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What have we learned from molecular biology of paragangliomas and pheochromocytomas?

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Abstract

Recent advances in molecular genetics and genomics have led to increased understanding of the aetiopathogenesis of pheochromocytomas and paragangliomas (PPGLs). Thus, pan-genomic studies now provide a comprehensive integrated genomic analysis of PPGLs into distinct molecularly defined subtypes concordant with tumour genotypes. In addition, new embryological discoveries have refined the concept of how normal paraganglia develop, potentially establishing a developmental basis for genotype-phenotype correlations for PPGLs. The challenge for modern pathology is to translate these scientific discoveries into routine practice, which will be based largely on histopathology for the foreseeable future. Here, we review recent progress concerning the cell of origin and molecular pathogenesis of PPGLs, including pathogenetic mechanisms, genetic susceptibility and molecular classification. The current roles and tools of pathologists are considered from a histopathological perspective, including differential diagnoses, genotype-phenotype correlations and the use of immunohistochemistry in identifying hereditary predisposition and validating genetic variants of unknown significance. Current and potential molecular prognosticators are also presented with the hope that predictive molecular biomarkers will be integrated into risk stratification scoring systems to assess the metastatic potential of these intriguing neoplasms and identify potential drug targets.

Keywords: molecular biology; pheochromocytoma; paraganglioma; immunohistochemistry

Introduction

Pheochromocytomas and paragangliomas (PPGLs) are neuroendocrine tumours originating from the adrenal medulla and extra-adrenal paraganglia, respectively. Pheochromocytomas (PCCs) are biochemically functional, producing epinephrine and/or norepinephrine, while sympathetic paragangliomas (PGLs) are almost always capable of catecholamine synthesis (i.e., norepinephrine and/or dopamine) but are often clinically silent. In contrast, parasympathetic PGLs are usually both biochemically and clinically silent given the usual lack of tyrosine hydroxylase, an enzyme required for catecholamine synthesis [1]. Parasympathetic PGLs are primarily located in the head and neck, while their sympathetic counterparts are located predominantly in the mediastinum, abdomen, and pelvis.

Paraganglionic tumours carry the highest degree of heritability among all human neoplasms, given that approximately 40% are associated with a germline pathogenic mutation as opposed to $\leq 10\%$ of other tumour types [2, 3]. In recent decades, there has been a considerable increase in knowledge of the genes responsible for hereditary predisposition. The expanded spectrum of genes highlights the roles of Krebs cycle in the development of many PPGLs (Table 1). Germline mutations in succinate dehydrogenase (*SDH-x*) genes (i.e. *SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2*) are causally related to pathogenesis of PPGL as well as gastrointestinal stromal tumour (GIST), renal cell carcinoma (RCC) and pituitary adenoma [4]. Relatively recently, *SDHC* epimutation and *SDHB* mosaic mutation added to the *SDH* genetic alterations associated with molecular pathogenesis of PPGL [2, 5]. Krebs cycle-related PPGLs are currently regarded as the most aggressive paraganglionic tumours, with germline *SDHB* mutations being among the strongest genetic risk factor for in development of metastatic PPGL. In addition, approximately 30% of ‘sporadic’ PPGL cases carry a somatic mutation in predisposing gene(s). Somatic mutations in other genes, i.e. *ATRX*, *TP53*, *KMT2D*, *SETD2*,

and *telomerase reverse transcriptase (TERT)* promoter, might have a synergistic impact in tumourigenesis and clinical progression of PPGL [6–12].

In this review, we present molecular genetic advances on the cell of origin of PPGLs and discuss pathogenetic mechanisms associated with germline and/or somatic mutations in these tumours. Aspects of genetic susceptibility are also discussed in the context of molecular clusters (i.e. pseudohypoxia, kinase signalling and Wnt altered subtypes) associated with tumour genotypes and pathway activation. Molecular prognosticators including somatic driver events considered to be important for defining the risk for metastasis (e.g. transcriptional telomerase activation, somatic *ATRX* mutations, *MAML3* fusions) are also presented in the context of a continuously evolving field of endocrine molecular pathology.

The roles of pathologists and tools available to them are changing in step with molecular discoveries. From a histopathological perspective, new immunohistochemical tools are potentially available for refining differential diagnosis, focusing on genotype-phenotype correlations, identifying hereditary predisposition and validating genetic variants of unknown significance (VUS). Current and potential molecular prognosticators also offer the hope that predictive molecular biomarkers will be integrated into risk stratification scoring systems to assess the metastatic potential of these intriguing neoplasms and identify potential drug targets.

Novel Concepts on Cell of Origin

Traditionally, PPGLs have been regarded as straightforwardly neural-crest derived neoplasms arising from the adrenal medulla and extra-adrenal paraganglia [13]. Novel experimental evidence has recently challenged the developmental background of the cell of origin of adrenal medulla and paraganglia, suggesting that peripheral nerves may serve as a stem cell niche for neuroendocrine system development [14]. Peripheral glial stem cells, termed nerve-associated Schwann cell precursors (SCPs), have been shown to generate chromaffin cells via an intermediate progenitor cell type characterized by a specific transcriptional program [14]. Not only do multipotent SCs propagate to chromaffin cells, but also to mesenchymal stem cells, Schwann cells, endoneurial fibroblasts, and melanocytes, which migrate along the peripheral nerves to reach specific sites within the developing embryo [15]. Utilising a combination of lineage tracing strategies with nerve- and cell type-specific ablations during embryonic mouse development, the role of SCs has been extended to organ of Zuckerkandl [16] and carotid body [17], but they seem to contribute less to the sympathetic paraganglia around the dorsal aorta [16].

Differences in origin of chromaffin cell lineage (i.e. SCP-derived adrenal medulla/organ of Zuckerkandl organ) and sympathetic cell lineage (i.e. mostly neural crest-derived sympathetic paraganglia) might indicate different cells of origin of PCC, PGL and neuroblastoma [14, 16]. This may also explain differences in biological behaviour and genetic backgrounds related to those intriguing neoplasms [15]. Nevertheless, susceptibility genes causally related both to paraganglionic and neuroblastic neoplasms (i.e. *KIF1B β* , *MAX*, *SDHA*; Table 1) [18–21], along with composite tumours that contain chromaffin cells and neurons [22], point towards a common or strongly related progenitor cell in a subset of cases [23]. Though the concept of SCP is a major modification of the traditional developmental model, the neural crest connection is not completely gone. Sympathetic neurons are derived from progenitors

that delaminate from the neural crest and migrate directly to their destinations. On the other hand, SCPs form from neural crest-derived precursors that essentially remain in place, then migrate along the axons of dorsal root ganglion neurons, join with preganglionic sympathetic axons emerging from the spinal cord and migrate along those axons to reach developing paraganglia [14].

Phenylethanolamine-N-methyltransferase (*PNMT*) gene encodes for phenylethanolamine-N-methyltransferase enzyme, catalysing the conversion of norepinephrine to epinephrine. Different chromaffin cell types (i.e. adrenergic versus noradrenergic) can be distinguished based on *PNMT* levels [24, 25]. Utilising *PNMT* mRNA expression as a marker of chromaffin cell type, the Cancer Genome Atlas (TCGA) PPGL cohort was investigated in the context of chromaffin cell biology [26]. Three PPGL subgroups subsequently emerged: (i) a *PNMT*^{high} subset almost exclusively occurring in the adrenal medulla and significantly associated with epinephrine/metanephrine production and subsequent secretion almost exclusively in the kinase-signalling mRNA subtype; (ii) a *PNMT*^{low}/*vesicle monoamine transporter* (*VMAT*)^{high} subset occurring in both adrenal and extra-adrenal sites and significantly associated with norepinephrine/normetanephrine production and subsequent secretion in the pseudohypoxia mRNA subtype; and (iii) a *PNMT*^{low}/*VMAT*^{low} subset occurring predominantly in the adrenal medulla and associated with production and secretion of a mixture of catecholamines and their metabolites which mainly but not exclusively with the Wnt-altered mRNA subtype (Table 2) [26].

