Updates on the genetics and the clinical impacts on phaeochromocytoma and paraganglioma in the new era

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Short title: genetics of phaeochromocytoma and paraganglioma

Biography of the corresponding author

The corresponding author is an internationally recognized authority in diagnostic and molecular pathology of endocrine cancer with more than 25 years of activities this field. He has published more than 250 articles in peer reviewed journals and has written book chapters in World Health Organization's classification of tumours of endocrine system. His publications have attracted high citations in the research field with the citation index (H-index) for his publications at 42 for 2015. He also serves on editorial boards for a few international peer reviewed journals.

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Abstract

Genetic mutations of phaeochromocytoma (PCC) and paraganglioma (PGL) are mainly classified into two major clusters. Cluster 1 mutations are involved with the pseudo hypoxic pathway and comprised of *PHD2, VHL, SDHx, IDH, HIF2A, MDH2 and FH* mutated PCC/PGL. Cluster 2 mutations are associated with abnormal activation of kinase signalling pathways and included mutations of *RET, NF1, KIF1Bβ, MAX* and *TMEM127*. In addition, *VHL, SDHx* (cluster 1 genes) and *RET, NF1* (cluster 2 genes) germline mutations are involved in the neuronal precursor cell pathway in the pathogeneses of PCC/PGL. Also, *GDNF, H-ras, K-ras, GNAS, CDKN2A (p16), p53, BAP1, BRCA1&2, ATRX* and *KMT2D* mutations have roles in the development of PCC/PGLs. Overall, known genetic mutations account for the pathogenesis of approximately 60% of PCC/PGLs**.** Genetic mutations, pathological parameters and biochemical markers are used for better prediction of the outcome of patients with this group of tumours. Immunohistochemistry and gene sequencing can ensure a more effective detection, prediction of malignant potential and treatment of PCC/PCLs.

Keywords: phaeochromocytoma; paraganglioma; mutations; immunohistochemistry; sequencing

1. Introduction

A neuroendocrine tumour that arises in the chromaffin cells in adrenal medulla is termed phaeochromocytoma (PCC). Extra-adrenal tumours arising from chromaffin cells are known as paraganglioma (PGL). PGL can occur in many different sites in the body and can be classified into sympathetic or parasympathetic depending on the type of paraganglia from which they originate [1, 2].

Incidence of PCC/PGL is around 2-5 patients per million per year. The collective incidence of PCC and PGL is about 1 per 100,000-300,000 in the general population where PCC is the most frequent tumour and PGL are much rarer (0.5 per million) [3, 4]. The highest incidence of the tumours occurs between 30 and 50 years, with an almost equal gender affinity [5-8]. However, some studies have reported a slight preference for females [9, 10]. Symptoms are produced due to excess catecholamine production in PCC and sympathetic PGL [3].

As both PCC and PGL originate from chromaffin cells, the histological features of these tumours are similar. Occasionally, unusual histological patterns like composite tumours or oncocytic change are noted [11-13]. Although scoring systems based on morphology as well as clinical features have been proposed to assess malignant potential of these tumours [14, 15], they have not been validated fully. Despite this, huge advances have been achieved in understanding the molecular pathogenesis of this group of lesions. Sporadic PCCs/PGLs are usually unicentric and unilateral while familial PCCs/PGLs are often multicentric and bilateral. Germline mutations and familial syndromes are known to be associated with 8-24% of sporadic phaeochromocytoma due to the advancement in genetics [7, 8]. More recent studies have indicated, however, that up to 40% of cases could be attributed to germline mutations in a growing list of susceptibility genes having interconnecting pathways [16]

Somatic mutations in inherited PCC/PGL genes can be detected in 25–30% of sporadic tumours. Also, somatic mutations in PCC/PGL can cause metastatic tumours in paediatric cases, and are mostly diagnosed before the age of 40 years [17]. Overall, germline and somatic mutations in a known PCC/PGL gene are present in 60% of tumours [18].

The current study provides a comprehensive review of the genetic mutations reported in PCC/PGL and focuses on the newly discovered genes. It is anticipated that improved understanding of the pathogenesis in PCC/PCL may provide hints to aid the prediction of malignant potential and detecting novel molecular targets for therapy of this group of tumours.

2. Molecular Genetics

2.1 Genetic Pathways involved in PCC/PGL

Transcriptome studies show that many PCC/PGLs and their inherent genetic mutations can be classified into two major clusters depending on their gene expression profile [19].

Cluster1 or the angiogenic cluster genes are involved with the pseudo hypoxic pathway of tumour development and they are *PHD2, VHL, SDHx, IDH, HIF2A, MDH2 and FH* mutated PCC/PGL [19]. The molecular pathways of these genes and downstream targets are listed in Figure 1. Favier et al. in 2012 reported that cluster 1 tumours showed a marked increase in vascularization and in the expression of vasoendothelial growth factor (VEGF) and its receptors [20]. VEGF proteins and receptors are the main factors in angiogenesis of cancers [21, 22]. Also, increased VEGF expression was observed in both benign and malignant tumours from cluster 1 [20]. In addition, characterization of the methylation profiles has revealed that *SDHX* mutated tumours in cluster 1 display a hypermethylated phenotype [23].

Cluster 2 or kinase signalling cluster involve genetic mutations associated with abnormal activation of kinase signalling pathways such as *PI3Kinase/AKT,* and the *mTOR* pathway [20]. The molecular pathway of these genes and downstream targets are listed in Figure 2.

The gene clusters are further subdivided based on transcription profiles. Cluster 1 can be divided into subcluster 1A and 1B. Cluster 1A contains PCC/PGL related to *SDHx and FH* while Cluster 1B contains tumours with HIF2A and *VHL* respectively [24]. Cluster 2 can be divided into groups 2A, 2B, 2C and 2D. Group 2A comprises *RET*, *MAX*, *NF1* and *TMEM127* mutated tumours whereas groups 2B and 2C are sporadic tumours [19, 25]. Group 2D tumours are lacking known mutations related to PCC/PGL. The subdivisions of cluster 1 and 2 molecular pathways of these genes in PCC/PGL are listed in Figure 3.

2.2 Cluster 1 genes

2.2.1 *Elegans Homolog of 1 (EGLN1) / Prolyl hydroxylase domain proteins (PHD2)*

Hypoxia-inducible factors (HIFs) function as key players in the cell response to hypoxia. Prolyl hydroxylase domain proteins (PHDs) initiate the degradation of the HIF-α protein through its hydroxylation [26]. There are three main isoforms of prolyl hydroxylase domain proteins namely PHD1, PHD2, and PHD3 which are encoded by the genes *EGLN2, EGLN1, and EGLN3*, respectively [26].

The PHD2–VHL–HIF-2α pathway plays a crucial role in erythropoiesis; a partial interruption of the pathway can cause erythrocytosis, whereas drastic alterations of the pathway are associated with the development of tumours [27]. Mutations in *PHD2* have been implicated in the pathogenesis of polycythaemia in humans [28]. Germline mutation of *PHD2* was first reported in a patient with erythrocytosis and recurrent PGL by Ladroue et al. in 2008 using direct sequencing [29].

Astuti et al. demonstrated the mutation analysis of *EGLN1 (PHD2*), *EGLN2 (PHD1*) and *EGLN3 (PHD3)* in 82 inherited PGL patients through PCR sequencing [30]. This study noted no germline mutations for *PHDs 1-3* and there was absence of mutation in known PCC/PGL susceptibility genes in the selected samples [30]. Welander et al. detected a novel germline mutation for the first time in *PHD2* in a PCC along with other known susceptibility genes in PCC after analysing 72 PCCs and 14 PGLs through targeted next generation sequencing [31]. Also, by using Sanger DNA sequencing, Yang and colleagues have reported a *PHD1* germline mutation in one patient along with a novel germline *PHD2* mutation in another patient presenting with recurrent PCC/PGL along with polycythaemia [27].

These studies showed that germline mutations in this group of genes are not a frequent event in PCC/PGLs. Nevertheless, *PHD1* mutation is the latest inherited abnormality that contributes to the development of PCCs/PGLs. The study by Yang et al. further provides evidence that the PHD2–VHL–HIF-2α pathway plays a role in the pathogenesis of PCC/PGL and their association with polycythaemia.

2.2.2 *Von Hippel Lindau (VHL)*

Von Hippel Lindau (VHL) is a tumour suppressor gene located on chromosome 3p25.3 consisting of 3 exons [32]. *VHL* was first identified by positional cloning in 1993 and is reported to have involvement in oxygen dependent regulation of hypoxia-inducible factor (HIF) [33]. Von Hippel-Lindau (VHL) syndrome is an autosomal dominant disorder caused by germline mutation of the *VHL* gene. The incidence of the syndrome is 1 in 36,000 births [4]. It is characterized by clear cell renal cell carcinomas, PCCs, PGLs, haemangioblastomas as well as cysts of the retina, cerebellum, kidney and pancreas [34].

