Pheochromocytoma and paraganglioma: molecular testing and personalized medicine

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ABSTRACT

Purpose of review:

Pheochromocytomas and paragangliomas (PPGL) are rare tumours, strongly associated with inherited susceptibility gene mutations, and presenting limited therapeutic options for patients with metastatic disease. This review discusses the recent developments in the characterization of PPGL genetic heterogeneity and associated tumourigenesis pathways, together with their potential clinical relevance.

Recent findings:

The mutational landscape of PPGL is now well defined, especially with the contribution of next generation sequencing (NGS). Up to 70% of these tumours harbour a germline or a somatic mutation in one of the numerous predisposing gene. In parallel, "omics" analyses have identified mutation-linked subsets of tumours substantially associated with molecular signatures suggesting new therapeutic targets for patients with a malignant transformation of the disease.

Summary:

In the near future, extended molecular testing of PPGL could be used to determine therapeutic approaches and assess diagnosis and prognosis biomarkers. Considering the current development of NGS-based genetic screening, this technology appears as a good option to improve both PPGL molecular diagnosis and patient management.

KEYWORDS

Paragangliomas; genetic testing; personalized medicine; next generation sequencing

INTRODUCTION

Pheochromocytomas (PCC) and paragangliomas (PGL) are rare neuroendocrine tumours that arise from neural crest cells and can develop either in the adrenal medulla (PCC), or in paraganglionic tissues (PGL) located from the skull base to the pelvic region.

Paraganglioma and pheochromocytoma (PPGL) can be classified as syndromic, familial or sporadic. The understanding of inherited forms of PPGL has dramatically changed over the past 15 years. Since the first description of mutations in the *SDHD* gene in patients with PPGL in 2000 [1], a dozen of susceptibility genes have been identified. It is currently accepted that roughly 40% of PPGL are associated with an inherited mutation [2]. Moreover, major advances in genomic analyses have shown that up to 30% of tumours actually carry somatic mutations in these known susceptibility genes [3, 4**] (Figure 1).

In contrast, the genetic events that drive the malignant progression of the disease are yet poorly understood, and malignancy is still defined by the presence of distant metastases. The risk of malignancy is about 10 to 20% with a 5-year survival rate estimated to 50% [5]. To date, no reliable predictors for malignant potential nor molecular or histological markers for malignancy exist. Therapeutic options are limited and mainly restricted to palliative management [6*].

The large genetic heterogeneity of PPGL is directly linked to the heterogeneity in subsequently activated cancer pathways. The identification of such pathways

paves the way to molecular targeted therapies and to the implementation of diagnosis and prognosis markers of malignancy.

Herein, we review the mutational landscape of PPGL at both germline and somatic levels. A molecular oriented strategy for targeted therapy will be discussed.

MAIN SUSCEPTIBILITY GENES AND ASSOCIATED TUMOURIGENIC PATHWAYS

Traditionally, pheochromocytomas were considered to have a syndromic presentation in about 10% of cases, as part of neurofibromatosis type 1 (due to mutations in the *NF1* gene), multiple endocrine neoplasia type 2 (associated with activating *RET* mutations) or von Hippel Lindau (caused by mutations in *VHL* gene) syndromic diseases. The description of familial form of PPGL and the improvement in molecular biology methods have led to the identification of *SDHx* [1, 7-10] and, more recently, *TMEM127* [11], *MAX* [12] and *MDH2* [13**] as PPGL tumour suppressor genes (Figure 1).

During the last few years, several large-scale genomic analyses, including CGH and SNP array, mRNA and microRNA expression studies and methylation profiling, have been conducted worldwide on independent series. Concordantly, these approaches have led to the description of well-defined tumour subtypes and their corresponding tumourigenic pathways.

Gene expression profiling initially revealed that PPGL could be separated in two main clusters (C1 and C2) each subdivided into sub-clusters (C1A and 1B; C2A, 2B and 2C) by unsupervised analysis [14-16]. These different groups were

defined according to their mutational status and strongly associated with specific tumourigenic pathways. In a same way, DNA methylation and miRNA profiling revealed a major influence of the main genetic drivers on the somatic molecular phenotype [4**, 17-19**].

Cluster 1-related genes

Unsupervised analyses distinguished two groups defined as C1A and C1B sub-clusters mainly corresponding to *SDHx*- and *VHL*-related tumours, respectively.