Accordingly, it has been postulated that subgroups (i) and (ii) have an adrenergic chromaffin cell-like and sympathetic neuron-like origin, respectively, while subgroup (iii) originates from earlier stages during chromaffin cell development, i.e. committed sympathoadrenal progenitor-like origin [23, 26]. From a biological and therapeutic perspective,

future research is warranted to elucidate and specify tumour-initiating cells, including cancer stem cells, in the tumourigenesis of PPGL.

Molecular Genetics and Genomics in PPGL Pathogenesis

Molecular mechanisms in PPGL tumourigenesis

PPGLs as neuroendocrine tumours are characterized by strong genetic determinism with approximately 40% of patients with PPGLs harbouring germline mutation and at least 30% of the remaining ‘sporadic’ cases carrying a somatic mutation in predisposing gene(s) [27–29].

Over the last 20 years, our knowledge of hereditary predisposition of PPGL has rapidly expanded from three (i.e. *VHL*, *RET* and *NFI*) to over 20 PPGL susceptibility genes. While only 17 predisposing genes are listed in the most updated version of World Health Organisation (WHO) Classification of Tumours of Endocrine Organs [22], the catalogue of genes implicated in the molecular pathogenesis is gradually expanding (Table 2). This encompasses tumour-suppressor genes and proto-oncogenes of low mutational frequencies reported only on the germline, germline and somatic, or only at the somatic level [2].

New DNA sequencing technologies have led to the discovery of several new predisposing genes implicated in mitochondrial metabolism, DNA methylation and mitogen-activated protein kinase (MAPK) signalling pathways [28–30]. These encompass *MDH2* gene encoding for malate dehydrogenase type 2, a mitochondrial enzyme of the Krebs cycle oxidizing L-malate to oxaloacetate; *DLST* gene encoding for dihydrolipoamide S-succinyltransferase, an E2 subunit of mitochondrial 2-oxoglutarate dehydrogenase complex catalysing the conversion of alpha ketoglutarate to succinyl-CoA; *SLC25A11* gene encoding for the 2-oxoglutarate/malate carrier involved in the malate aspartate shuttle; *DNMT3A* gene encoding a DNA methyltransferase establishing DNA methylation patterns; *H3F3A* gene encoding for Histone H3.3, a protein essential in maintaining genome integrity during mammalian development; *MERTK* gene encoding for proto-oncogene tyrosine-protein kinase MER, an enzyme regulating cell survival and phagocytosis of apoptotic cells; and *MET* gene encoding for c-Met protein, which possesses tyrosine kinase activity.

In parallel, a genomic approach based on whole-genome DNA methylation assessment with subsequent exome sequencing of Krebs cycle genes has identified new candidate genes, namely *IDH3B* and *GOT2*. *IDH3B* encodes the beta subunit of NAD-specific isocitrate dehydrogenase 3 (IDH3) catalysing the oxidation of isocitrate to α -ketoglutarate, whereas *GOT2* encodes glutamic-oxaloacetic transaminase 2 (GOT2), a mitochondrial enzyme converting oxaloacetate into aspartate by transamination, with the consequent conversion of glutamate to α -ketoglutarate [31].

An additional screening strategy based on metabolome-guided genomics (*or* energy pathway metabologenomics [32]) revealed the first ever identified patient with an *Isocitrate Dehydrogenase 2 (IDH2)*-mutated PPGL accompanied by a large increase in 2-hydroxyglutarate in neoplastic tissue among others with *Isocitrate Dehydrogenase 1 (IDH1)*- and *Fumarate Hydratase (FH)*-mutated counterparts [33]. In fact, targeted metabolomics (i.e. succinate:fumarate ratio, fumarate:malate ratio and 2-hydroxyglutarate levels in tumour tissue) could be a valuable tool to identify *SDH-x*, *FH* and *IDH* pathogenic variants, respectively. Mass spectrometric analysis of biochemical imbalances by liquid Chromatography (LC)-mass spectrometry displayed a higher succinate:fumarate ratio in SDH-deficient PPGLs, GISTs and RCCs compared to their SDH-proficient counterparts [34]. Detection of succinate utilising magnetic resonance spectrometry (^1H -MRS) also appears a highly specific and sensitive hallmark of SDH-deficiency in PPGLs [35].

Even though paraganglionic tumours are characterised by a low mutational load and mutual exclusion between most pathogenic mutations, whole exome sequencing (WES) and/or targeted next generation sequencing (NGS) have revealed double germline mutations (i.e. Multilocus Inherited Neoplasia Alleles Syndrome [36]). To exemplify, *SDHC* and *PTEN* germline mutations were documented in a patient suffering from carotid PGLs and multifocal papillary thyroid carcinoma [37]. In addition, another patient with head and neck PGL

harboured *SDHB* and *CHEK2* germline mutations; the latter known to be causally related to hereditary breast and ovarian carcinoma [38]. Furthermore, a comprehensive cancer-predisposition gene testing in an adult multiple primary tumour series revealed an individual carrying *MAX* and *FH* mutations in the germline and suffering from bilateral PCCs without any reported familial history of neoplasia [39]. Simultaneous occurrence of *SDHB* and *TP53* germline mutations has been recently reported in a patient with metastatic PCC [40].

At the somatic level, *HRAS* mutations do not co-occur with other germline mutations [38, 41–44], whereas other somatic variants have been documented to co-segregate, e.g. *ATRX* with *SDH-x/FH/VHL/RET/NF1* mutations [7, 8, 38, 45, 46], *MYO5B/VCL/MYCN* with *SDHB* mutations [47], *KDM2B/TP53* with *SDHB* mutations [8, 9] and *TERT* promoter mutations with *SDH-x* mutations [10, 11]. Such co-occurrence(s) might have a synergistic effect on driver mutations and hence co-segregation of susceptibility mutations and/or overlapping structural alterations among tumours of different genetic backgrounds could play a role in tumour progression [2]. Somatic *ATRX* mutations as well as genomic alterations related to transcriptional *TERT* activation (i.e. *TERT* promoter mutations or hypermethylation and structural *TERT* rearrangement or amplification of *TERT* locus) confer information on metastatic progression and poor prognosis of patients with Krebs cycle-associated paraganglionic tumours [48]. Moreover, somatic *MYO5B* mutations leading to deregulated Rab and Rac/Rho GTPase pathways which appear to be preferentially enriched in PPGL-affected patients with metastatic disease and germline *SDHB* mutations [47, 49].

In addition to germline and/or somatic mutations, diverse mechanisms influence tumourigenesis of PPGL through a broad range of biological pathways i.e. mitochondrial metabolism, chromatin remodelling, cell cycle regulation as well as MAPK, mTOR, MYC and Wnt/b-catenin signalling pathways [28, 30]. These mechanisms encompass recurrent copy number changes (i.e. 9q, 17q, 19p13.3 and 20q gains; 1p/3q, 11p, 11q, 6q, 17p, 22 losses),

fusions, epigenetic aberrations in *SDH*- and *FH*-related tumours, aberrant hypermethylation of the promoter region of *SDHC* gene (or *SDHC* epimutations), post-zygotic mosaicism as well as transcriptional dysregulations [8, 9, 53–55, 22, 31, 41, 45, 46, 50–52].