VHL was classified into type 1 (without phaeochromocytoma) and type 2 (with phaeochromocytoma) by Neumann and Wiestler in 1991 [35]. Brauch et al. further subdivided VHL type 2 into type 2A (with phaeochromocytoma), type 2B (with phaeochromocytoma and renal cell carcinoma) and type 2C (patients with isolated phaeochromocytoma without haemangioblastoma or renal cell carcinoma) [36].

Regarding the prevalence, PCCs occur in 6%-9% of individuals with Von Hippel-Lindau syndrome type 1 and the prevalence rises to 40%-59% in persons with Von Hippel-Lindau syndrome type 2 [37, 38, 39]. The mean age of onset of PCC in patients with Von Hippel-Lindau syndrome is approximately at 30 years [4]. However, some individuals may also present with this neoplasm before the age of 10 years [34]. VHL-associated PCCs are frequently bilateral and multiple. These tumours characteristically produce only norepinephrine. This is because of the lack of the enzyme phenyl ethanolamine-N-methyl transferase, an enzyme responsible for the conversion of norepinephrine into epinephrine in adrenal medulla [37].

In PCC/PGLs, *VHL* mutations noted were often missense mutations [39]. Also, these tumours are rarely malignant and a younger age group is commonly affected [40]. Somatic *VHL* mutations in head and neck paragangliomas were reported for the first time by Merlo et al. in 2013 [41]. The *VHL* gene was analysed in 53 PGL tissues by gene sequencing, multiplex-ligation–dependent probe amplification, and quantitative PCR. *VHL* somatic mutation was found in 50% (2/4) of non-SDH mutated head and neck PGLs [41].

These results indicate that *VHL* mutation can predict the clinical diagnosis of PCCs and plays an important role in the pathogenesis of sporadic head and neck paragangliomas.

2.2.3 *Succinate Dehydrogenase Complex (SDHX)*

Succinate dehydrogenase (SDH) is a mitochondrial enzyme complex which is part of both the Kreb's cycle and electron transport chain [42]. SDH oxidizes succinate to fumarate in the Kreb's cycle (citric acid cycle) and transports electrons to coenzyme Q in the electron transport chain [42].

Succinate dehydrogenase consists of four subunits, SDHA, SDHB, SDHC and SDHD. Subunits A and B form the core of the SDH protein, whereas the other two subunits C and D, are often referred as the anchoring subunits [43]. In 2009, two factors involved in the assembly of the SDH complex were discovered, SDHAF1 [44] and SDHAF2 [45], which may play a role in the development of cancers associated with this pathway. In practice, the mutation of any of the SDH members (SDHX) can be detected by the loss of protein SDHB as determined by immunohistochemistry. This is because that these subunits are linked together. Mutations of any of these genes cause defects in the structure of these subunits and faulty assembly, which will result in loss of the SDHB protein [46].

In year 2000, *SDHD* mutations were discovered in sporadic and familial PGL/PCC [47-49]. Germline mutations in the *SDHX* genes give rise to familial PCC/PGL syndrome. Familial paraganglioma syndromes are autosomal dominant inherited diseases caused by mutations in SDHX genes. They have been classified into 5 paraganglioma syndromes, PGL1, PGL2, PGL3, PGL4 and PGL5 caused by mutations in SDHD, SDHAF2, SDHC, SDHB and SDHA respectively [4]. SDHD protein positivity can also be used to detect the mutation of *SDHD* in cases where the interpretation of SDHB protein by immunohistochemistry is difficult [50].

Hensen et al. determined the mutation frequency of the four SDH genes in 1,045 patients from 340 Dutch families with PCC/PGLs. The most commonly mutated gene was *SDHD* (87.1%), followed by *SDHAF2* (6.7%), *SDHB* (5.9%), and *SDHC* (0.3%) [51].

2.2.3.1 *Succinate Dehydrogenase Complex Subunit A (SDHA)*

PGL5 is caused by mutations in the *SDHA* gene. Homozygous germline mutations in *SDHA* are linked to Leigh syndrome. Heterozygous germline mutation of *SDHA* was first reported by Burnichon et al. in a patient presenting with a catecholamine-secreting abdominal PGL [52]. Also, *SDHA* germline mutations in PCCs were reported by Kopershoek et al. after analysing 316 PCC/PGL tissues by immunohistochemistry and sequence analysis [53]. The study reported the importance of the use of SDHA immunohistochemistry in detecting patients with germline *SDHA* mutations [53]. Also, Welander et al. in 2014 reported 2 PCCs with *SDHA* mutations using targeted next generation sequencing on 86 PCC/PGL tissues. These 2 cases with mutations were males and the tumours were benign [31].

2.2.3.2 *Succinate Dehydrogenase Complex Subunit B (SDHB)*

SDHB mutation in four PGLs was reported for the first time by Astuti et al [49]. Another study reported *SDHB* germline missense mutation in a case of sporadic malignant PCC in 2002 [54]. Among *SDHX* genes, SDHB mutations in PCC/PGL are associated with higher morbidity and mortality [55, 56].

PGL4 is transmitted in an autosomal dominant pattern with incomplete penetrance. *SDHB* mutation carriers were more likely to develop PGL4 and malignant disease [49, 57]. PGL4 usually presents with PGLs although it can be also present as PCC, head and neck PGLs or both types of tumour simultaneously [57]. The mean age of onset for patients with PGL4 is 25–30 years [57, 58]. *SDHB* mutation in PGL4 is associated with an increased risk of malignancy [55, 58]. The morbidity associated with these tumours is related mainly to its metastatic potential [58, 59].

2.2.3.3 *Succinate Dehydrogenase Complex Subunit C (SDHC)*

SDHC mutations were first reported in PGLs in 2000 by Niemann et al [60]. PGL3 is an autosomal-dominant syndrome without maternal imprinting caused by mutation in the SDHC gene [61]. This syndrome is characterised by head and neck PGLs. PGL3 usually develops with solitary head and neck paragangliomas. Nevertheless, some cases coursing with adrenal PCC and PGLs in other sites have been reported [4]. SDHC-associated tumours are not likely to be PCC. Also, they are more likely to be benign and do not have multiple tumours. [61]. The mean age at diagnosis of these tumours is 38 years [59].

SDHC mutations were detected in 4% (22/492) of patients with parasympathetic PGL in a study reported by Schiavi et al. but there was no case of PCC having *SDHC* mutation [61]. Mannelli et al. reported the association of *SDHC* mutation with PCC [62]. Later, Peczkowska et al. also reported the association [63]. All patients with *SDHC* mutations had PGLs and no PCCs were detected [61, 64]. .

2.2.3.4 *Succinate Dehydrogenase Complex Subunit D (SDHD)*

Germ line mutations in *SDHD* were reported in PGL by Baysal et al. in 2000 using direct sequencing [47]. Pigny et al., in 2008 reported the first case of a paraganglioma with maternal transmission of the mutated *SDHD* gene [65]. Piccini et al. revealed that *SDHD* was the most mutated 80.5% (29/36) gene among the genes coding for the *SDHX* complex after analysis of samples from 79 patients with head and neck paragangliomas by polymerase chain reaction and sequencing [66].

PGL1 is the autosomal dominant syndrome caused by mutation in the *SDHD* gene. Clinically this syndrome is characterised by multiple head and neck PGLs with an age of onset of 28–31 years. The PCCs can also be manifested either unilateral or bilateral tumours [55, 58, 67].

2.2.3.5 *Succinate Dehydrogenase Complex Assembly Factor 2(SDH5/ SDHAF2)*

Correct flavination of SDHAF2 is essential for a fully functional succinate dehydrogenase complex [45]. Loss of SDHAF2 results in loss-of-function of succinate dehydrogenase and a reduction in stability of the enzyme complex, leading to diminished amounts of all subunits [45]. Mutations in *SDHAF2* were detected in unrelated large Dutch and Spanish families with hereditary PGLs [45, 68, 69]. *SDHAF2* mutated tumours have been mostly identified as parasympathetic PGLs, and no metastases have been described [68, 69].

PGL2 is an autosomal-dominant syndrome caused by mutation in the *SDH5/SDHAF2* gene characterised by hereditary paragangliomas [45]. To date, no PCC has been reported in PGL2 syndrome. The average age of onset for patients with PGL2 is 33 years [68].

