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4 Genetics of apparently sporadic pheochromocytoma and paraganglioma in a Chinese  
5 population

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## Abstract

### Objective

Identification of germline mutation in patients with apparently sporadic pheochromocytomas and paragangliomas is crucial. Clinical indicators, which include young age, bilateral or multifocal, extra-adrenal, malignant or recurrent tumours, predict the likelihood of harbouring germline mutation in Caucasian subjects. However, data on the prevalence of germline mutation, as well as the applicability of these clinical indicators in Chinese, are lacking.

### Design and methods

We conducted a cross-sectional study at a single endocrine tertiary referral centre in Hong Kong. Subjects with pheochromocytomas and paragangliomas were evaluated for the presence of germline mutations involving 10 susceptibility genes, which included *NF1*, *RET*, *VHL*, *SDHA*, *SDHB*, *SDHC*, *SDHD*, *TMEM 127*, *MAX* and *FH* genes. Clinical indicators were assessed for their association with the presence of germline mutations.

### Results

Germline mutations, two being novel, were found in 24.4% of the 41 Chinese subjects recruited and 11.4% among those with apparently sporadic presentation. The increasing number of the afore-mentioned clinical indicators significantly correlated with the likelihood of harbouring germline mutation in one of the 10 susceptibility genes. ( $r = 0.757$ ,  $p = 0.026$ ). The presence of 2 or more clinical indicators should prompt genetic testing for germline mutations in Chinese subjects.

### Conclusions

In conclusion, our study confirmed that a significant proportion of Chinese subjects with apparently sporadic pheochromocytoma and paraganglioma harboured germline mutations and these clinical indicators identified from Caucasians series were also applicable in Chinese

subjects. This information will be of clinical relevance in the design of appropriate genetic screening strategies in Chinese populations.

## Introduction

The 2014 Endocrine Society Clinical Practice Guideline for pheochromocytoma and paraganglioma (PPGL) focuses on the multidisciplinary yet personalized management of PPGLs. In particular, the guideline reflects a major paradigm shift in the clinical management of these traditional “10% tumours”, by recommending that genetic testing be considered in each patient with PPGL, especially those with bilateral, malignant, or extra-adrenal disease, even if they do not have a positive family history of PPGL [1]. These recommendations have certainly recognized the increased prevalence of germline mutations among patients with PPGL, as well as the distinct genotype-phenotype correlations in hereditary PPGL syndromes, which could impact on the therapeutic approach for the disease, especially in metastatic PPGLs <sup>2</sup>.

To date, more than 14 susceptibility genes have been identified that are implicated in the pathogenesis of PPGL, accounting for a prevalence of approximately 40% of hereditary PPGLs as reported from Caucasian series [<sup>3</sup>]. However, the prevalence of germline mutations among patients with apparently sporadic PPGLs (i.e. absence of positive family history of PPGL or features suggestive of hereditary PPGL syndromes) is lower. In a recent systematic review of 31 studies, which involved 5031 subjects mostly from European populations, the prevalence of germline mutations was around 11-13%, if only apparently sporadic PPGLs were included in the analysis [<sup>4</sup>]. Nonetheless, although with much geographical variations, studies on the overall prevalence of germline mutations among apparently sporadic PPGLs are mostly from Caucasian populations. On the other hand, comprehensive genetic study in Chinese with apparently sporadic PPGLs is lacking [<sup>5-7</sup>]. Previous genetic studies in Chinese subjects with PPGL were either limited to one single susceptibility gene or to head and neck paragangliomas only [<sup>8-10</sup>]. Furthermore, until next generation sequencing (NGS) becomes readily available, universal screening of all subjects with PPGL remains a laborious process. In fact, even if NGS gains favour in future as a cost-effective and efficient method of genetic screening in PPGL [<sup>11</sup>], there are still shortcomings and technical limitations with NGS [<sup>12</sup>].