SDHx genes comprise *SDHA*, *SDHB*, *SDHC* and *SDHD* genes encoding the four subunits of succinate dehydrogenase (SDH, mitochondrial complex II), and *SDHAF2*, which encodes an SDH assembly factor, responsible for the flavination of SDHA protein. Germline mutations in these tumour suppressor genes cause hereditary paragangliomas with familial or sporadic presentation. It is worthy of note that mutation in *SDHB* is a risk factor for malignancy and poor prognosis [20, 21]. *SDHD* and *SDHAF2* mutations are mainly found in patients with a family history of head and neck paragangliomas in the paternal branch while mutations in *SDHA* have been described in sporadic form of the disease only. *SDHA*, *SDHAF2* and *SDHC*-related PPGL remain unfrequent. Somatic mutations in *SDHx* are extremely rare, if ever [22].

Germline *VHL* mutations predispose to the von Hippel Lindau disease, a systemic cancer syndrome that gives rise to clear-cell renal cell carcinoma, PPGL and other tumours in many organs including central nervous system, eyes and pancreas. *VHL*-associated PPGL are frequently early-onset pheochromocytomas that can be bilateral and/or recurrent. Head and neck paragangliomas have been more rarely described.

Very high similarities in gene expression profiles inside each subgroup led to the discovery of germline mutations in *FH* [17] and *MDH2* [13**] genes in apparently sporadic tumours clustering with C1A and the identification of somatic mutations in *VHL* [16] and *EPAS1* [23] in sporadic C1B-tumors.

FH and *MDH2* encode the fumarate hydratase and the malate dehydrogenase, respectively, two enzymes belonging to the tricarboxylic acid cycle. Mutations in *FH* predispose to hereditary leiomyomatosis and renal cell carcinoma (HLRCC), associating cutaneous and uterine leiomyomatosis and type 2 papillary renal carcinoma. The incidence of *FH* mutation in PPGL is estimated at 1%. Interestingly, about 40% of cases carrying germline *FH* mutation presented a metastatic disease [24, 25*]. To date, only one germline *MDH2* mutation has been identified in a patient with multiple malignant PPGL. Larger international cohort studies are now required to determine the prevalence of *MDH2* mutations and to confirm a possible association with malignancy, as already demonstrated for *SDHB* and *FH*.

Gain of function mutations of *EPAS1* (encoding the hypoxia-inducible factor 2α) have been described recently in PPGL at the somatic level [26, 27*]. Additional studies revealed that these hot spot mutations could actually be mosaic or germline mutations [28]. In that case, affected patients can present associated polycythemia and somastinoma.

Germline or somatic mutations in cluster 1 genes lead to a pseudo-hypoxic signature [14-16] with overexpression of angiogenesis factors as one consequence. Thus, antiangiogenic drugs are promising candidates for targeted therapies in malignant PPGL, especially those belonging to the C1 cluster. Several phase II clinical trials evaluating tyrosine kinase inhibitors sunitinib and axitinib

are currently in progress and should determine the efficacy of such therapies [6*].

Gene expression and methylation profiling studies have allowed differentiating tumorigenesis mechanisms in C1A from C1B. Glycolysis is activated in *VHL*-related tumours while DNA and histone hypermethylation is observed in *SDHx*-, *FH*- and *MDH2*-linked tumours. For therapeutic purpose, the hypermethylator phenotype of C1A tumours suggests that DNA-demethylating drugs such as 5-aza-2'-deoxycytidine or histone methyl transferase inhibitors could be an effective approach. Moreover, temozolomide, an alkylating agent, has been shown, in a limited cohort, to be more effective in patients with *SDHB* malignant tumours compared with non-*SDHB* patients. This increased response is probably explained by the extinction of the repair enzyme MGMT which promoter is highly methylated in this subgroup of tumours [29**].

Cluster 2-related genes

Cluster 2 includes tumours carrying mutations in *NF1*, *RET*, *TMEM127* and *MAX* genes as well as a large set of sporadic tumours.

Mutations in the *NF1* tumour suppressor gene lead to neurofibromatosis type 1 (NF1), a frequent autosomal dominant syndrome (prevalence estimated to 1/3,000). PPGL occurrence is rare in NF1 and is in general restricted to unique pheochromocytoma. Mutation analysis of the large *NF1* gene is not indicated in the majority of cases as clinical diagnosis is mostly obvious. Interestingly, *NF1* is the most somatically mutated gene in sporadic pheochromocytomas with a somatic mutation rate estimated between 20 and 40% [30, 31].