2.2.3.6 Carney-Stratakis dyad and Carney triad

Carney–Stratakis syndrome (Carney dyad) is an autosomal dominant syndrome associated with mutations in *SDHB*, *SDHC* and *SDHD* [70]. Both genders are affected equally in this syndrome and the mean age of onset is 23 years [4]. This syndrome is characterised by PGLs and gastrointestinal stromal tumours (GISTs) but not pulmonary chondroma, in contrast to the Carney triad. Carney triad is a rare disorder that primarily affects young women and the mean age of onset is 20 years [71]. The classic Carney triad includes extra-adrenal sympathetic paraganglioma, gastrointestinal stromal tumours and pulmonary chondroma. Adrenal cortical adenoma, oesophageal leiomyoma and PCCs were also shown to be associated with this syndrome [71]. No genetic mutations are reported so far in hereditary PGLs in Carney triad [71, 72]. However, hyper-methylation of *SDHC* was reported by Haller and colleagues in 3 of 4 patients with Carney triad [73].

2.2.4 *Isocitrate Dehydrogenase (IDH)*

In the Kreb's cycle, IDH catalyses the oxidative decarboxylation and converts isocitrate into alpha keto glutarate (αKG) [74]. Mutation in the *isocitrate dehydrogenase 1* gene was first discovered by sequencing in colorectal cancers [75]. Since then, mutations in *isocitrate dehydrogenase 1 and 2 (IDH1/2)* have been discovered in tumours of neural origin [76].

IDH1/2 mutations are heterozygous point mutations and are suggestive of activating, oncogenic mutations. IDH mutants are not able to competently carry out the normal oxidative reaction, which results instead in the conversion of αKG to 2hydroxyglutarate (2HG) [77]. 2HG is not present in normal cells. The accumulation of 2HG in cells with *IDH1/2* mutations is a form of oncometabolite [78] which results in the activation of pseudo-hypoxic pathway [79]. The consequences of activation this pathway may contribute to the pathogenesis of PGL/PCC [80].

Gaal et al. in 2010 for the first time reported *IDH1* mutation in paraganglioma after analysing 365 PCC/PGLs (253 paraffin embedded tissues and 112 frozen tissues). A somatic heterozygous *IDH1* p.Arg132Cys mutation was detected in a sporadic carotid paraganglioma diagnosed in a 61-year-old woman. On the other hand, no mutation was detected in PCC [81]. However, a study by Yao et al. did not find any mutation of *IDH1* after analysis of 104 PCC/PGL tissue samples [82]. This suggests that pathogenic mutations in these genes do not account for the majority of PCC/PGLs that display a pseudo-hypoxic profile [82].

2.2.5 *Endothelial Pas Domain Protein 1 (HIF2A/EPAS1)*

Wang et al. in 1995 described hypoxia-inducible factors (HIFs), which are transcription factors that respond to changes in tissue oxygen concentration [83]. These

proteins are composed of α and β subunits [83]. The HIF-β subunit is constitutively expressed whereas the HIF-α subunit is inducible by hypoxia [84]. Abnormal HIF-α function enhances cell proliferation and growth [85]. Recently, several groups have established that mutations in *HIF2A*/*EPAS1* that lead to HIF2 stabilisation are implicated in the pathogenesis of PCC/PGL. This could be the second most frequently affected oncogene in PCC/PGL after *RET* [18, 31, 86, 87].

The first report of somatic *HIF2A* mutations in paragangliomas and somatostatinomas that were clinically associated with polycythaemia was published in 2012 using PCR assay in two patients [88]. Pacak et al. indicated the existence of a new syndrome with multiple PGLs and somatostatinomas associated with polycythaemia and suggested this new syndrome resulted from somatic gain-of-function *HIF2A* mutations, which cause an upregulation of hypoxia-related genes [89]. In 2013, the first association of a somatic *HIF2A* mutation in PCC and congenital polycythaemia was reported [87].

Germline *EPAS1/HIF2A* mutation with congenital polycythaemia associated with multiple PGLs was first reported by Lorenzo et al. using Sanger sequencing [90]. Also, Mendez et al. assessed 41 PCC/PGL tissues for mutations in *EPAS1*. Of these tumours, 17% (7/41) were found to carry somatic *EPAS1* mutations. Three of them also had congenital erythrocytosis, whereas 3 were single sporadic PCC/PGL cases. This study reported that *HIF2A* mutations can cause sporadic PCC/PGL in the absence of polycythaemia [86].

Welander et al. identified both somatic and germline mutations in the *EPAS1* gene after analysing 42 patients with sporadic PCCs [31]. All *EPAS1*-mutated tumours displayed a pseudo-hypoxic gene expression pattern, suggestive of an oncogenic role of the identified mutations [31].

Buffet et al showed that *HIF2A*-related tumours were caused by post zygotic mutations occurring in early developmental stages. They suggested that germline mosaicism

can lead to PCC/PGL, polycythaemia and somatostatinoma in the same patient and should be considered during the familial genetic counselling in persons diagnosed with *HIF2A*-related polycythaemia-paraganglioma syndrome [91].

2.2.6 *Fumarate Hydratase (FH)*

Fumarate hydratase (FH) is a homotetramer which catalyses the hydration of fumarate to malate in Kreb's cycle and is located on chromosome 1q42 [92]. Germline mutations in *FH* gene cause hereditary leiomyomatosis and renal cell carcinoma [93]. Pollard et al. showed that in cells deficient in fumarate hydratase, the cells accumulated fumarate and succinate which stabilized HIF1A, leading to pseudo-hypoxia resulting in tumorigenesis in paragangliomas, leiomyoma and renal cell carcinoma [92]. Selak et al. also showed that succinate and fumarate can activate the oncogenic HIF pathway by inhibiting HIF prolyl hydroxylases [94].

Letouze et al. in 2013 identified for the first time a germline mutation in *FH* causing PGL after exome sequencing of one hyper-methylated sample without *SDHX* mutation among 145 PCC/PGL tissue samples [23].

Clark and colleagues did exome resequencing studies in a child with phaeochromocytoma and identified a candidate *FH* missense mutation (p.Cys434Tyr). Then, analysis of mutation of *FH* was done in 71 patients with PCC/PGL. They detected another candidate missense mutation (p.Glu53Lys) by candidate gene sequencing. *In vitro* analyses demonstrated that both missense mutations (p.Cys434Tyr and p.Glu53Lys) were catalytically inactive and extended the clinical phenotype associated with *FH* mutations to paediatric PCC [95].

Castro et al. identified germline *FH* mutations in 0.83% (5/598) of patients with PCC/PGL after collecting blood and tissue samples from 598 PCC/PGL patients through

direct Sanger sequencing [96]. The mean age at diagnosis of the patients with mutation was 45 years. Three out of the 5 (60%) tumours with the mutation showed metastasis [96].

FH mutations should be considered as a new rare source of predisposition to PCC/PGLs. *FH* inactivation causes PGL/PCC by establishing a hypermethylator phenotype [23]. FH deficient PCC/PGLs display a similar genetic pathway of development as *SDHB*-mutated malignant PCC/PGL. Therefore mutation screening for *FH* mutation should be included in PCC/PGL genetic testing and also for PCC/PGL with malignant behaviour [96].

2.2.7 *Malate dehydrogenase2 (MDH2)*

Malate dehydrogenase 2 (MDH2) is a tumour suppressor gene located on chromosome 7q11.23 [97]. It helps in the reversible oxidation of malate to oxaloacetate, in the Kreb's cycle [98]. The protein encoded by this gene is localized to the mitochondria and may play a key role in the malate-aspartate transport that operates in the metabolic coordination between cytosol and mitochondria [98]. Diseases associated with *MDH2* mutation include 1-2-hydroxyglutaric aciduria and sleeping sickness [99].

Cascon et al. for the first time reported germline *MDH2* c.429+1G>T mutation in paraganglioma through whole-exome sequencing, using tumour DNA from a male patient diagnosed with multiple malignant paragangliomas [100]. Study also reported that *MDH2* mutations are associated with a methylator phenotype and had a transcriptional profile similar to SDH gene mutated tumours. Genetic study of five asymptomatic relatives of the patient showed that two of them carried *MDH2* mutation thus showing *MDH2* to be a new familial paraganglioma gene [100].

2.2.8 Genetic mechanism in Cluster 1 genes

The pathway for the manifestation of cluster 1 genes is the pseudo-hypoxic pathway. Hypoxia is a state which occurs when oxygen concentration drops below 21%. On the other hand, pseudo-hypoxia is a state where cellular oxygen is present in sufficient amounts and this oxygen cannot be processed further due to a disruption in oxygen-sensing pathways [101]. Hypoxia Inducible Factors (HIFs) are transcription factors which are activated under hypoxic or pseudo-hypoxic conditions and are composed of oxygen-sensitive α-subunit and stable β-subunit. HIFs consist of three isoforms HIF-1α, HIF-2α, and HIF-3α [102]. HIF-1α is activated during short periods of severe hypoxia, while HIF-2α is activated under mild hypoxia for more prolonged periods of time [101].