Therefore, various algorithms have been developed from Caucasian series in order to prioritize the screening of different susceptibility genes on the basis of clinical indicators, which include age at disease presentation and different tumour characteristics [<sup>13</sup>]. However, the applicability of these clinical indicators to predict the presence of germline mutations in Chinese patients with apparently sporadic PPGLs is still not known.

Therefore, we conducted this study to examine the genetics of Chinese patients with apparently sporadic PPGLs at a tertiary endocrine referral centre in Hong Kong. Our study evaluated the local prevalence of germline mutations in 10 susceptibility genes of PPGL: the neurofibromin 1 (*NF1*) tumour suppressor gene in neurofibromatosis type 1; the rearranged during transfection (*RET*) proto-oncogene; the von Hippel-Lindau (*VHL*) tumour suppressor gene, genes encoding the four subunits (A, B, C and D) of the succinate dehydrogenase (SDH) complex (*SDHA*, *SDHB*, *SDHC* and *SDHD*); the transmembrane protein 127 (*TMEM127*) gene, the MYC-associated factor X (*MAX*) tumour suppressor gene and the fumarate hydratase (*FH*) genes. We also analysed the application of the 5 generally agreed clinical indicators for the presence of germline mutation in these subjects: age at disease onset younger than 45 years old, bilateral or multifocal disease, extra-adrenal involvement, and having recurrent, or malignant tumours.

## **Materials and methods**

This was a cross-sectional study involving subjects with PPGL managed at the Queen Mary Hospital in Hong Kong. The study protocol was approved by the institutional review board of the University of Hong Kong / Hospital Authority Hong Kong West Cluster.

In the study, the 2004 World Health Organization (WHO) definition of PPGL was applied [<sup>14</sup>]. Pheochromocytoma is defined as a tumour arising from catecholamine-producing chromaffin cells in the adrenal medulla, while closely related tumour of extra-adrenal sympathetic and parasympathetic paraganglia is classified as paraganglioma [<sup>15</sup>]. Diagnoses

of PPGL were based on urinary levels of catecholamines or catecholamine metabolites (including normetanephrines, metanephrines, vanillylmandelic acid and homovanillic acid) and surgical histology, with or without iodine 131-labelled metaiodobenzylguanidine scintigraphy (MIBG). Head and neck paraganglioma was diagnosed on the basis of imaging findings (computed tomography, magnetic resonance imaging or ultrasonography, as appropriate) or surgical histology [16]. Malignancy was defined based on the WHO classification, as the presence of frank loco-regional invasion or metastases to non-chromaffin sites [17].

In the study, apparently sporadic presentation was defined as the absence, at disease presentation, of a family history of PPGL, or syndromic features (personal history of medullary thyroid carcinoma or oral mucosal neuromas, or haemangioblastoma at disease presentation, or phenotypic features of neurofibromatosis type 1), which were suggestive of multiple endocrine neoplasia (MEN) type 2, von Hippel Lindau (VHL) disease or neurofibromatosis type 1 (NF1), respectively.

All subjects with PPGL from the medical and surgical endocrine clinics of Queen Mary Hospital were invited to participate in this study from March 2011 to December 2012. In known familial cases of PPGL, only the probands were included.

Among a total of 60 subjects with PPGLs being followed up at our clinics, 11 known asymptomatic germline mutations carriers were excluded, as they were family members of probands with familial diseases (10 subjects with MEN2, and 1 subject with VHL disease). Of the remaining 49 subjects, 8 refused to participate. Therefore, 41 unrelated eligible subjects with PPGLs were finally enrolled into our study.

Demographic and clinical data were collected from the computer-based clinical management system of the Queen Mary Hospital, and through reviewing their medical records. Except for

proband in familial cases with known germline mutations, all recruited subjects underwent genetic testing for germline mutations involving all coding exons of *FH*, *MAX*, *SDHA*, *SDHB*, *SDHC*, *SDHD*, *TMEM127*, *VHL* genes and exons 10, 11 and 16 of *RET* genes. Neurofibromatosis type 1 was diagnosed on the basis of phenotype alone as generally accepted [18]. All subjects gave written informed consent prior to any study related procedures.