Activating mutations in the *RET* proto-oncogene cause multiple endocrine neoplasia type 2 (MEN2). MEN2A, which accounts for 95% of MEN2 cases, can associate medullary thyroid carcinoma (MTC), PPGL and parathyroid adenomas while MEN2B is characterized by MTC, PPGL and clinical abnormalities such as ganglioneuromas of the lips, tongue and colon but without hyperparathyroidism. Causative mutations are hot-spot missense mutations classified into three risk of developing MTC categories according to the recently revised American Thyroid Association guidelines [32**]: highest risk (MEN2B and the *RET* p.Met918Thr mutation), high risk (MEN2A and the *RET* codon Arg634 mutations) and moderate risk (other mutations). Pheochromocytomas associated with *RET* mutations are frequently bilateral while paragangliomas are reported to be rare [33]. Somatic *RET* gain-of-function mutations have been reported in about 5% of sporadic PPGL [16].

Mutations in *TMEM127* and *MAX* genes are found in about 1-2% of cases [11, 12, 34**], primarily in PPGL with family history but also in apparently sporadic forms of the disease. Somatic mutations have been described in *MAX* [35] but not in *TMEM127*.

PPGL presenting mutations in *RET*, *NF1*, *TMEM127* and *MAX* but also sporadic tumours classifying in cluster 2A share the overexpression of the RAS/MAPK and PI3K-AKT-mTOR signalling pathways. The use of mTOR inhibitors or other drugs targeting the RAS-RAF pathway might be of interest in cluster 2 malignant PPGL but needs to be evaluated in adapted clinical trials.

CONTRIBUTION OF HIGH THROUGHPUT SEQUENCING

Multiple technological advances in molecular genetics have allowed the development of high throughput sequencing, referred to as "next generation sequencing" (NGS). Among them, whole exome sequencing (WES) revealed *MAX*, *FH* and *MDH2* as new susceptibility genes [12, 13**, 17] (Figure 1).

With the aim of identifying novel disease causing genes or new driver mutations, several WES studies have been performed on PPGL tumour tissue during the last years.

In 2013, WES conducted on 4 cases of benign apparently sporadic tumours revealed 2 tumours with hotspot mutations in *H-RAS* [36]. Targeted *H-RAS* direct sequencing in larger cohorts showed a somatic mutation rate of 5-15% in sporadic PPGL [4, 36, 37*, 38*].

Fishbein and colleagues [39**] recently published WES of 21 matched tumour/ germline DNA pairs. They confirmed the high prevalence of somatic *NF1* mutations in sporadic PPGL (3 of 7) and reported somatic *ATRX* mutations. *ATRX* encodes a SWI/SNF chromatin remodelling protein playing a role in telomere maintenance and chromosome integrity. In a validation cohort, 12.6% of tumours exhibited a somatic *ATRX* mutation, comprising several tumours with germline *SDHx* mutations. Again, this finding suggests association between epigenetic regulation and clinically aggressive features in PPGL.

WES of a set of 30 tumour-normal DNAs pairs and one trio (including the primary tumour and a metastasis) allowed identifying somatic mutations in various cancer genes including *TP53* (10%), *CDKN2A* (7%), *MET* (2.5%), *CDH1*, *MLL2*, *ATRX*, *GNAS* and *FHIT* [4**]. Recurrent mutations were also found in *CLPTML, SYNE1, CAPN2* and *RFPL4A* genes, which have not been related to

cancer yet. Additional studies are required to determine the role of these genes in PPGL tumourigenesis and the prevalence of their mutations.

Somatic mutations in *ATRX* and *TP53* genes have been confirmed in an independent WES analysis of 40 PPGL [40**]. Additional known cancer genes have been involved such as *STAG2*, *PALB2* and *STAT3* genes but still with low frequencies.