Under normoxic conditions, HIF-1α and HIF-2α are degraded via the ubiquitin-proteasome pathway. This pathway is controlled by the VHL protein which ubiquitinates HIF-α and leads to degradation of HIFs by the proteasome enzyme complex [101, 102]. This activity requires proline hydroxylation of HIF-1α and this process is mediated by members of the EGLN/PHD family [101]. However, hypoxic or pseudo-hypoxic conditions lead to HIF-1α stabilization which in turn leads to activation of HIF target genes including those associated with angiogenesis, haematopoiesis, cell growth and cell migration [101].

Mutations within the *VHL*, *PHD2* genes result in the absence of functional VHL protein. This VHL protein deficiency will further induce pseudo-hypoxia in cells and as a result of this, HIF-1α is allowed to accumulate and bind to HIF-1β. Following this, transcription of several target genes associated with angiogenesis, energy metabolism, survival, and growth will occur and these will in turn leads to the pathogenesis of PCC/PGLs [33, 103].

Mutated *SDH* complex has also been associated with pseudo-hypoxic response [104]. Inactivation of SDH complex causes accumulation of succinate and this inhibits EGLN/PHD

enzyme activity, thereby leading to HIF-stabilization and activation of HIF target genes resulting in the development of PCC/PGLs [94].

FH mutations in PCC/PGL hypoxic pathways results in loss of fumarate hydratase activity [101]. This loss of FH enzymatic activity results in the accumulation of intracellular fumarate which competitively inhibits EGLN1/ PHD, thus stabilizing the HIF complex and similarly activating its oncogenic target genes resulting in tumour development [105].

Hydroxylation and stabilization of HIF-2α protein is mediated by *HIF2A* mutations. Also, this *HIF2A* mutation prevents recognition of HIF-2α protein by VHL and thus results in increased HIF-2α stabilization. This in turn leads to the activation of many hypoxia-related genes and may result in development of PCC/PGL [29, 101]

Studies have shown that HIF-1α and HIF-2α are overexpressed in *SDH* and *VHL* mutated PCCs and PGLs [104, 106]. Also a study by Castro et al. showed that *FH*-mutated PCCs/PGLs displayed the same epigenetic changes as SDHB-related tumours [96]. Thus, cluster1 tumours cause pseudo-hypoxic response by stabilizing HIFs under normoxic conditions and result in PCC/PGL pathogenesis.

2. 3. Cluster 2 genes

2.3.1. *Kinesin Family Member1B (KIF1B)*

Kinesin family member 1B *(KIF1B)* is a member of the kinesin 3 family of genes which have a specific cellular role in energy transport [107]. It is a large gene of 50 exons located at chromosome 1p36.22 and encodes two isoforms, *KIF1Bα* and *KIF1Bβ* [107].

The function of *KIF1Bβ* is to act as a tumour suppressor gene which is necessary for neuronal apoptosis [108]. *KIF1Bβ* act as a haplo-insufficiency tumour suppressor and its allelic loss is believed to be involved in the pathogenesis of neuroblastoma and other cancers [109].

In 2008, Schlisio et al. reported *KIF1B β* mutations for the first time in phaeochromocytoma without any other inclining mutations [108]. The group sequenced 52 PCC tissues and identified *KIF1B β* missense variants in two PCC samples [108]. A family with germline mutations of *KIF1B β* predisposing to neuroblastoma, ganglioneuroma, PCC and lung carcinoma was also reported [110]. A study by Welander and colleagues on 72 PCC and 14 PGL tissue samples using targeted next generation sequencing reported a novel *KIF1Bβ* germline mutation in a PCC patient [31]. In the same study, another PCC with a somatic missense mutation of *KIF1Bβ* in combination with a germline *NF1* mutation was also reported [31]. No case of PGL associated with *KIF1B* mutation has been reported in the literature.

KIF1B β is necessary for neuronal apoptosis when nerve growth factor becomes limiting and is frequently deleted in neural crest-derived tumours. Also, inherited loss-of-function *KIF1Bβ* missense mutations in PCC prove that *KIF1Bβ* is a genetic target of these diseases.

2.3.2. *Rearranged during Transfection Proto oncogene (RET)*

RET is an oncogene which is counted among the PCC/PGL susceptibility genes. The *RET* gene is involved in cell growth and differentiation and the protein is mainly expressed in urogenital and neural crest precursor cells. *RET* activation is essential for the development of kidneys as well as the sympathetic, parasympathetic, and enteric nervous system [111]. Oncogenic activation of *RET* has been shown to activate both PI3K/AKT and RAS/RAF/MAPK dependent pathways [112].

Multiple endocrine neoplasia 2 (MEN2), an autosomal dominant disorder, occurs as a result of germ line activating missense mutations of *RET* with an incidence of 1:35000 in the general population [4, 113]. Based on the presence of phaeochromocytoma,

hyperparathyroidism, and characteristic physical features, MEN 2 can be grouped into MEN2A, MEN2B and familial medullary thyroid carcinoma. Among the three types, MEN2B is the most aggressive form. Patients with MEN2A or MEN2B have an approximately 50% chance of developing PCC [113, 114]. PCC associated with *RET* mutations is mostly bilateral and have high chances of recurrence, though low risk of malignancy [113]. PGL is reported to be rare in MEN2 [115, 116]. In MEN2, patients with PCCs usually present between the ages of 30 and 40 years [37, 58].

2.3.3. *Neurofibromin 1 (NF1)*

The *NF l* gene serves as a tumour suppressor gene as its main function is to suppress cell proliferation by converting RAS protein into its inactive form, thereby inhibiting the oncogenic RAS/RAF/MAPK signalling pathway [117]. It also inhibits the PI3K/AKT/mTOR pathway via suppression of RAS [118].

Neurofibromatosis type I (NF1), or von Recklinghausen disease, is an autosomal dominant disorder caused by mutation in the *NF1*gene, characterized by cafe-au-lait spots, lisch nodules in the eye, axillary and inguinal freckling and fibromatous tumours of the skin [4]. The worldwide incidence of NF1 is 1 in 3000-4000 people [4].

Incidence of PCCs in NF1 patients is around 0.1%-5.7%. Patients with NF1 have hypertension and show a relatively high frequency of PCCs. The tumours in these patients occur mostly unilaterally and are seldom PGLs [4, 58]. NF1 associated PCCs are frequently malignant. Also, the average age of tumour occurrence in these patients is 40 years [4, 37, 58]. The diagnosis of this syndrome is usually achieved during childhood and the diagnosis is based on its clinical presentation. Genetic testing for *NF1* mutations is not routinely performed as the *NF1* gene is large and there is no known discrete mutation hot spots associated with development of PCC [119].

About 20% of sporadic PCC/PGL has mutations in the *NF1* gene. The *NF1* gene is the most frequent target of somatic, truncating mutations in sporadic phaeochromocytoma [40, 119]. Burnichon et al., using direct sequencing of the *NF1* gene and single nucleotide polymorphism array, reported a high level of *NF1* somatic alterations in 41% (25/61) of phaeochromocytoma tissues. However, the alleged higher rate of somatic *NF1* mutations in this study is due to a selection bias since Burnichon et al. studied only those sporadic tumours belonging to cluster 2A [119]. In another study carried out by Welander et al. using high-density single nucleotide polymorphism (SNP) microarrays, 23.8% (10/42) of unselected sporadic PCCs were found to exhibit somatic *NF1* mutations [40]. Welander et al. also noted that *NF1* mutated neuroendocrine tumours were often PCCs, rarely malignant and often noted in middle aged patients [40]. Thus, approximately one-fifth of sporadic PCCs may have somatic NF1 mutations. A majority of the tumours in both studies displayed loss of heterozygosity at the *NF1* locus, indicating that deletion may be a major mechanism of *NF1* alteration in PCCs and PGLs.

2.3.4. *Transmembrane protein 127 (TMEM 127)*

Transmembrane protein 127*(TMEM 127)* was identified as a PCC/PGL susceptibility gene through a family with an autosomal dominant inheritance of phaeochromocytoma [120]. It is a tumour suppressor gene of 4 exons identified by positional cloning on chromosome 2q11 [120]. Qin et al. identified germline *TMEM127* mutations in a population of 103 tissue samples of PCC. The PCCs bearing mutations comprised 30% of the tested familial tumours and about 3% of the tested sporadic phaeochromocytoma. Qin et al. demonstrated that PCCs with *TMEM127* mutation showed hyper-phosphorylation of mammalian target of rapamycin (mTOR) effector proteins and indicated that *TMEM127* is a negative regulator of mTOR [120]. mTOR is a member of the phosphatidylinositol-3kinase (PI3K)-related kinase family

which modulate cellular growth, angiogenesis and cell survival. Elevated mTOR signalling has been detected in many common human cancers and clinical trials are underway for mTOR inhibitors in multiple cancers [121, 122].

