	VHL mutation present ($n=2$)	VHL mutation absent ($n=40$)	*P*
Age (Years, Mean±SD)	23.5±12.0	35.3±16.1	0.313
Size (cm, Mean±SD)	7.1±1.6	7.0±2.8	0.977
Weight (g, Mean±SEM)	38.5+4.5	156.5±29.5	0.383
Malignant disease	1/2	1/40	0.094
Urine Metanephrines (mcg/24 h, Mean±SEM)	92±14	803±210	0.460
Urine Normetanephrines (mcg/24 h, Mean±SEM)	5250±450	4080±378	0.498

cm=centimeters, g=grams, SD=Standard Deviation, SEM=Standard Error of Mean, mcg=micrograms

Table 6: Comparison of clinico-pathological attributes between patient groups with and without SDH mutation

Attribute	SDH mutation present ($n=2$)	SDH mutation absent ($n=40$)	*P*
Age (Years, Mean±SD)	29±14.1	35.3±16.1	0.588
Size (cm, Mean±SD)	5.0±1.4	7.0±2.8	0.316
Weight (g, Mean±SEM)	50+6.0	156.5±29.5	0.431
Urine Metanephrines (mcg/24 h, Mean±SEM)	1012±58	803±210	0.828
Urine Normetanephrines (mcg/24 h, Mean±SEM)	2983±1157	4080±378	0.527

cm=centimeters, g=grams, SD=Standard Deviation, SEM=Standard Error of Mean, mcg=micrograms

SDH genes. PCC/PGL patients with genetic mutations not only require more stringent follow-up, but also screening of family members. A limitation of this study was that it did not include other uncommon genes associated with PCC/PGL, such as, transmembrane domain protein 127 (TMEM127), MYC-associated factor X (MAX), fumarate hydratase (FH) and malate dehydrogenase 2 (MDH2). Also, due to the number of specific gene mutations being relatively small, genotype-phenotype correlation was not performed. We have reported the genotype-phenotype correlation in Indian patients with MEN2-associated pheochromocytoma in an earlier study.[5] Management of Indian PCC/PGL patients is challenging due to the late presentation, and the complex socio-economic issues. This is further compounded by illiteracy and social stigma regarding genetic/familial diseases impeding counselling and genetic testing of other family members. Our study adds to the genetic mutation data for the Indian PCC/PGL patients which can aid in understanding their profile, clinical presentation and emphasising need for genetic testing.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Conflicts of interest

There are no conflicts of interest.

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