Finally, the most recent publication reporting PPGL exome sequencing identified recurrent *MLL2* (=*KMT2D*) variants in 14 of 99 explored tumours with 6 of them co-occurring with somatic or germline mutations in *NF1*, *RET* or *TMEM127* [41**]. As occurrence of *KMT2D* mutation has not been confirmed in a cohort of 13 abdominal PGL, future studies are needed to elucidate whether identified *KMT2D* missense variants are activating, deleterious or without pathogenic significance and to determine the implication of *KMT2D* in PPGL tumourigenesis [42].

All these WES studies indicate that PPGL harbour limited somatic single nucleotide variants (SNV), counting for less than 40 mutations in coding regions per tumour. They concordantly show very low mutation frequencies in multiple genes suggesting that no major underlying driver is responsible for PPGL tumourigenesis or malignant transformation in addition to mutations occurring in known PPGL susceptibility genes. However, further studies exploring non coding regions such as whole genome sequencing, could detect recurrent promoter, intronic or intergenic variants of interest. To date, only *TERT* promoter C228T mutations have been reported in metastatic *SDHx*-deficient tumours, including extra-adrenal PGLs but also adrenocortical carcinomas and gastrointestinal stromal tumours (GIST) [43*, 44].

Next generation sequencing in clinical practice

Recent published guidelines on PPGL recommend that genetic testing should be considered in all patients with PPGL, given that about 40% of all patients with PPGL have disease-causing germline mutations [34**]. A sequential strategy using a clinical feature-driven diagnostic algorithm is generally applied to prioritize the analysis, as conventional Sanger sequencing remains expensive and time consuming.

The use of NGS now allows a simultaneous screening of all PPGL genes of interest. Recent publications demonstrated that targeted NGS using gene panels but also whole exome sequencing are sensitive, time efficient and cost-effective methods, which can be used as a reliable alternative to Sanger sequencing [45-49**]. Such a strategy is now relevant in laboratory routine and should rapidly lead to a more widespread PPGL diagnostic genetic testing in all at-risk individuals.

Regarding diagnosis, using an NGS-based assay including the less commonly involved genes should improve the knowledge of mutation prevalence for all genes and associated phenotypes. As NGS test is feasible on germline DNA but also on DNA extracted from frozen or from formalin-fixed paraffin embedded (FFPE) tissues, this method will be relevant from a clinical perspective. Indeed, it will certainly be helpful to identify appropriate targeted therapy as indications are usually based on somatic DNA sequencing results. Finally, when biomarkers for response and resistance to therapy will be identified, NGS analysis would be decisive for follow-up and treatment adjustment if required. Moreover, although it is still technically challenging, detection and characterization of circulating

tumour cells and cell free circulating tumour DNA might play a role in malignant PPGL management in the next future. These precise technologies could help defining and estimating the degree of inter- and intra-tumour heterogeneity in PPGL of the same patient as recently demonstrated [4**, 40**, 50**]. At the beginning of the development of precision medicine in patients with aggressive disease, the implication of genetic PPGL heterogeneity needs to be clarified, as it would have a direct impact on the interpretation of detected biomarkers for diagnosis, prognosis and response to therapy.

CONCLUSION

During the past 15 years, mutations associated with PPGL have been described in more than 15 genes, at germline and/or somatic levels with very different mutational frequencies according to the nature of the gene. Despite this complex mutational landscape, different "omics" analyses have shown that mutations in major susceptibility genes are driver events in PPGL tumourigenesis revealing at least three groups of tumours exhibiting distinct molecular profiles. The genetic and genomic hallmarks characterizing each tumour could ultimately be used as diagnosis and prognosis biomarkers and should allow personalized treatment. In that perspective, next generation sequencing testing in germline and tumour DNA should be used in PPGL patient management as a major tool.

KEY POINTS

- PPGLs are characterized by a high rate of heritability and a strong genetic heterogeneity.
- Using genomic hallmarks, PPGLs can be classified in tumour subtypes associated with distinctive and targetable tumourigenic signatures.
- Genomics-driven therapy is a pertinent perspective for patients with malignant PPGL, comprising antiangiogenic drugs for cluster 1-tumours and mTOR or RAS-RAF pathway inhibitors for tumours belonging to cluster 2.
- Next generation sequencing recently contributed to the identification of new susceptibility genes and, in a near future, should be commonly used to detect driver mutations at germline and somatic levels and to determine biomarkers useful for patient care.

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3. Conflicts of interest: None

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FIGURE TITLE AND LEGEND

Figure 1: Identification of the major genes involved in PPGL tumourigenesis