Neuman et al. reported germline mutations of *TMEM 127* in 4% (2/48) of patients with multiple PGLs [123]. Thus, both phaeochromocytoma and paraganglioma have now been identified in association with germline *TMEM127* gene mutations.

In a multi-institutional study comprising of 990 patients with PCC/PGL, using multiplex polymerase chain reaction in DNA from blood and tissue samples, *TMEM127* mutations were identified in 2% (20/990) of the cases, all of which had PCC [82]. A clear family history of phaeochromocytoma was present in only 25% (5/20) of cases which indicates that *TMEM127* mutation could be associated with sporadic PCC [82].

With regards to cellular mechanisms, the tumour suppressive properties of *TMEM127* cause variation of mTOR function in the endolysosome. Also, mutations of *TMEM127* lead to increase in mTOR signalling, a feature that may help in the development of PCC/PGL [120, 123].

2.3.5. *Myc Associated Factor X (MAX)*

Myc associated factor X *(MAX)* is a gene of five exons, associated with regulation of cell proliferation, differentiation and death [124]. *MAX* plays a crucial role in the control of the MYC/MAX pathway; the deregulation of which contributes to numerous neoplastic conditions, including neuroblastoma [125, 126, 127].

Next generation exome sequencing helped to discover germline mutations of *MAX* in 12 patients with PCC [128]. Segregation of *MAX* mutation, lack of MAX protein in the tumours by immunohistochemical analysis and loss of the wild-type *MAX* alleles in the tumours indicated that *MAX* is a tumour suppressor gene [128].

Burnichon et al. in 2012 sequenced *MAX* from DNA obtained from the blood leucocytes of 1,694 patients with PCC/PGL using a multiplex PCR-based method. For the first time, *MAX* mutation was noted in PGL [127]. The study also confirmed the presence of somatic mutations of *MAX* in 1.65% (4/245) of PCC/PGL [127].

A study by Comino-Mendez et al. showed 25% of their patients (3/12) had PCCs with *MAX* mutation that showed metastasis at diagnosis [126]. These findings supported the idea that functional loss of MAX protein is correlated to metastatic potential suggesting that *MAX* mutations are associated with a high risk of malignancy. A study by Burnichon et al, however, did not support the above theory as only 7% (2/28) of *MAX* mutation bearing tumours developed metastasis [127].

In the literature, *MAX* mutations have been studied in different series with a total of 2041 cases of PCC/PGLs [66, 127, 128, 129]. Overall, the frequency of *MAX* mutation in the population is 1.9% (40/2041). Also, mutations were noted in cases with multiple tumours. In the literature, *MAX* mutation was also noted in 5 cases of malignant phaeochromocytoma [126, 127]. Thus, the prognostic importance of the MYC/MAX pathway in the development of both hereditary and sporadic forms of PCC/PGL should not be overlooked. In addition, mutation of *MAX* as reflected by loss of MAX protein expression was found to be detectable by immunohistochemistry [126]. In general, the frequency of *MAX* mutation in PCC/PGLs is low, so targeted genetic screening should be considered after the more common genes have been excluded.

2.3.6. *Menin* (*MEN1*)

Multiple endocrine neoplasia type I is an autosomal dominant disorder characterized by tumours of the parathyroid, pancreatic islets, duodenal endocrine cells, and the anterior

pituitary [130]. Some patients may also develop adrenal cortical tumours, carcinoid tumours, facial angiofibromas, collagenomas and lipomas [131, 132]

The tumour suppressor gene, *Multiple endocrine neoplasia 1 (MEN1),* mutations in which cause multiple endocrine neoplasia type I was discovered by positional cloning [130]. The *MEN1* gene with its product, menin, is involved in transcriptional regulation, genome stability, and cell proliferation [132].

Interestingly, seven cases of PCC associated with MEN1 syndrome have been reported in the literature [131, 133]. In all cases, the tumours were unilateral with malignancy noted only in one patient [131]. The first association of PGL with MEN1 syndrome revealed a new missense mutation of the *MEN1* gene. The genetic mutation was reported by Jamilloux et al. in 2014 in a 58-year-old woman presenting with three features of MEN1 syndrome (hyperparathyroidism, pancreatic neuroendocrine tumour, and adrenocortical adenoma) along with PGL. Screening for other genes causing PGL was negative. The new germline mutation p.Arg275Lys of the *MEN1* gene was identified by direct sequencing and has not been previously reported [134].

Screening of patients with *MEN1* mutations in PCC/PGL is potentially important. If the result is negative, it proves that the disorder is not associated with a germline abnormality of MEN1. On the other hand, a positive result would benefit the family members as there is a rationale for appropriate screening for early detection of MEN1 syndrome [135].

2.3.7. Genetic pathway for Cluster 2 genes

The genes in cluster 2, *RET* /*NF1/TMEM127/MAX/* KIF1Bβ, are connected with oncogenic kinase signalling pathways.

Oncogenic activation of *RET* prompts an activation of the tyrosine kinase receptor which activates PI3 kinase (PI3K)/AKT/mTOR and RAS/RAF/ERK signalling pathway

[112]. Both pathways promote cell proliferation, growth, and survival leading to PCC/PGL development [136, 137].

The *NF1* gene product neurofibromin activates an enzyme, ras GTPase, to inhibit *Ras* and downstream growth signals which results in the activation of RAS/RAF/MAPK and Akt/mTOR signalling pathways, thus loss of function in this gene results in PCC/PGL formation [117, 118].

TMEM127 mutations promote pathogenesis of PCC/PGL by cessation of negative regulation of *mTOR* signalling through membrane interactions [120]. Activation of *mTOR* is deregulated in many human cancers and is a downstream signal of both *RET* and *NF1* mutations via the PI3K/AKT pathway [120].

MAX protein is a cofactor of the proto-oncogene *MYC* and functions as a transcription factor in association with *MYC* and *MXD* [126]. A link between the MYC/MAX/MXD1, PI3K/AKT/mTOR and RAS/RAF/MAPK signalling cascades has been reported and consequently, alterations in MAX-MYC signalling can in turn promote the development of tumours in PCC/PGLs [138, 139,140].

KIF1Bβ mutant tumours cluster with PCCs carrying *RET* or *NF1* mutations, and independently from those with mutations in *VHL* or *SDH* genes [110]. This two-cluster structure triggers overlapping features of the respective mutations, comprising the activation of RAS/RAF/ERK signalling in *RET*- and *NF1*-mutant PCC [19]. Also, *KIF1Bβ* mutant PCC tumours are significantly enriched in genes related to amino acid metabolism, such as glutamate and glutamine, as well as in genes linked with oxidative stress response [110]. In addition, *KIF1Bβ* has also been found to facilitate the pro-apoptotic effect of PHD3, whose loss of function may therefore prevent apoptosis and promote PCC/PGL development [108].

Thus *RET*, *NF1* and *TMEM127* mutations are associated with hyper-phosphorylation of *mTOR* targets and the MYC-MAX signalling pathway is also linked to PI3K/AKT/ mTOR

signalling [120,138]. Hence the mTOR signalling pathway may be a point of convergence for all signalling pathways associated with PCC/PGL development [137].

2.4. Activation of neuronal precursor cells pathway

Lee et al. has shown that the different susceptibility genes converge into a single common pathway, the *neuronal precursor cells* pathway, in PCC/PGLs [141]. *VHL, NF1, SDHX (cluster 1 genes) and RET (cluster 2 gene)* germline mutations in this pathway can cause a defect in the apoptosis of neuronal progenitor cells.

Neuronal apoptosis is induced by a protein called c-Jun, which is in turn activated by loss of nerve growth factor [142]. The NF1 gene product, neurofibromin, can inhibit the nerve growth factor receptor and loss of neurofibromin promotes the survival of embryonic sympathetic neurons in the absence of nerve growth factor [143].

Lee et al showed that elevated levels of the transcription factor JunB can block apoptosis in the PCC cell line, PC12, and suggested inhibition of c-Jun by JunB [141]. They proposed that the accumulation of succinate acts not through HIF-1 but via inhibition of PHD3 mediated apoptosis. This study has also demonstrated that PHD3 induces neuronal apoptosis and that accumulation of succinate due to SDH inactivation inhibits PHD3 which in turn leads to survival of embryonic neurons leading to tumour formation in PCC/PGLs [141].