The TCGA project [26, 46] identified novel genomic alterations, i.e. somatic *cold shock domain containing E1 (CSDE1)* mutations and somatic fusions involving *Mastermind Like Transcriptional Coactivator 3 (MAML3)*, *NF1*, *BRAF* and *NGFR* genes; confirmed biomarkers, i.e. pseudohypoxia subtype, germline *SDHB* mutations, and somatic *ATRX* mutations; and classified PPGLs into four molecularly defined groups: (i) pseudohypoxia subtype, (ii) kinase signalling subtype, (iii) Wnt-altered subtype, driven by *MAML3* fusion and *CSDE1* mutation, and (iv) cortical admixture subtype, most likely representing adrenal cortical contamination [6]. However, all paraganglionic tumours harbouring germline *MAX* mutations were detected in the latter subgroup suggesting that cortical admixture subtype might be driven by distinct biology [26]. The subtypes do not share common cellular or biological behaviour and with genome doubling occurring preferentially in pseudohypoxic *VHL*- and *EPAS1/HIF2A*-related tumours and pseudohypoxic Krebs cycle-related tumours being the most clinically aggressive PPGLs respectively [6, 26].

The TCGA study followed a comprehensive integrated genomic analysis which classified PPGLs into five molecularly defined groups with distinct driver mutations, expression signatures, copy number changes, DNA methylation alterations, and miRNA dysregulations, as follows: (i) cluster C1A including *SDH-x*- and *FH*-mutated tumours; (ii) cluster C1B comprising *VHL*-mutated tumours; (iii) cluster C2A encompassing tumours harbouring *RET*, *NF1*, *TMEM127*, *MAX*, and *HRAS* mutations; (iv) cluster C2B comprising *MET*-mutated tumours; and (v) cluster C2C with clusters C2B and C2C being enriched in sporadic tumours [8, 27]. This expanded the initial transcription profile-based classification, which was composed of a pseudohypoxic cluster 1 and a kinase receptor-signalling cluster 2

[56]. A strong concordance between gene-expression of PPGL subtypes and tumour genotype has been subsequently confirmed [57] with *EPAS1/HIF2A*-mutated tumours exhibiting a characteristic expression signature within the pseudohypoxic cluster 1 [58]. Nevertheless, a considerable proportion of tumours analysed at the transcriptomic level lacks any susceptibility mutation(s) in known PPGL genes [29].

Transcriptome-based classification of PPGLs into pseudohypoxic cluster 1, kinase signalling cluster 2, and Wnt altered cluster 3 impacts on distinct molecular pathways and down-stream targets (Table 2) [6, 14, 63, 26, 28, 29, 46, 59–62].

- Cluster 1 is characterized by activation of pseudohypoxic pathways with resultant activation of HIF- α target genes implicated in angiogenesis, cell proliferation, survival and epithelial-mesenchymal transition (Figure 1A). In the SDH-/ FH-deficient and mutant IDH state, oncometabolite accumulation lead to inhibition of the ten-eleven translocation (TET) enzymes and Jumonji domain histone demethylases causing overall DNA hypermethylation among other epigenetic modifications, which impact on several genes involved in epithelial-mesenchymal transition as well as chromaffin differentiation. Synergistic roles of TET repression and pseudohypoxia have been recently revealed in the acquisition of metastatic traits.
- Cluster 2 is associated with abnormal activation of kinase signalling pathways, i.e. the RAS/RAF/ERK, PI3Kinase/AKT/mTOR and MYC/MAX/MXD1 pathways (Figure 1B), and DNA hypomethylation linked to a higher number of somatic copy number aberrations. Haploinsufficient tumour suppressor gene *KIF1B β* acts downstream of the nerve growth factor (NGF) pathway and appears to play a significant role in intrinsic mitochondria-mediated apoptosis via the regulation of structural and functional dynamics of mitochondria. Dysregulation of *KIF1B β* / *mitochondrial metalloprotease YME1L1*/ *mitochondrial GTPase OPA1* mechanism might be involved in malignant

potential of neural crest-derived neoplasms including paraganglionic and neuroblastic tumours.

- Cluster 3 is characterized by Wnt/b-catenin pathway activation, which along with Sonic Hedgehog pathway expression is associated with chromaffin cell differentiation and DNA hypomethylation.

RNA sequencing (RNAseq) analysis data from TCGA and Therapeutically Applicable Research to Generate Effective Treatments (TARGET) cohorts including 3,319 tumour samples originating from 35 different tumour types have challenged the current clinicopathological classification as PPGLs clustered with pancreatic neuroendocrine tumours and neuroblastomas [64]. Nevertheless, in-depth characterization of long intergenic noncoding RNA (lincRNA) expression profiles has recently displayed not only a specific lincRNA profile in PPGLs from a pan-cancer perspective but also five emerging molecular subtypes corresponding to the established molecular classification of PPGL[52]. Another study identified four long noncoding RNA-based (lncRNA) subtypes which strongly correlated with mRNA expression clusters and accordingly extending the spectrum of transcriptional dysregulations in PPGLs to long non-coding RNAs [53].

Circular RNA (circRNA) expression profiling has been explored in paraganglionic tumours with findings indicating that altered circRNAs regulate histone methylation processes. circRNA-miRNA-mRNA coding-noncoding gene co-expression (CNC) networks were mapped. Accordingly, interactions of circRNAs with their target miRNAs were shown to regulate histone methylation and hence influence PPGL pathogenesis [54].

High-throughput miRNA profiling analyses have identified miRNAs that have molecular and functional roles in tumourigenesis and/or clinical progression of PPGL. An integrative study of miRNome, transcriptome and proteome identified a signature of 6 miRNAs

(i.e. miR-21-3p, miR-183-5p, miR-182-5p, miR-96-5p, miR-551b-3p, and miR-202-5p) being associated with metastatic risk and time to progression as well as highlighted combined expression of miR-21-3p/miR-183-5p with the highest predictive power for metastasis in PPGL. This expression was also associated *in vitro* with pro-metastatic features, that is neuroendocrine-mesenchymal transition phenotype and increased cell migration rate [55]. The study further supported the findings emerging from previous miRNA analyses on the role of the miR-183 cluster (miR-96/182/183) in metastatic progression of PCCs [65, 66].

Genetic susceptibility

An autosomal dominant pattern of inheritance is linked to germline mutations in almost all PPGL susceptibility genes [67]. Of note is a parent-of-origin expression phenotype documented via the paternal line in *SDHD*-, *SDHAF2*- and *MAX*-related cases [68]. Rare reports of *SDHD*-related PCC dependent on inheritance via the maternal line are also noted [69, 70]. Gain-of-function mutations in *RET*, *MET*, *MERTK*, *EPAS1/HIF2A*, *GOT2* and *DNMT3A* oncogenes do not require a second hit, while inactivation or loss of the wild-type allele is present in the setting of tumour suppressor gene(s).