Genomic DNA samples were extracted from 10ml ethylenediaminetetraacetic acid (EDTA) peripheral whole blood using the QIAamp DNA mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Forward and reverse primers were designed based on previously published information [16, 19-24]. All coding exons and exon-intron boundaries of *FH*, *MAX*, *SDHA*, *SDHB*, *SDHC*, *SDHD*, *TMEM127*, *VHL* genes and exons 10, 11 and 16 of *RET* gene were screened for mutations by Sanger sequencing, using an ABI 3730xl DNA analyser (Life Technologies, USA) at the Centre for Genomic Sciences, the University of Hong Kong. The sequence results were analysed with the ABI Sequence Scanner Software v1.0 (Life Technologies, USA). In-silico analysis was used for the assessment of their pathogenicity. Prediction software modules used for in-silico analysis included Mutation Taster (<http://www.mutationtaster.org/>), PolyPhen-2 (<http://genetics.bwh.harvard.edu/pph2/>) and KGGSeq (<http://statgenpro.psychiatry.hku.hk/limx/kggseq/>). Furthermore, a control group of DNA samples from 100 healthy Hong Kong Chinese subjects from the Hong Kong Cardiovascular Risk Factor Prevalence Study (CRISPS) cohort [25] was used as reference samples for population allele frequency estimation in the evaluation of novel missense variants.

All data were analysed with SPSS Statistics Version 20.0 (SPSS, Chicago, IL). Descriptive statistics were calculated for all variables. Comparison of categorical data was performed by Chi-squared test or Fisher's exact test as appropriate. Continuous data were compared using Mann-Whitney-U test. Ordinal data were analysed using the Gamma test. Logistic regression

models were used to evaluate the association among various clinical parameters with the likelihood of subject harbouring germline mutations. Receiver operating characteristic (ROC) curve was used to evaluate the optimal number of clinical indicators in our study cohort to indicate the presence of germline mutation. Optimal cut-off was derived from Youden index criterion [maximum of (sensitivity + specificity) - 1] [26]. All tests were two-sided, and p value of less than 0.05 was considered statistically significant.

## Results

In our cohort of 41 subjects, 9 subjects were found to harbour germline mutations and 1 subject had NF1, which translated into a prevalence of 24.4% for hereditary PPGL. On the other hand, 6 of these 10 subjects had syndromic features suggestive of hereditary disease at disease presentation. Therefore, 35 subjects had PPGL with apparently sporadic presentation, and the prevalence rate of germline mutations was 11.4% (4 of 35 subjects).

Among the 9 mutations identified on sequencing, 7 were located on the *RET* gene, 1 on the *SDHB* gene and 1 on the *SDHD* gene. Both the deletion mutation in the *SDHB* gene and the insertion mutation on the *SDHD* gene were novel pathogenic variants that had not been previously reported (Table 1). A sequence variant was also found on exon 2 of the *TMEM127* (c.53C>T) gene in 3 subjects, including the subject with an *SDHB* germline mutation. All 3 subjects were heterozygous for this sequence variant. However, in-silico analyses suggested that the sequence variant was not associated with any change in protein structure. Furthermore, screening of 100 healthy controls also revealed 2 subjects with the same heterozygous variants, yielding a minor allelic frequency of 1%. Hence, this sequence variant in *TMEM127* gene was likely a polymorphism rather than a pathogenic mutation.

Of the 35 subjects (18 men and 17 women) with apparently sporadic PPGL in our study cohort, 27 and 8 had pheochromocytoma and paraganglioma respectively. Among those paragangliomas, 1 was head and neck paraganglioma, 3 were in the abdomen and 4 were in

the bladder. Their mean age of diagnosis was  $45 \pm 13$  years. Other biochemical and tumour characteristics are summarized in Table 2. About 77% of the tumours were larger than 3cm, 28.6% were either multifocal or bilateral tumours, and 89.3% showed avidity on MIBG scan. The prevalence of malignancy was 11.4% and the recurrence rate was 22.9% over a median follow-up of 5 years.