Growth of c-Jun activity induced by the *MEN1* gene product, menin, and blocking of c-Jun up regulation by MYC also suggest potential roles for *MEN1* and *MAX* mutations in this pathway [132,144].

2. 5. Other genes associated with PCC/PGL

2.5.1. *Glial Cell Line Derived Neurotrophic Factor (GDNF)*

Glial cell line-derived neurotrophic factor (GDNF) is a member of the transforming growth factor beta superfamily [145]. *GDNF* mutations have been associated with the pathogenesis of Hirschsprung disease [146]

GDNF was shown to be the functional RET ligand [145] and it was suggested that point mutations in *GDNF* which alter GDNF function in terms of RET binding capacity could be involved in the genesis of PCC [147]. Woodward et al. identified a *GDNF* sequence variant (R93W) mutation in one patient with sporadic phaeochromocytoma out of 28 tumours. They suggested that the R93W mutation could function as a susceptibility mutation for PCC of low penetrance ([147]. On the other hand, Dahia et al. also investigated the role of *GDNF* mutation in 22 sporadic PCC tissues using semi quantitative PCR, but no disease causing somatic *GDNF* mutations or gross gene amplification were detected in these tumours. The result indicated that *GDNF* plays a minor role in the origin of PCC [148]. However, *GDNF* allelic variants may influence the susceptibility of a patient to PCC and only small cohorts of PCC have been interrogated for *GDNF* mutation at present [147].

2.5.2. *Ras genes*

Ras genes (*H-ras, K-ras, N-ras, M-ras* and *R-ras)* regulate signalling pathways that control many cellular responses such as proliferation, survival and differentiation [149]. Ras signalling is regarded as a major event in cancer pathogenesis and they are the most common targets for somatic gain-of-function mutations in human cancers [150,151]. Activating *Ras* mutations occur in ~30% of human cancers [151].

Lin et al indicated that activation of *Ras* signalling pathways favours PCC formation in-vitro [152]. However, earlier studies by Moley et al. and Moul et al. found no evidence

for *Ras* mutations in primary human PCC [153,154]. In 1992, Yoshimoto et al. have screened 169 endocrine tumours for *H-ras* mutations and identified for the first time a mutation in one patient with PCC among 19 PCC cases by polymerase chain reaction-single strand conformation polymorphism detection [150].

Among the ras family genes, only *H-ras* and *K-ras* mutations have been reported so far in PCC/PGLs. The specific role of these genes and their mutations in the pathogenesis of PCC/PGLs are detailed below.

2.5.2. 1. *Harvey Rat Sarcoma Viral Oncogene (H-ras)*

Harvey Rat Sarcoma Viral Oncogene belongs to the *Ras* oncogene family. Located on chromosome 11p15.5, the gene is involved in signal transduction pathways [155,156].

Germline mutations in this gene cause Costello syndrome, a disease characterized by increased growth at the prenatal stage, growth deficiency at the postnatal stage, predisposition to tumour formation, mental retardation, skin and musculoskeletal abnormalities, distinctive facial appearance and cardiovascular abnormalities [157,157].

Recently Crona et al. identified recurrent somatic *H-ras* mutations in PCC/PGL through exome sequencing. *H-ras* mutations was noted in 6.9% (n = 4/58) of the tumours. The *H-ras* mutation positive tumours were 3 PCCs and 1 PGL [159]. PCC/PGL with *H-ras* mutations showed activation of the RAS/RAF/ERK signalling pathway [159]

In 2014, Oudijk et al. by means of Sanger sequencing determined the prevalence of *Ras* mutations in a cohort of 271 PCC/PGLs. *H-ras* mutations were detected in 5.2% (14/271) of cases and were confined to sporadic PCCs. In this large series, *H-ras* mutations in PCCs lacked any significant correlation with pathological or basic clinical end-points [160].

About 5% of sporadic tumours have somatic mutation in *H-ras.* Mutations in *H-ras* occur mutually exclusively from upstream pathogenic activators and germline defects in *Ras* result in established syndrome-phenotypes [159,160]. Thus, the existence of *H-ras* mutations in otherwise healthy patients with PCC/PGLs is expected to be somatic. The identification of *H-ras* mutations as a new pathogenetic driver in sporadic PCC opens up the possibility of new therapeutic approaches.

2.5.2. 2. *Kirsten Rat Sarcoma Viral Oncogene (K-ras)*

Kirsten ras (*K-ras)* is a proto oncogene from the mammalian ras gene family located on chromosome 12p12.1 [161]. *K-ras* plays a vital role in normal tissue signalling, including proliferation, differentiation, and senility. Also, *K-ras* is noted to be one of the most activated oncogenes, with 17 to 25% of all human tumours harbouring an activating *K-ras* mutation [161]. Similar to *H-ras*, germline mutations for *K-ras* mutations have only been reported in disease syndromes and they include Noonan syndromes and in cardio facio-cutaneous syndromes [151].

Hrasćan et al. reported point mutations of *K-ras* for the first time in 62% (8/13) of PCCs after paraffin embedded samples were analysed by the polymerase chain reaction using restriction fragment length polymorphism and dinucleotide repeat polymorphism methods [162]. Also, this study has noted that *K-ras* mutations were homogenous in PCC compared to insulinomas in which *K-ras* mutations were found to be heterogeneous [162].

Future studies need to be focused on other, less-characterized *Ras* family members, such as *M-ras* and *R-ras*, in order to reveal novel insights in the role of Ras signalling pathways in PCC/PGLs.

2.5.3 *Guanine Nucleotide Binding Protein (GNAS)*

GNAS is a complex imprinted locus that produces multiple transcripts through the use of alternative promoters and alternative splicing [163].

Somatic mutations of *GNAS* in PCC/PGL were first reported by Williamson et al. in 1995 after examining tissues from patients having more than one organ affected by an endocrine disorder and patients having separate distinct endocrine diseases for G protein gene mutations [164].

Integrative epigenomic and genomic analysis of malignant phaeochromocytoma by Sandgren et al. also showed that GNAS is a potential candidate gene that can cause PCC/PGL [165]. Further studies are needed to establish this hypothesis.

2.5.4. *Cyclin Dependent Kinase Inhibitor (CDKN2A/p16)*

Cyclin-dependent kinase inhibitor (*p16*) encodes proteins that regulate two critical cell cycle regulatory pathways, the p53 and retinoblastoma1 pathway [166]. Neural system tumours and melanoma syndrome are some of the syndromes associated with mutation of *p16* [167].

Aguiar et al. used semi-quantitative multiplex PCR to search for *p16* deletion in 26 phaeochromocytomas but was not able to find any instances of *p16* deletion. They concluded that *p16* does not play a role in the pathogenesis of PCC. [168]. Also, Dammann et al. studied the methylation status of the *p16* gene in both hereditary and sporadic PCC by methylation-specific PCR, and noted hyper-methylation of the *p16* gene in 24% (6/25) of the tested specimens [169].

Using tissue microarray and immunohistochemistry, Muscarella et al. evaluated the expression of p16 in 31 phaeochromocytoma tumour specimens and found that the p16 protein expression was down regulated in 30 PCC specimens. In contrast, high expression of

p16 protein was observed in the majority of non-tumour control specimens (5/7). These findings suggested that down regulation of p16 protein could play an important role in the development of PCC. The primary cause for such down regulation is inactivation of *p16* gene, with the results from Dammann et al, indicating that epigenetic deactivation rather than deletion may be the mechanism by which *p16* influences PCC development [169,170].