Most *EPAS1/HIF2A* mutations associated with PPGL tumourigenesis are somatic or mosaic [71], while *SDHB* mutations occur almost always in the germline, with somatic [4] or mosaic counterparts [5] being rather rare genetic events. A sporadic genetic disorder potentially characterized by mosaicism is the Carney Triad, characterised by tumours affecting at least five organs, but always encompassing gastric GIST, PPGL, and pulmonary chondroma. In some patients, adrenocortical adenoma and oesophageal leiomyoma may occur [2, 72, 73]. Even though 10% of Carney Triad patients harbour germline *SDHA*, *SDHB* or *SDHC* variants [74], most cases display SDH down-regulation through *SDHC* promoter hypermethylation [51, 75, 76]. Germline and/or somatic mosaicism for site-specific aberrant *SDHC* hypermethylation has

been reported both in patients suffering from PCC and GIST [31] as well as PGL and adrenocortical adenoma [50]. Postzygotic mosaic mutation in *H3F3A* gene has recently been recognised to be associated with paraganglionic tumours and aggressive giant cell tumour of bone in a non-familial PPGL syndromic context [9]. The aforementioned PPGL related genes mutated postzygotically, but prior to terminal differentiation, and expand the mosaicism-related spectrum of endocrine tumour syndromes such as McCune-Albright syndrome and neurofibromatosis type 1 (NF1) [22]. Identifying germline or somatic mosaic disruptions has implications for risk assessment as well as genetic testing and counselling in PPGL-affected families.

The diverse genetic backgrounds correlate with differences not only in modes of inheritance and penetrance, but also in clinical presentation, tumour distribution and biological behaviour [2]. This is in accordance with the aforementioned cell of origin model based on the TCGA cohort [26]. To exemplify, extra-adrenal sympathetic PGLs are most frequently associated with *SDHD* and *SDHB* mutations, while head and neck parasympathetic PGLs usually harbour *SDHD*, *SDHB* or rarely *SDHC* mutations. On the other hand, PCCs are more often associated with *RET*, *NF1*, *TMEM127*, *MAX*, and *VHL* mutations [77]. Within the pseudohypoxic cluster 1, recent evidence also supports differences between cases of *EPAS1/HIF2A* mosaicism and somatic *EPAS1/HIF2A* mutation(s) from a clinical standpoint. Patients with somatic *EPAS1/HIF2A* mutations tend to be associated with late onset of the disease, milder clinical phenotype, and an improved prognosis compared with patients who have *EPAS1/HIF2A* mosaicism associated often with metastatic disease, multiplicity and recurrence [78].

Genotype-phenotype correlations in PPGL

As mentioned, 40% of patients carry a germline mutation in >20 susceptibility genes and at least 30% of sporadic cases are related to somatic mutations [27–29]. Familial PPGLs differ from their sporadic counterparts in that they are often multifocal or bilateral and are characterised by clinical presentations at young age. Since the late 90's, several hereditary PPGL syndromes have been described (Table 1). Eighty percent of the inherited PPGLs are caused by *VHL* and *SDH-x* germline mutations [79].

Multiple Endocrine Neoplasia Type 2

Multiple Endocrine Neoplasia type 2 (MEN2) is an autosomal dominant syndrome with an incidence of 1 in 30,000 [80]. MEN2 syndrome is causally related to activating germline mutations of the *RET* proto-oncogene. *RET* gene encodes for a tyrosine kinase receptor and is located on chromosome 10q11.21 [81, 82]. MEN2 syndrome is subdivided into MEN2A and MEN2B. Over 90% of the patients with MEN2A had a classic tumour triad: medullary thyroid carcinoma, PCC and parathyroid lesion giving rise hyperparathyroidism [83]. MEN2B is characterised by medullary thyroid carcinoma and PCC as well as mucocutaneous neuromas, gastrointestinal ganglioneuromatosis, cutaneous lichen amyloidosis and a marfanoid habitus. PCC are diagnosed in approximately 50% of MEN2 patients and often develop between the ages of 30-40 [84]. Fifty percent of the patients develop bilateral PCCs with low metastatic potential [84, 85].

von Hippel-Lindau disease

von Hippel-Lindau disease (VHL) is another autosomal dominant syndrome with an estimated incidence of 1 in 36,000 [86]. The VHL disease is causally related to germline mutations in the *VHL* tumour suppressor gene located on chromosome 3p25-26. The VHL

disease has a very high penetrance with more than 90% of the patients developing one or more of the clinical sequelae at age 65 [87–89]. VHL is characterized by a diverse tumour spectrum of clear cell RCC and renal cysts, retinal and central nervous system hemangioblastomas, tumours of the endolymphatic sac, papillary cystadenoma of the epididymis, broad ligament and mesosalpinx, pancreatic neuroendocrine tumours and pancreatic cysts, and PPGL [90]. Approximately 20% of the patients carrying a *VHL* mutation develop PCC, sometimes with bilateral PCC, and with a mean age at presentation of 30-year-old [91].

Neurofibromatosis type 1

Neurofibromatosis type 1 (NF1) is caused by mutations in the *NF1* tumour suppressor gene on chromosome 17q11.2, which encodes for neurofibromin [92]. NF1 is an autosomal dominant condition with high penetrance and prevalence estimated at 1 in 3000 [93]. Patients with NF1 develop neurofibromas in more than 90% of the cases. PCC is an uncommon manifestation with an incidence of 5% [90]. Although rare and sometimes bilateral, the risk of metastatic disease is relatively high (up to 12%) in patients with NF1 [94, 95].

Familial PPGL syndromes

Familial PPGL syndromes are relatively new syndromes compared to MEN2, VHL and NF1 syndromes. They comprise a group of hereditary genetic disorders caused by germline mutations in *SDH-x* tumour suppressor genes. All *SDH-x*-related syndromes are autosomal dominant and have varying penetrance rates. The genotype-phenotype correlations differ between *SDH-x* pathogenic variants. *SDHD* gene was the first to be identified in a PPGL-affected family [96]. Phenotype of these patients differs according to transmitting parent. In case of paternal transmission, patients will develop head and neck PGLs in more than 97% of the cases [97]. Until recently, it was considered that maternal transmission was not responsible

for the development of PPGL and new insights show that these patients may have a lifetime risk of 5% of developing a PPGL [98]. The median age at first tumour presentation of patients carrying *SDHD* mutations is 33-year-old. These patients have low risk of metastatic disease [99]. *SDHB* mutations are found in up to 10% of the patients with PPGL and cause the development of sympathetic PGL in 60% of the cases, and head and neck PGL in 40% respectively [28, 83]. Median age for developing PPGL in patients with *SDHB* mutation is like patients with *SDHD* mutation. However, patients with *SDHB* mutated tumour have a worse prognosis and with a metastatic risk of approximately 30% [100]. *SDHA*, *SDHC*, and *SDHAF2* mutations are less common in patients with PPGL (Table 1) [77, 101, 102]. *SDHC* mutations often cause single head and neck PGL with a very low risk of metastasis [99]. Patients who develop PPGL due to *SDHA* pathogenic variants have a relatively high proportion of metastatic disease estimated approximately at 20% [77, 103]. In addition, patients with *SDHA*, *SDHB*-, *SDHC*- or *SDHD*-mutated tumours have increased risk of developing GIST, SDH-deficient RCC, and pituitary adenoma [4]. On the other hand, germline *SDHAF2* mutations are only associated with head and neck PGLs without any documented non-paraganglionic tumours noted in other sites (Table 1) [104].

As discussed above, a pattern of co-existing tumours of GIST, extra-adrenal PGL and pulmonary chondroma in individuals first described by Carney *et al.* and later referred to as the Carney triad. Carney triad is a non-hereditary genetic disorder potentially associated with currently unknown somatic mosaicism and/or SDH genetic defects including aberrant DNA hypermethylation of *SDHC* or, rarely, *SDH-x* pathogenic variants [2, 72, 73, 105]. In 2002, Carney and Stratakis identified a new syndrome within the group of patients with the Carney triad, who suffered from a combination of GIST and PGL [106]. This familial condition was later referred to as Carney-Stratakis dyad and is caused by germline *SDH-x* mutations [107].