In our study, the frequency of germline mutations in subjects with syndromic presentation (4 subjects with a personal history of medullary thyroid carcinoma or oral mucosal neuromas, 1 subject with a family history of PPGL and 1 subject with phenotypic features of NF1) was 100%. However, in those subjects with apparently sporadic presentation, we found that only “age of disease onset younger than 45 years old” ( $p = 0.023$ ) and having “bilateral or multifocal tumours” (100% versus 19% in subjects without mutations,  $p < 0.004$ ) were statistically significant clinical indicators of presence of germline mutations (Table 3). Furthermore, we have shown that, with an increasing number of these clinical indicators, the subjects were more likely to harbour germline mutations ( $r = 0.757$ ,  $p = 0.026$ ; Gamma test). In addition, with the ROC curve, we demonstrated that in those subjects with an apparently sporadic presentation of PPGL, the presence of 2 or more of the clinical indicators provided the optimal cut-off to initiate genetic testing, with a 100% sensitivity of picking up germline mutation in 1 of the 10 susceptibility genes of PPGL, coupled with a specificity of 77.4%. The area under the curve was 0.85 (95% CI = 0.72-0.99) (Figure 1).

## Discussions and conclusions

Our study is the first comprehensive series studying the prevalence of germline mutation involving a substantial number of susceptibility genes in Chinese subjects with apparently sporadic PPGLs. Since 2002, there have been several Caucasian studies on the prevalence of hereditary PPGL [<sup>16, 27-29</sup>], although their results differed owing to geographical variations, differences in inclusion and exclusion criteria, and the number of susceptibility genes being studied. Our group demonstrated that the prevalence of germline mutation in Chinese subjects

with apparently sporadic PPGL was in keeping with published Caucasian data, and the previous notion that only 10% of PPGLs were hereditary was no longer valid worldwide. Given the rapid advancement in molecular biology, and the fact that known germline mutations are still not detected in a significant proportion of familial PPGL cases worldwide, it is anticipated that the prevalence of germline mutations in PPGLs, including in Hong Kong, may be higher and will likely continue to rise in the future.

Furthermore, in this study, we have demonstrated that the 5 generally agreed clinical indicators for the presence of germline mutation in Caucasian patients with apparently sporadic PPGLs: age at disease onset younger than 45 years old, bilateral or multifocal disease, extra-adrenal involvement, and having recurrent, or malignant tumours, were also applicable in the clinical assessment in Chinese populations. Although in our cohort, only “age younger than 45 years old” and “bilateral or multifocal disease” were significant clinical indicators in univariate analysis, we did show that with an increasing number of these clinical indicators, Chinese subjects with apparently sporadic PGLL were more likely to harbour germline mutations in 1 of the 10 susceptibility genes examined. In other words, our findings were in line with the recommendations of the 2014 Endocrine Society Clinical Practice Guideline for PPGL, which recommended that genetic testing should be considered in each patient with PPGL, especially those with bilateral, malignant, or extra-adrenal disease, even if they do not have a positive family history of PPGL. In fact, subjects with a family history of PPGL or a personal history of syndromic lesions (e.g. medullary thyroid carcinoma or VHL disease associated tumours) should inevitably be offered genetic testing of the corresponding specific gene. Nonetheless, after-all, the majority of PPGLs are still sporadic tumours without a positive family history. In addition, hereditary diseases could have been missed, due to de novo mutations, genomic imprinting, unexhausted family history, and incomplete disease penetrance [<sup>30</sup>]. However, the list of susceptibility genes of PPGL is anticipated to keep growing, whilst universal screening of all PPGL subjects for all susceptibility genes is still technically or financially not feasible in most countries. Before NGS or whole exome

sequencing becomes a widely accessible genetic screening method at a reasonable cost, our findings might perhaps provide an alternative algorithm to guide targeted genetic testing in Chinese patients with apparently sporadic PPGL. In our cohort, we found that genetic testing was clearly indicated in Chinese subjects with 2 or more of the afore-mentioned clinical indicators. With this, the sensitivity of picking up a germline mutation in a Chinese subject with apparently sporadic PPGL was 100%, and the specificity was 74.2%. These translated to a positive predictive value of 33.3% and a negative predictive value of 100%. Since pheochromocytoma carries significant mortality if they are not diagnosed early and properly treated<sup>31</sup>, there is no doubt that the detection of a germline mutation would pose a significant impact on both disease management and tumour surveillance of not only the index patient, but also their as yet unaffected family member. With a sensitivity and negative predictive value up to 100%, the risk of missing a hereditary PPGL could certainly be minimized.