2.5.5. *Transformation related protein 53 (p53)*

Transformation related protein 53 (p53) translates a tumour suppressor protein containing transcriptional activation, DNA binding, and oligomerization domains [171]. *p53* mutation is the most common mutation in many human cancers [172-176]. In addition, germline mutations in this gene are associated with hereditary cancers such as Li-Fraumeni syndrome [177] and adrenocortical carcinoma in children [178]

Lin et al. in 1994 performed mutation studies for *p53* abnormalities in 23 cases with adrenal neoplasms [179]. The immunohistochemical study demonstrated overexpression of p53 protein in the tumour cells of adrenal neoplasms. Five of six PCCs showed an apparent electrophoretic mobility shift between the tumour and its paired adjacent normal adrenal tissue. The study was done using the polymerase chain reaction-single strand conformation polymorphism method. Thus, *p53* gene mutation may play a role in the tumorigenesis of benign and functional human adrenal tumours [179]. Lam and colleagues detected p53 protein over-expression in 4 PCC/PGL (3 phaeochromocytomas and one paraganglioma) out of 65 (6%) patients with PCC/PGL [180]. Two of the p53 positive tumours were bilateral. However, Petri et al. showed no p53 mutations or protein over expression in PCC tumours from 48 patients (including 63 samples - 13 paraffin blocks and 50 frozen blocks) [181]. On the other hand, Luchetti et al. demonstrated the presence of somatic *TP53* missense gene mutation (c.1010G>A; p.R337H) in 2.35% of sporadic PCC/PGL (2/85) samples using next

generation sequencing followed by direct DNA sequencing [17]. Overall, the prevalence of p53 mutation is low in PCC/PGL

2.5.6. *Breast Cancer Associated Protein 1 (BAP1)*

Breast cancer associated protein 1(BAP1) is a tumour suppressor gene located on chromosome 3p21 and was initially identified as a protein that binds to *BRCA1* [182]. *BAP1* helps in the regulation of key cellular pathways, including the cell cycle, cellular differentiation, cell death, gluconeogenesis and the DNA damage response [182]. Germline mutations in *BAP1 are* associated with tumour predisposition syndrome (TPDS), which involves increased risk of malignant mesothelioma, uveal and cutaneous melanoma [183]. Somatic *BAP1* mutations were reported in cutaneous melanocytic tumours (epithelioid atypical spitz tumours and melanoma), uveal melanoma, mesothelioma, clear cell renal cell carcinoma, and other tumours [184]. However, the complete tumour spectrum associated with germline *BAP1* mutations is not yet known.

Using whole exome sequencing, Wadt et al. reported a patient with paraganglioma carrying a germ line *BAP1* mutation in a Danish family with multiple uveal malignant melanoma and suspected mesothelioma cases, as well as several other cancers including cutaneous malignant melanoma and breast cancer [185]. Also, somatic loss of *BAP1* wild-type allele was confirmed in the tumour tissues from this patient with uveal malignant melanoma and paraganglioma [185]. Thus, as a broad-spectrum tumour suppressor gene *BAP1* may be involved in the formation of PCC/PGL [185]

2.5.7. *Breast Cancer 1 and Breast Cancer 2 (BRCA1 and BRCA2)*

BRCA1 and *BRCA2* are tumour suppressor genes located on chromosome 17 and chromosome 13 respectively. They play a critical role in DNA repair, cell cycle checkpoint

control, and maintenance of genomic stability [186]. Germline mutations in *BRCA1* and *BRCA2* are most commonly associated with familial breast and ovarian cancer and the clinical syndrome seen in *BRCA* mutation carriers is referred to as the hereditary breast-ovarian cancer syndrome [187]. Mutations in *BRCA1* and *BRCA2* also increase the risk of several other cancers such as fallopian tube cancer, peritoneal cancer, prostate cancer and pancreatic cancer [188].

Barak et al. reported an unusual association between *BRCA1* and *BRCA2* mutations in blood samples from two patients with PCC using restriction enzyme digest of amplified PCR product [189]. It is hard to draw conclusions from two cases, but they raise the possibility of an increased risk for developing PCCs in *BRCA1* and *BRCA2* mutation carriers. More studies are needed in larger series to reveal the exact role of *BRCA1* and *BRCA2* in the carcinogenesis of PCC/PGLs.

2.5.8. *Alpha Thalassemia/Mental Retardation Syndrome X-linked (ATRX)*

ATRX is a member of the SWitch /sucrose non fermentable (SWI/SNF) family of chromatin remodelers, which play an important role in telomere maintenance and chromosome integrity [190]. It is a large gene of 300 kb on the X chromosome and germline mutation in *ATRX* leads to X-linked alpha thalassemia mental retardation syndrome whereas somatic mutations are associated with neuroblastomas and gliomas [191].

Fishbein and colleagues reported somatic mutations of *ATRX* in PCC/PGL using whole exome sequencing in 21 fresh frozen tumours/matched germline DNA samples and identified somatic *ATRX* mutations in 9.5% (2/21) of cases. Both these tumours with positive *ATRX* mutations had inherited *SDHB* mutation. Immunohistochemistry was done to confirm the effect of the mutations on the ATRX protein in the tumour tissue. ATRX protein was found to be absent in the tumour cells [191]. To determine the frequency of somatic *ATRX*

mutations in PCC/PGL, the group sequenced the *ATRX* coding region in 2 validation sets of PCC/PGL using amplicon sequencing. Validation set 1 included PCC/PGLs from 52 patients with known inherited mutation status whereas validation set 2 comprised tumours from 51 patients without clinical genetic testing. Overall, 12.6% (13/103) of PCC/PGL harboured *ATRX* somatic mutations. Of these, 31% had truncating mutations and 69% had missense mutations [191].

Although PCC/PGL with *ATRX* variants is too small a sub-group to identify statistically significant associations, some of them had inherited *SDHx* mutations, suggesting an interaction between the somatic and inherited genomes in solid cancers. This needs to be studied further and to identify other somatic alterations involved in PCC/PGL tumorigenesis [191].

2.5.9. *Lysine (K)-Specific Methyltransferase 2D (KMT2D)*

KMT2D (Lysine (K)-Specific Methyltransferase 2D), also known as *mixed-lineage leukaemia 2 (MLL2)*, is a protein coding gene located on 12q13.12 that regulates DNA accessibility. Germline mutations in this gene have been associated with Kabuki syndrome, a developmental disorder characterized by postnatal dwarfism, specific facial features and intellectual disability. Somatic mutations of the gene are linked with medulloblastoma and lymphoma [192].

Whole exome sequencing identified two somatic mutations and one constitutional variant of *KMT2D* in a discovery cohort of 16 fresh frozen PCCs. A validation cohort of 83 fresh frozen PCCs revealed missense variants in 13% (11/83) of the PCCs studied [192]. The study also reported that tumours with *KMT2D* mutations were found to be significantly larger in size than tumours with other known PCC susceptibility gene mutations.

3. Application of immunohistochemistry

It is important that some of the mutated genes in PCC/PGL can be detected by immunohistochemistry. This will allow an economical way of screening for mutations in PCC/PGL after surgical resection. Loss of SDHB protein expression is seen in PCC/PGL harbouring a mutation in *SDHA*, *SDHB, SDHC or SDHD* genes. SDHB is the most common stain being used for screening mutations in the group of *SDH* genes. Also, loss of SHDA protein can be detected by immunohistochemistry. SDHA immunostaining is negative in SDHA-mutated tumours only. The problems of interoperation variability of negative staining and inter-observers' interpretation in immunohistochemical detection of SDHX have been tested by a multi-institional study from seven expert endocrine pathologists in Europe. They have validated the use of SDHB/SDHA immunohistochemistry to identify patients with *SDHX* mutations in 15 centres with a 351 phaeochromocytoma/paraganglioma cohort [193]. In addition, SDHD protein staining is also available [50].

Negative immunohistochemical staining for MAX protein is observed in tumours harbouring only truncating *MAX* mutations [127].

Positive immunohistochemical staining of 2 succinyl cysteine protein can be indirectly used to detect *FH* mutated PCC/PGL [194]. *FH* mutated tumours accumulate fumarate which favours the covalent alteration of cysteine to 2 succinyl cysteine.

ATRX protein in the tumour cells can be detected by immunohistochemistry [191]. Merinoni et al. reported that tumours with somatic *ATRX* mutations showing negative immunohistochemical staining were associated with pooer prognosis than tumours without a mutation [195].

Positive immunostaining using anti-KMT2D antibody can be used to detect the *KMT2D* mutation in PCCs. Juhlin et al. demonstrated positive nuclear staining for KMT2D protein in all the cases of PCCs showing increased copy number of *KMT2D* [192].

4. Utility of Next Generation Sequencing in PCC/PGL

Genetic testing is important in patients with PCC/PGL because one-third of all patients with PCC/PGL have disease causing germline mutations and establishing a hereditary syndrome in the proband may effect in earlier diagnosis and treatment of PCC/PGL in families [196]. Hence genetic testing is recommended in those individuals who are at high risk for susceptibility, which includes positive family history, presence of syndromic features, multifocal tumours, malignancy, PCC/PGL location, or a combination of some of these characteristics [196]. Genetic testing has limited value in patients with unilateral phaeochromocytoma/paraganglioma, non-syndromic, no malignant features and negative family history [196].

In the previous decades, genetic testing for germline mutations in genes predisposing to PCC/PGL involved testing single gene and prediction by clinical features [197]. This testing method is expensive and took a long time to get the results. Advances in massive parallel sequencing technologies such as next-generation sequencing have transformed the practice of DNA sequencing. The new technique allows simultaneous sequencing of multiple genes in a single run at a much lower cost than conventional DNA-sequencing techniques [197]

The application of targeted next generation sequencing methods and whole exome sequencing methods has already been reported in PCC/PGL [18]. Laboratories in America have developed a phaeochromocytoma and paraganglioma panel where next generation sequencing technology is used to detect mutations in 13 PCC/PGL susceptibility genes *(EGLN1, EPAS1, KIF1B, MEN1, MAX, NF1, RET, SDHA, SDHAF2, SDHB, SDHC, SDHD, TMEM127, VHL)* simultaneously.