Hereditary leiomyomatosis and renal cell carcinoma syndrome

Hereditary leiomyomatosis and renal cell carcinoma (HLRCC) or *FH* tumour predisposition syndrome is caused by germline *FH* mutations. As the name states, patients with HLRCC syndrome develop cutaneous and/or uterine leiomyomatosis as well as *FH*-deficient RCC. More recently, germline *FH* mutations have been identified in patients with PPGL and subsequently these tumours expanded the HLRCC-related tumour spectrum [108, 109]. *FH*-associated PPGLs are often multiple and of high metastatic potential [108].

Emerging roles of molecular genetics and molecular pathology

Over the past decade, conventional single gene sequencing has been replaced by NGS, using systems in which many genes can be analysed simultaneously. This progress is highly relevant for PPGL, in which several susceptibility genes are related to aetiopathogenesis. The American Endocrine Society and the European Society of Endocrinology both agree that patients with PPGL should be referred for genetic screening [110, 111]. In addition, the NGS in PPGL Study Group published a consensus statement in which specific recommendations are proposed for the use of NGS in hereditary PPGL [112]. The main goal of screening is to identify patients who carry a disease-causing mutation and to test tumour tissue for the applicability of targeted therapies. NGS technologies are capable of sequencing large numbers of different DNA sequences in a single/parallel reaction [113, 114]. An often-used method for genetic screening is targeted NGS, which only targets the coding regions of genes of a specific disease using a ‘panel’ [115]. Now, three types of targeted NGS panels have been recommended in the diagnosis of PPGL. The first panel, described by Pillai *et al.*, includes genes mutated at germline level and associated with hereditary disease (*VHL*, *SDH-x*, *FH*, *MAX*, *NF1*, *RET*, and *TMEM127*) [114]. The second is a more extended panel encompassing genes functionally relevant in PPGL (*EGLN1*, *EPAS1*, *SDHAF2*, *KIF1B*, and *MET*). The third is the most

comprehensive panel, as described by Toledo *et al.*, which includes recently identified genes at germline and/or somatic level [112]. Targeted NGS panels have made genetic screening in PPGL in individual laboratories more affordable, easy and straightforward [112]. However, this has limitations such as difficulty in adding new genes to existing panels, as well as instrument-based errors [116]. Even though targeted NGS has been established in highly specialised centres, it is not yet widely available in developing countries or less advanced clinical centres.

Whole Genome Sequencing (WGS) is a method for sequencing the whole human genome whereas whole exome sequencing (WES) is a method for sequencing only the coding regions of DNA. They are comprehensive techniques for genetic screening. WGS has the sensitivity to detect driver mutations even when they are present at very low frequency (1%) in tumour cells [117]. However, WGS creates large amounts of data which need extensive filtering in a rather costly and complex bioinformatics analysis. WES, on the other hand, detects only sequences in the desired coding regions of DNA: the exome. It is expected that 85% of the disease-causing mutations occur within the exome, which only comprises 1% of the whole-genome [118]. WES is used to analyse all potential disease-causing genes including known susceptibility genes as well as genes unrelated to a disease. Thereby, WES can be used to identify driver mutation in PPGL with a previous negative genetic testing [9, 112]. WES has contributed to the identification of several PPGL susceptibility genes, such as *MAX*, *FH*, *MDH2*, *HRAS*, *ATRX*, and *KMT2D*. A limitation of WES is that promoters, enhancers, transcription factor binding sites and deep intronic sequences are not included in the analysis and hence relevant pathogenic variants would have been missed [119, 120]. This highlights the need for WGS implementation into clinical practice.

Novel NGS techniques are RNAseq and DNA methylation profiling. RNAseq, as the term states, uses RNA instead of DNA for the analysis, which also yields expression profile

and mutational status [114]. RNAseq can be utilised in the detection of alternative gene-spliced transcripts, posttranscriptional modifications, gene fusions, mutations/single-nucleotide polymorphisms, and alterations in gene expression. This might provide new insights in the molecular basis of PPGL, as others already have shown [45, 121]. A downside of this technique is that analysis is more complex than targeted NGS or WES and, therefore, the implementation of RNAseq in molecular diagnostics of PPGLs is still limited [112]. Fishbein *et al.* analysed tumour samples using RNAseq and detected in-frame RNA fusion transcripts spanning the 5' portion of UBTF (upstream binding transcription factor) on 17q21.31 and the 3' portion of *MAML3* on 4q31.1 [46]. These fusions are implicated in the pathogenesis of PPGL and appear to constitute a marker of aggressive disease.

DNA methylation profiling focuses on DNA methylation aberrations often present in neoplastic cells. These can display DNA hypomethylation and hypermethylation of promotor CpG islands with resultant transcriptional silencing of tumour suppressor genes [122]. A major advantage of DNA methylation profiling is that such DNA methylation alterations are reversible [123]. This reversibility allows for therapeutic options such as targeted drug therapies. So far, Letouze *et al.* and de Cubas *et al.* performed genome-wide DNA methylation profiling using NGS techniques in PPGLs [59, 124]. The study by Letouze and colleagues showed that the origin of metastasis in *SDHB*-mutated PPGL was due to silencing of *PNMT* and *KRT19* genes, which are involved in cell differentiation and epithelial-mesenchymal transition. De Cubas and co-workers found 48 novel molecular predictive markers based on DNA methylation profiling. *SDHB*-mutated tumours showed the highest levels of DNA methylation along with *PNMT* hypermethylation, whereas *VHL*- and *EPAS1*-mutated counterparts show intermediate levels of DNA methylation.

Molecular Predictive and Prognostic Biomarkers

While much attention has been directed at the identification of genes that are causally related to the occurrence of PPGL, there is still a lack of predictive or prognostic biomarkers, whether tissue- or serology-based [6]. In the updated WHO classification, PPGLs are now referred to as ‘metastatic’ or ‘non-metastatic’ instead of ‘malignant’ or ‘benign’ respectively, and all paraganglionic tumours are considered to have metastatic potential [125].

In a multi-disciplinary context, endocrine-related specialists need to take into consideration all the clinical, biochemical, pathological, and genetic information to make a metastatic risk prediction on an individualised basis. Ideally, the genetic profile of PPGL could be used as prognostic marker for metastatic disease with Krebs cycle gene dysregulation being highly relevant in this context. In past decade(s), many mutations have been associated with metastatic PPGL, such as *SDHB*, *SDHA*, *SDHD*, *FH*, *MDH2*, *SLC25A11*, *MAX*, and *MAML3* [46, 108, 126–131]. Of these, *SDHB* is the strongest genetic risk factor for metastatic PPGL, accounting for 40-50% of the cases [128, 132]. Nevertheless, PPGL-affected patients harbouring *SDH-x*-mutations develop a metastatic phenotype only in 50% of the cases, suggesting that additional molecular mechanism(s) might play a role in metastatic progression as previously detailed in the session on *molecular mechanisms in PPGL tumourigenesis* [128].

As a recent example, Wilzén *et al.* identified recurrent mutations in *MYCN*, *MYO5B* and *VCL* genes, which co-occurred with *SDHB* mutations in metastatic paraganglionic tumours [47]. Telomerase activation and *ATRX* mutations were also associated with metastatic PPGL. Fishbein and colleagues reported an association between *ATRX* mutations and alternate lengthening of telomeres in metastatic tumours [7]. Liu *et al.* and Dwight *et al.* showed that high *TERT* expression, which is associated with telomerase re-expression, is detected in metastatic PPGLs [10, 133]. In this context, Job and co-workers displayed that transcriptional *TERT* activation and *ATRX* mutations appear to be more accurate than *SDHB* mutations in

discriminating metastatic PPGLs from non-metastatic counterparts, particularly in the pseudo-hypoxic cluster [48]. *MAML3* fusion can also be utilised as predictive marker for aggressive disease with a tendency of high mutational rate in older patients [3, 46, 129].