Our study has several limitations. First, referral bias exists as this study was carried out at a tertiary referral centre, and patients with more difficult tumours, for example extra-adrenal and multifocal involvement, or malignant or recurrent disease might get referred and hence increased the likelihood of having subjects potentially harbouring germline mutations. However, this is not entirely true, as more than 50% of subjects in our cohort suffered from isolated unilateral benign pheochromocytomas only. Nonetheless, even if significant referral bias does exist, this might perhaps further justify the use of a targeted approach in genetic testing, as universal screening in Chinese patients with apparently sporadic PPGL is unlikely to have added benefits, assuming an even lower prevalence of germline mutations outside our study cohort. On the other hand, the prevalence of germline mutations in our cohort could also have been underestimated, as evaluation of other known susceptibility genes of PPGL (the gene encoding SDH assembly factor 2 [*SDHAF2*] [<sup>32</sup>], the hypoxia inducible factor 2A [*HIF2A*] gene [<sup>33</sup>], the kinesin family member 1B [*KIF1B*β] gene [<sup>34</sup>] and the propyl-hydroxylase domain 2 [*PHD2*] gene [<sup>35</sup>]) were not included in our current study. However, in a recent study involving 68 subjects with apparently sporadic PPGLs, there was only 1 subject

each positive for germline mutation of *KIF1B* and *PHD2* genes respectively, suggesting the rarity of these germline mutations [36]. *HIF2A* mutations are usually found as sporadic mutations in tumour samples [33]. Furthermore, *SDHAF2* germline mutations are often associated with multiple head and neck paragangliomas<sup>3</sup> and there were only 2 subjects with head and neck paraganglioma in our cohort with one of them already found to be positive for *SDHD* germline mutation. In fact, the few head and neck paragangliomas included in our study cohort is also one of the main limitations in this study such that a more comprehensive review covering all types of PPGL in Hong Kong is not possible. Other limitations include the relatively short duration of follow-up in this study. As malignant tumours might occur many years after surgery of the primary PPGL, with a median follow-up of 5 years in this study, the prevalence of malignancy could also have been under-estimated. Furthermore, in our study, measurement of calcitonin level was not required when defining “apparently sporadic presentation” in the study subjects. Indeed, in places where genetic testing is not readily available, calcitonin measurement is useful, especially in the context of young patients presenting with bilateral pheochromocytomas. However, given that calcitonin assays are not always reliable, genetic testing should remain the gold standard in the evaluation, especially when serum calcitonin level is equivocal. Last but not least, the relatively small sample size in our study rendered more detailed analysis of genotype-phenotype correlations in these germline mutations not possible. In fact, the biochemical profile is one of the major discriminants for prioritizing the genetic testing. However, this prioritization of genetic testing was not carried out in our study and hence we were also unable to address the issue of cost effectiveness.

Nonetheless, this is the first study on genetic screening and the applicability of clinical indicators predicting germline mutations in a Chinese population with apparently sporadic PPGL, covering a significant number of causative genes in this disease. More extensive research involving a larger cohort of Chinese subjects is definitely warranted to further characterize the genetics of this disease in our population.

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**Declaration of interest**

My co-authors and I declare no conflicts of interest.