The National Institutes of Health (NIH)-sponsored TCGA (The Cancer Genomic Atlas) includes whole genomic sequencing analysis of at least 500 samples of PCC/PGL. The data is certain to provide new insights into genomic alterations and will contribute to estimating the added value of whole genome sequencing for diagnostic purposes in PCC/PGL [197]

Next-generation sequencing based on nanopore technology could be used in future for screening of PCC/PGL susceptibility genes. This technology promises affordable and fast genome sequencing by providing long read lengths and does not require additional DNA amplification or enzymatic incorporation of modified nucleotides. This could help health care providers and researchers to decode a genome within hours at a low cost [198].

The implementation of these techniques is challenging as next generation sequencing technology generates large amount of sequence data. Because of this, interpreting the results of next generation sequencing is more complex. Also, addition of newly discovered genes in the targeted screen design can pose technical and economic challenges for implementation [197]. Furthermore, tests to detect mosaic epi-mutations may need to be developed as the current next generation sequencing platforms do not identify hyper methylated areas on target genes. Importantly, these events are reported in the tumorigenesis of PCC/PGL [197].

The final report of a next generation sequencing test should be the result of careful analysis, discussion with geneticists, pathologists and extensive literature searches to determine the classification of variants [18]. Regardless of technical improvements in design of next generation sequencing, some variants still require confirmation by conventional sequencing due to poor coverage. Positive tests should be validated by Sanger sequencing before results are reported to the patient. Hence, no single platform currently fulfils the requirements of an ideal PCC/PGL screening test [197]

5. Potential genetic markers for malignancy

PCC/PGL is usually benign, but about 10% of cases may be malignant. There is a lack of reliable prognostic makers for PCC/PGL [199]. However, some molecular biomarkers such as the Ki67 labelling index, loss of cell adhesion molecules (CD44) and human telomerase reverse transcriptase expression have been proposed as useful markers in detecting malignancy in PCC/PGL [15]

Thompson advocated the first scoring system for predicting malignancy in phaeochromocytoma, known as the PASS (Pheochromocytomas of Adrenal gland Scaled Score) system compiling histological findings to distinguish benign from malignant phaeochromocytoma [14]. The classification was controversial as it comprised of too many histological factors that covered the general features of malignancy rather than specific features of PHEO/PGL [15]. Kimura et al used a system known as GAPP (grading system for adrenal phaeochromocytoma and paraganglioma) to predict the clinical behaviour of PHEO/PGL. The system used parameters of histology, proliferative index and biochemical profiles. Also, GAPP could be used together with the SDHB marker for the prediction of metastasis in PHEO/PGL [15]. The group also demonstrated the ability of GAPP classification to differentiate low-grade malignancies from moderate to high-grade malignancies with different rates of metastasis [15]. Nevertheless, GAPP needed to be validated by pathologists in other centres.

DNA methylation and microRNA expression profiles have also revealed new prognostic markers in PCC/PGL. DNA methylation profiling discovered that RDBP (negative elongation factor complex member E) is related to the presence of metastasis in PCC/PGL. Thus, RDBP could be used for stratifying patients according to the risk of developing metastases [199]. Also, Patterson et al. analysed miRNA expression in benign and malignant phaeochromocytoma tumour samples using whole genome microarray

profiling and found that miR-483-5p, miR-183, and miR-101 had significantly higher expression in malignant tumours as compared to benign tumours. In addition, these miRNAs could be detected in phaeochromocytoma patient serum [200]. Also, study by Castro et al. reported significant upregulation of the miRNA cluster 182/96/183 in metastatic PCC/PGL [201]. Overall, mi-RNAs may be useful for distinguishing malignant from benign phaeochromocytomas

6. Remarks and conclusions

Phaeochromocytoma and paragangliomas are the result of a blend of genetic syndromes and epigenetic changes. This study has for the first time reviewed the roles of all driver genes reported in the pathogenesis of PCC/PGLs. The timelines of discovery of role of these genes in phaeochromocytoma/paraganglioma are summarized in Table 1. The discovery of these genes leads to clarification of many syndromes [202]. Genetic mutations of PCC/PGLs are classified into two major clusters depending on their gene expression profile. The pseudo-hypoxic pathway involving the *PHD2, VHL, SDHX, IDH, HIF2A, MDH2 and FH* gene mutations are attributed to cluster 1 genetic profiling in PCC/PGLs. Genetic pathways in cluster 2 are associated with abnormal activation of kinase signalling pathways and include mutations of *RET*, *NF1*, *KIF1Bβ*, *MAX* and *TMEM127*. In addition, many other genes including *GDNF, H-ras, K-ras, GNAS, CDKN2A, p53, ATRX, BAP1 and BRCA1&2* have also been reported in the development of PCC/PGLs. Mutations in *succinate dehydrogenase B (SDHB)* have been emerging as the most clinically relevant mutation as PCCs and PGLs carrying them have relatively high rates of malignancy, as high as 50%, which has been validated in many studies.

The identification of a germline mutation in patients with PCC/PGL could lead to the early diagnosis of multiple tumours in the settings of syndromic neoplasia. Also, relatives at

risk could be screened for mutations. The identification of a somatic mutation helps in avoiding familial screening and also helps in exploring the possibility of new therapeutic approaches if surgery is not curative [203]. Germline mutations are seen only in *SDHA, SDHC, SDHAF2, FH, KIF1β and TMEM127* whereas somatic mutations are found only in *HRAS*. Both germline and somatic mutations are found in *SDHB, SDHD*, *NF1*, *RET*, *VHL* or *MAX*. Somatic mutation or somatic mosaicism is noted in *EPAS1*. Mutations in other genes, such as *MEN1, EGLN1, EGLN2, MDH2* and *IDH1, BAP1* have been reported in single cases or families suggesting that their role in PCC/PGL is uncertain. In addition, somatic mutations in *ATRX, TP53* and *KMT2D* have been described, but their role is yet to be established.

Advances in sequencing methods such as next generation sequencing as well as familiarity of these genetic mutations and syndromes can help in promoting correct diagnoses and effective genetic advice. Thus, patients with PCC/PGL could be considered for offering genetic screening in appropriate clinical settings. Other genetic changes, pathological parameters and biochemical markers are used for better prediction of the outcome of patients with this group of tumours. Furthermore, this will further help in the development of novel/modified therapeutic approaches in the treatment of PCC/PGLs. In future, knowledge of these genes and advances in gene sequencing method can ensure a more effective detection, prediction of malignant potential and treatment of PCC/PCLs.

Figure legends

Figure 1

Genetic pathways and interactions of cluster 1genes in phaeochromocytoma/ paragangliomas (PCC/PGLs):

Cluster 1 genes are involved with pseudo-hypoxic pathways and are comprised of *PHD2, VHL, SDHX, IDH, HIF2A and FH*. Inactivation of SDH, IDH, or FH are believed to cause a pseudo-hypoxic response due to accumulation of oncometabolites which in turn leads to the activation of HIF-1α target genes such as *EPO*, *VEGF*, *GLUT1* and *P21 ras* resulting in the development of PCC/PGLs. Mutations within the *VHL* and *PHD2* genes result in the absence of functional VHL protein and this can further activate HIF target genes.

Figure 2

Genetic pathways and interactions of cluster 2 genes in phaeochromocytoma/ paragangliomas (PCC/PGLs):

Cluster 2 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 (activating in the case of *RET* and inactivating in the others) in PCCs/PGLs include *NF1, KIF1Bβ*, *MAX/MXD*, *RET*, *TMEM127*. Activation of mTOR may constitute a common mechanism for tumour development caused by mutations in *RET, MAX*, or *TMEM127*. The role of p53 in the development of PCC/PGL is poorly understood and the most likely mechanism would be evasion of apoptosis.

Figure 3

The subdivisions of cluster 1 and 2 molecular pathways of these genes in PCC/PGL

In PCC/PCL, Cluster 1 genes could be divided into 2 groups. Cluster 1A contains PCC/PGL related to *SDHx and FH* while Cluster 1B contains tumours with HIF2A and *VHL* respectively. Cluster 2 can be divided into subcluster 2A, 2B, 2C and 2D. Subcluster 2A comprises *RET*, *MAX*, *NF1* and *TMEM127* mutated tumours whereas subcluster 2B and 2C are sporadic tumours. Cluster 2D are tumours lacking known mutations related to PCC/PGL.

Declaration of interest

There is no conflict of interest in the publication of the manuscript.

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