Several studies estimated the median overall survival (OS) of patients with metastatic PPGL at 5.3 to 24.6 years (5-year OS = 62-85% and 10-years OS = 71-73%), with significantly longer survival in case of metachronous metastases compared to synchronous ones [134–138]. Favourable prognosis has been associated with surgical resectability, female gender, younger age at diagnosis, small tumour size, head and neck PGL, low proliferative index, and less than five-fold increase in catecholamine levels.

From a genetic perspective, germline *SDHB* mutations, somatic *NF1* and *MAML3* fusions, somatic *SETD2* or *ATRX* mutations and high somatic mutation rate have been associated with unfavourable prognosis [46, 139, 140]; although the precise contributions to clinical outcome are difficult to parse. A recent meta-analysis of twenty-one studies and 703 patients with PPGL showed independent association of *SDHB* mutation with metastasis. The median survival time for the cohort of PPGL was 240 months. In univariate analysis, survival of the patients with PPGL was correlated with both *SDHB* mutation and metastatic stage. However, in multivariate analysis, only the presence of metastases independently correlated with patient prognosis [141]. Poor clinical outcome has also been associated with global hypermethylation, high Ki-67 labelling index [46] and aberrant *TERT* expression [48].

Several new types of markers have recently emerged. lncRNA evaluation analysis revealed one putative lncRNA with major reduced expression in metastatic tumours, which accurately discriminated metastatic from non-metastatic *SDH-x*-related tumours, while being an independent risk factor associated with poor clinical outcome in case of an *SDH-x* mutation [53, 142]. Another analysis on miRNA in PPGL identified a six-miRNA signature that correlated with metastatic risk and time to progression, independent of *SDHB* mutation status

[55]. Hypermethylation of the *RDBP* (negative elongation factor complex member E) promoter also appeared to be a promising biomarker of metastatic PPGL as this correlated with time to progression [124, 143].

Currently, cell free (cf) DNA-based methods are increasingly employed as diagnostic or screening tools for cancer detection [144]. In the context of PPGL, with extensive candidate genetic markers, cfDNA detection maybe relevant once a causative germline or somatic mutation has been identified in the primary tumour, especially in patients with increased risk of metastatic disease. However, no such studies have been performed in this patient group.

Roles of Pathology in the new era of genomic advances

ICCR guideline and risk stratification for PPGL

Many therapeutic management decisions are based on an accurate pathology report, which is best done via standardized structured reporting and, therefore, can be applied and interpreted objectively in all patient settings [145]. The International Collaboration on Cancer Reporting (ICCR) was founded to aid and improve international benchmarking in cancer management. An ICCR Pheochromocytoma and Paraganglioma Histopathology Reporting Guide specific for resection specimens and biopsies has recently been published (<http://www.iccr-cancer.org/datasets/published-datasets/endocrine/phaeochromocytoma>) in 2019 and further elaborated in subsequent papers [146, 147]. In the development of the ICCR guideline, the expert panel made a distinction between “core elements” and “non-core elements”. Core elements are regarded as minimum reporting requirements and are defined as essential for clinical management, staging or prognosis. Non-core elements are not considered mandatory for reporting but may be of clinical importance and recommended as good clinical practice, though not validated. The ICCR guideline consists of 16 core elements and 5 non-core elements, which are all clinical or histopathological parameters [147]. One non-core element, designated ‘adverse features’, takes into account risk stratification scoring systems such as the Pheochromocytoma of the Adrenal gland Scoring Scale (PASS) [148] and the Grading system for Adrenal Pheochromocytoma and Paraganglioma (GAPP) [149]. The former is specifically developed for PCC, incorporating 12 histological parameters, whereas the latter, which is more recent, is applicable for both PCC and sympathetic PGL, incorporates 4 histological parameters, immunohistochemical assessment (Ki-67 labelling index) and biochemical data (i.e. catecholamine type) and was generated for predicting metastases and grading of malignancy. A comparison study of the 2 scoring systems using 106 PCC and 37 PGL showed that higher GAPP scores were associated with aggressive PPGL [150]. On the

other hand, PASS score was not associated with metastatic disease and demonstrated significant interobserver variability.

Although these risk stratification scoring systems are not universally adopted and not endorsed in the latest edition of World Health Organisation's volume on Endocrine Tumours, both models are potentially helpful, especially in suggesting that any particular tumour has low metastatic potential [22, 151]. However, it is generally agreed that histological parameters are best considered in conjunction with biochemical and molecular markers. Examples of molecular markers, which have varying levels of evidence in predicting metastatic disease, include germline *SDHB* mutations, somatic *ATRX* and/or *SETD2* mutations, *TERT* gene aberrancies and *MAML3* fusions [151]. Pseudohypoxia Krebs cycle-related and/or Wnt-altered molecular clusters, hypermethylation phenotype and high somatic mutational burden could also confer predictive and prognostic information [46, 151]. These markers, except for *SDHB*, are not yet used widely in routine clinical diagnostic setting and should be further clinically validated in prospective studies before they could be applied in risk stratification model(s) predicting metastatic potential and/or clinically aggressive disease.

The current edition (8th Edition) of the American Joint Committee on Cancer (AJCC) introduced a TNM staging system for neuroendocrine tumours of the adrenal gland which could be used for staging of PCC and sympathetic PGL [152]. The T staging depends on the size of the tumour, location of PPGL and invasion (T1: PCC less than 50mm and with no extra-adrenal invasion; T2: PCC of 50 mm or more with no extra-adrenal invasion as well as sympathetic PGL of any size; T3: tumours of any size with invasion of surrounding tissue). Apart from the standard TNM parameters, the AJCC manual recommends the collection of data such as catecholamine secretion, chromogranin A level, mitotic count and germline mutational status (*SDHB* mutations). Thus, some of the new pathological and molecular parameters are important in the clinical management of patients with PPGL.

Current and future roles of immunohistochemistry

The roles of immunohistochemistry (IHC) in the pathology work-up of paraganglionic tumours are evolving [153]. Currently, these apply mostly to differential diagnosis and initial diagnostic workup, which includes Ki-67 quantitation according to the ICCR guidelines. However, genetic and predictive biomarkers are increasingly of interest.

Differential diagnosis has generally relied on demonstration of neuroendocrine markers, of which tyrosine hydroxylase is arguably the most specific, and exclusion of epithelial markers. However, keratins can be expressed focally in head and neck PGLs, which are often negative for tyrosine hydroxylase and can also be negative for generic neuroendocrine markers including chromogranin A and synaptophysin. GATA3 (GATA binding protein 3), which is a transcription factor contributing to lineage determination for both neurons and chromaffin cells, can be useful in some cases but is also expressed in parathyroid, breast, and urothelial tumours [154]. Immunoreactivity for dopamine beta-hydroxylase has been reported in a high percentage of tumours that are tyrosine hydroxylase negative [155], but this intriguing observation has not been universally observed and requires further investigation [1].