**Author contributions.**

C.H.L. researched the data and wrote the manuscript. W.S.C, Y.C.W., C.Y.Y., and B.H.H.L. researched the data. C.H.Y.F. performed statistical analyses. C.Y.Y.C., K.H.M.K., S.P.L.C. and C.M.M. performed genetic analyses. K.C.B.T. critically reviewed and edited the manuscript. K.S.L.L. initiated and supervised the study, critically reviewed and edited the manuscript, and is the guarantor of this work and as such had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis

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## 525 **Figure legends**

526 Figure 1 Receiving operator characteristics curve showing the number of clinical  
527 indicators in relation to the presence of germline mutations in subjects with apparently  
528 sporadic PPGL  
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Table 1: Genotype and phenotype of study subjects with germline mutations

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Table 2: Biochemical and tumour characteristics of study subjects

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Table 3:

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Comparison of clinical indicators by the presence of germline mutations in subjects

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with apparently sporadic phaeochromocytoma and paraganglioma

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Table 1: Genotype and phenotype of study subjects with germline mutations

Gene	Exon	Mutation	Age at diagnosis	Phenotype
SDHB	2	NM_003000.2:c.112delC*#	30	Recurrent metastatic bladder paraganglioma
SDHD	3	NM_003002.2:c.213_242dup (p.Val72_Pro81dup)*	45	Head and neck paraganglioma
RET	11	NM_020975.4:c.1902C>G# (p.Cys634Trp)	38	Bilateral pheochromocytomas
	11	NM_020975.4:c.1900T>G (p.Cys634Gly)	51	Bilateral pheochromocytomas
	11	NM_020975.4:c.1900T>C# (p.Cys634Arg)	36	Bilateral pheochromocytomas
	11	NM_020975.4:c.1901G>A (p.Cys634Tyr)	39	Bilateral pheochromocytomas
	11	NM_020975.4:c.1900T>C# (p.Cys634Arg)	35	Bilateral pheochromocytomas
	16	NM_020975.4:c.2753T>C (p.Met918Thr)	25	Bilateral pheochromocytomas
	11	NM_020975.4:c.1900T>C (p.Cys634Arg)	28	Metastatic, bilateral pheochromocytomas
NF1	-	NA	49	Right pheochromocytoma

\* Denotes novel mutations

# Denotes subjects with apparently sporadic presentation

Table 2: Biochemical and tumour characteristics of subjects with apparently sporadic pheochromocytoma and paraganglioma

Baseline parameters	Frequency	Percentage (%)
Biochemical characteristics		
Elevated 24-hour urine catecholamines or fractionated metanephrine or VMA*		
Yes	29	93.5
No	2	6.5
Tumour characteristics		
Size of primary tumour (Largest dimension in cm)		
<1cm	0	0
1-3cm	8	22.9
3-6cm	20	57.1
>6cm	7	20.0
MIBG avidity#		
MIBG-avid	25	89.3
Non MIBG-avid	3	10.7
Bilateral or multifocal tumours	10	28.6
Malignant tumours	4	11.4
Recurrent tumours	8	22.9

\*Missing data in 4 subjects; #Missing data in 7 subjects

Table 3:  
Comparison of clinical indicators by the presence of germline mutations in subjects with apparently sporadic pheochromocytoma and paraganglioma

		Subjects with apparently sporadic pheochromocytoma and paraganglioma (N = 35)		
Clinical indicators		Mutation positive	Mutation negative	p-value
N		4 (11.4%)	31 (88.6%)	-
Age at diagnosis (Year)		32 ± 6	46 ± 13	<b><u>0.023</u></b>
At or younger than 45 years old		4 (100%)	13 (42%)	<b><u>0.045</u></b>
Older than 45 years old		0 (0%)	18 (58%)	
Extra-adrenal disease		1 (25%)	7 (23%)	1.000
Bilateral or multifocal tumours		4 (100%)	6 (19%)	<b><u>0.004</u></b>
Malignant tumours		1 (25%)	3 (10%)	0.399
Recurrence		2 (50%)	6 (19%)	0.218

Statistically significance with p-value <0.05 were underlined and bolded  
Data are expressed as mean ± SD or median with inter-quartile range whichever is appropriate

673 Figure 1 Receiving operator characteristics curve showing the number of clinical  
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