All around the world, the availability of genetic testing to identify mutations in PPGL is increasing. However, testing is currently time-consuming and expensive. The use of IHC may help in identifying underlying mutations in a cost-effective and as feasible screening approach [156]. IHC is utilised as screening tool to serve as surrogate marker for the presence of germline mutations. Dahia *et al.* were the first to demonstrate that SDHB protein expression is lost in hereditary PGL associated with *SDHB* or *SDHD* mutations [157]. Thereafter, multiple studies showed that this effect occurs in any of the hereditary tumours caused by germline *SDH-x* mutations [158–160]. As the SDH subunits are linked to form the functional SDH enzyme, different *SDH-x* mutations may cause destabilization of the complex and degradation of SDHB.

Tumours with *SDHB*, *SDHC*, *SDHD*, or *SDHAF2* mutations show negative stain to SDHB protein and retain immunoreactivity for SDHA (Figure 2). On the other hand, tumours harbouring *SDHA* mutations lose immunoreactivity for both SDHA and SDHB. Thus, SDHA could be added into the immunohistochemical panels for detection of SDH-x mutations. SDHD IHC is generally positive in *SDH-x* mutated tumours and may help in the interpretation of inconclusive SDHB immunoreactivity patterns in order to predict or validate *SDH-x* genetic variants [161].

Another example of use of IHC is in HLRCC syndrome in which a germline *FH* mutation leads to loss of FH protein expression and S-(2-succinyl) cysteine (2SC) overexpression [162]. In addition, the use of Carbonic Anhydrase IX (CAIX) immunostaining has added to the value of IHC in patients harbouring *VHL* mutations. Favier *et al.* reported positive CAIX staining in 88% of tumours with *VHL* mutations and negative CAIX staining in 91% of tumours without *VHL* mutation [163]. In studies of tumours with other mutations, Korpershoek and colleagues showed loss of MAX protein expression (by IHC) in *MAX* mutated tumours [164] (Figure 3). It is worth noting that Cheung and co-workers reported ambiguous results with regard to MAX immunoreactivity patterns in PPGLs [165].

NF1 protein immunoreactivity has also been studied in relation to *NF1* mutation and was absent in the majority of the PCCs, providing a sensitivity of 66% in the detection of *NF1* mutations [166]. However, negative NF1 immunoreactivity was also seen in 63% of the NF1 wild-type PCCs and, consequently, NF1 IHC had low specificity, making it an inefficient screening tool for *NF1*-mutated tumours. RET IHC does not seem to be of great additional value in routine diagnostic practice, as it was shown to be variably expressed in a minority of PCC tumour cells with no preference for PCC with a specific genetic background [167]. Along these lines, loss of BAP1 protein expression was demonstrated to be unrelated to genetic mutations in PPGLs [168].

Thus, patients with PPGL can be indirectly screened for mutations utilizing IHC complementary to the use of genetic testing. In this setting, immunohistochemical markers need to be validated in large series to become valuable in indirect genetic screening and histopathologists should be aware of limitations and pitfalls in their applicability [153]. Although NGS is the standard approach for genetic screening, IHC likely plays a vital role in validating genetic variants of unknown significance (VUS) emerging from NGS analysis. VUS is common finding in NGS and immunohistochemistry can confirm those variants with functional changes in protein expression. Papathomas *et al.* emphasized this need for assessing pathogenicity of VUS and further strengthened the role of SDHB/SDHA IHC in determining the functionality of VUS [159]. In addition, Wallace and colleagues showed that the combination of metabolite profiling with SDHB IHC has complementary utility in this context [169]. IHC might be indicative of whether a VUS is of any biological significance but does not assess functionality at the enzymatic level. Hence, formal evidence should be provided via functional assays for definitive categorisation.

IHC has both current and potential new roles to play both in assessing prognosis and guiding patient management. Currently used markers are the proliferative marker, Ki-67 and SDHB. SDHB is useful for both screening for mutation and risk stratification. Loss of immunoreactivity for ATRX has been reported in some PGLs and may be an immediate marker for further study [7]. Furthermore, chromogranin B has recently been shown to have a strong correlation to both the PASS score and metastasis/local recurrence. Low mRNA expression levels of chromogranin B, as well as low plasma levels and absent or weak chromogranin B immunostaining were all associated with metastasis/local recurrence [170]. IHC for somatostatin receptor 2A, which is expressed by most PGLs but at varying levels, can be useful in deciding whether ^{68}Ga PET/CT will be useful in screening for tumor metastases [171]. An important consideration regarding IHC is that most of the molecular characterization of PPGL

clusters has been done at the level of the transcriptome. RNA and protein levels often do not match [172], and proteins are the ultimate determinant of function. Pathologists working with IHC may therefore in many cases continue to have “the final word”.

Conclusions

The past decade has seen a rapid increase in our knowledge of the molecular pathogenesis of PPGL, spurred by the advent of whole genome-based techniques and international collaborations such as The Cancer Genome Atlas project and the International Collaboration on Cancer Reporting [173]. The number of candidate genes with germline or somatic mutations has now grown to over 20, allowing detection of germline mutations in almost 40% of patients with PPGL and somatic mutations in another 40%. Thus, almost all PPGL have a known molecular basis for PPGL. In addition, with the ability to cluster these genes in subgroups, much has been learned about molecular pathogenesis of the tumours. New embryological techniques have added greatly to understanding the complex development of the human adrenal medulla and extra-adrenal paraganglia. These details may provide mechanistic insights into how the mutated genes lead to tumour development.

Now the challenge is to translate these findings into clinical practice and combine traditional pathology-based classification systems with clinical, immunohistochemical, and molecular findings to allow prognostic classification and prediction-based treatment. Overall, in upcoming years, tumour biomarkers may play a central role in PPGL. The use of molecular prognosticators will help in the identification, prognosis, and treatment of patients with metastatic PPGL.

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Authors' contributions:

T. G. Papathomas, D. P.D. Suurd, A.K. Lam and R. R. de Krijger drafted the design of the work;

T. G. Papathomas and D. P.D. Suurd performed the literature search and drafted the initial text;

K. Pacak and M. R. Vriens criticised the manuscript;

A. K. Lam and R. R. de Krijger and A.S. Tischler gave input on the concepts and finalised the manuscript;

All authors contributed to the final manuscript.

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Table 1. Genotype-phenotype correlations of pheochromocytoma (PCC) and paraganglioma (PGL) associated with germline mutations and/or mosaicism

Gene	Molecular Cluster	Mutation Rate	Tumour Type	Syndromes, Tumours & Related Features
<i>VHL</i>	Pseudohypoxia non-Krebs cycle related	5-10%	PCC>>PGL	von Hippel-Lindau disease
<i>EPAS1/ HIF2a</i>	Pseudohypoxia non-Krebs cycle related	1%	PGL>PCC	PPGL-somatostatinoma- polycythaemia syndrome (or Pacak–Zhuang syndrome) Haemangioblastoma, Ocular Manifestations i.e. dilated capillaries, retinal neovascularization and fibrosis overlying the optic disc
<i>PHD1/ EGLN2</i>	Pseudohypoxia non-Krebs cycle related	<1%	PCC/PGL	Polycythaemia with borderline or mildly elevated erythropoietin levels
<i>PHD2/ EGLN1</i>	Pseudohypoxia non-Krebs cycle related	<1%	PGL/PCC	Polycythaemia with borderline or mildly elevated erythropoietin levels
<i>SDHA</i>	Pseudohypoxia Krebs cycle related	<3%	PGL>>PCC	Familial PPGL <i>SDHA</i> -related Gastrointestinal Stromal Tumour, Pituitary Adenoma, SDH-deficient Renal Cell Carcinoma, Neuroblastoma?
<i>SDHB</i>	Pseudohypoxia Krebs cycle related	5-10%	PGL>PCC	Familial PPGL <i>SDHB</i> -related Gastrointestinal Stromal Tumour, SDH-deficient Renal Cell Carcinoma, Pituitary Adenoma
<i>SDHC</i>	Pseudohypoxia Krebs cycle related	<5%	PGL>PCC	Familial PPGL <i>SDHC</i> -related Gastrointestinal Stromal Tumour, SDH-deficient Renal Cell Carcinoma, Pituitary Adenoma
<i>SDHD</i>	Pseudohypoxia Krebs cycle related	5-10%	PGL>PCC	Familial PPGL <i>SDHD</i> -related Gastrointestinal Stromal Tumour, SDH-deficient Renal Cell Carcinoma, Pituitary Adenoma, Pancreatic Neuroendocrine Tumour?
<i>SDHAF2</i>	Pseudohypoxia Krebs cycle related	<1%	PGL>PCC	Familial PPGL <i>SDHAF2</i> -related
<i>FH</i>	Pseudohypoxia Krebs cycle related	<5%	PCC/PGL	Hereditary Leiomyomatosis and Renal Cell Carcinoma (or <i>FH</i> tumour predisposition syndrome)

<i>MDH2</i>	Pseudohypoxia Krebs cycle related	<1%	PGL	
<i>SLC25A11</i>	Pseudohypoxia Krebs cycle related	<1%	PGL	
<i>GOT2</i>	Pseudohypoxia Krebs cycle related	<1%	PGL	
<i>IDH3B</i>	Pseudohypoxia Krebs cycle related	<1%	PGL	Acute Myeloid Leukaemia
<i>DNMT3A</i>	Pseudohypoxia Krebs cycle related	<1%	PGL	Acute Myeloid Leukaemia
<i>DLST</i>	Pseudohypoxia Krebs cycle related	<1%	PCC/PGL	
<i>RET</i>	Kinase Signalling	5%	PCC>>PGL	Multiple Endocrine Neoplasia type 2 (MEN2) syndrome
<i>MET</i>	Kinase Signalling	<1%	PCC>PGL	Hereditary Papillary Renal Carcinoma
<i>MERTK</i>	Kinase Signalling	<1%	PCC/PGL	Medullary Thyroid Carcinoma
<i>NF1</i>	Kinase Signalling	<5%	PCC>>PGL	Neurofibromatosis 1 (NF1) syndrome
<i>TMEM127</i>	Kinase Signalling	<5%	PCC>PGL	Familial PPGL <i>TMEM127</i> -related Clear Cell Renal Cell Carcinoma
<i>MAX</i>	Kinase Signalling	<5%	PCC>PGL	Familial PPGL <i>MAX</i> -related Renal Oncocytoma, Renal Cell Carcinoma, Pituitary Adenoma? Pancreatic Neuroendocrine Tumour? Neuroblastic Tumour(s)?
<i>KIF1Bβ</i>	Kinase Signalling	<1%	PCC	Neuroblastoma
<i>H3F3A</i>	Kinase Signalling	<1%	PCC/PGL	Giant Cell Tumour of the Bone
<i>BAP1</i>	Kinase Signalling	<1%	PGL	<i>BAP1</i> tumour predisposition syndrome
<i>MEN1</i>	Kinase Signalling	<1%	PCC>PGL	Multiple Endocrine Neoplasia type 1 (MEN1) syndrome

Table 2. Molecular clusters and associated genetic features in pheochromocytoma (PCC) and paraganglioma (PGL)

Molecular Custer	Phenylethanolamine-N-methyltransferase (PNMT) / Vesicle monoamine transporter (VMAT) mRNA expression	Gene	Pathway Activation	Risk of Metastatic Disease
Pseudohypoxia Krebs cycle-related	Low / High	<i>SDHA, SDHB, SDHC*, SDHD, SDHAF2, FH, MDH2, IDH1**, IDH2**, IDH3B, GOT2, SLC25A11, DNMT3A, DLST</i>	Hypoxia signalling pathway and epigenetic modifications impacting on genes involved in epithelial-mesenchymal transition and chromaffin cell differentiation and the presence of oncometabolites	High
Pseudohypoxia non Krebs cycle-related	Low / High	<i>VHL, EPAS1/HIF2a***, PHD1/EGLN2, PHD2/EGLN1, IRP1**</i>	Hypoxia signalling pathway and its target genes including erythropoietin (EPO) and its receptor (EPOR) and genes related to tumorigenesis	Intermediate
Kinase signalling	High/?	<i>RET, MET, MERTK, NF1****, BRAF*****, NGFR*****, TMEM127, MAX, HRAS**, FGFR1**, KIF1Bβ, H3F3A*****, BAP1, MEN1</i>	RAS/RAF/ERK, PI3Kinase/AKT/mTOR and MYC/MAX/MXD1 signalling pathways	Low
Wnt altered	Low / Low	<i>MAML3*****, CSDE1**</i>	Wnt/b-catenin and Sonic Hedgehog signalling pathways	Intermediate

* epimutation is also documented in addition to germline and less frequently somatic mutation

** documented only at the somatic level

*** documented at the somatic, postzygotic mosaic and germline level

**** germline and somatic mutations/fusions

***** fusions documented at the somatic level

***** documented only at the postzygotic mosaic level

Figure Legends

Fig. 1 Genetic mechanisms in PPGLs in clusters 1 and 2: Panel (A) cluster 1 genes are involved with pseudo-hypoxic pathways including *VHL*, *PHD2*, *HIF2A*, *SDH-x*, *FH* and *IDH*. Inactivation of *SDH*, *FH* and *IDH* are considered to cause a pseudo-hypoxic response due to accumulation of oncometabolites which in turn leads to the activation of HIF-1 α target genes such as *EPO*, *VEGF*, *GLUT1* and *P21ras* implicated in the development of PPGLs. *VHL* and *PHD2* mutations result in the absence of functional VHL protein and this further activates HIF target genes; Panel (B) cluster 2 gene mutations are associated with abnormal activation of kinase signalling pathways such as the PI3Kinase/AKT, RAS/RAF/ERK and mTOR pathways. Proteins that have been found to be altered by germline mutations in PPGLs include *NF1*, *KIF1B β* , *MAX/MXD*, *RET*, *TMEM127*. Activation of mTOR might constitute a common mechanism for tumour development caused by *RET*, *MAX*, or *TMEM127* mutations. The role of p53 in the development of PPGL is poorly understood and the most likely mechanism would be evasion of apoptosis. These figures were published in “Updates on the genetics and the clinical impacts on pheochromocytoma and paraganglioma in the new era”; 100; Pillai S, Gopalan V, Smith RA, Lam AK; Updates on the genetics and the clinical impacts on pheochromocytoma and paraganglioma in the new era; Crit Rev Oncol Hematol. 100; 190-208; Copyright (2016), with permission from Elsevier and the corresponding author.

Fig. 2 *SDHB*-mutated paraganglioma. Panel A shows the histology of a paraganglioma under haematoxylin and eosin stain. Panel B shows IHC stain for *SDHB* in a paraganglioma showing loss of *SDHB* protein in tumour cells (T) and being retained as granular immunoreactivity in endothelial cells (black arrow) and inflammatory cells (blue arrow) (which acts as intrinsic

controls). This tumour has particularly delicate blood vessels which are positive to. Panel C shows granular immunoreactivity for SDHA in tumour cells and endothelial cells.

Fig. 3 IHC stain for MAX in a pheochromocytoma from a patient with a germline *MAX* mutation. Characteristic nuclear staining of MAX is present in adrenal cortex (left upper portion) and in endothelial cells within the tumour, while the tumour cells are negative for MAX protein.