Recent Advances in Histopathological and Molecular Diagnosis in Pheochromocytoma and Paraganglioma: Challenges for Predicting Metastasis in Individual Patients

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Pheochromocytomas and paragangliomas (PHEO/PGL) are rare but occasionally life-threatening neoplasms, and are potentially malignant according to WHO classification in 2017. However, it is also well known that histopathological risk stratification to predict clinical outcomes has not yet been established. The first histopathological diagnostic algorithm for PHEO, "PASS", was proposed in 2002 by Thompson et al. Another algorithm, GAPP, was then proposed by Kimura et al. in 2014. However, neither algorithm has necessarily been regarded a 'gold standard' for predicting post-operative clinical behavior of tumors. This is because the histopathological features of PHEO/PGL are rather diverse and independent of their hormonal activities, as well as the clinical course of patients. On the other hand, recent developments in wide-scale genetic analysis using next-generation sequencing have revealed the molecular characteristics of pheochromocytomas and paragangliomas. More than 30%–40% of PHEO/PGL are reported to be associated with hereditary genetic abnormalities involving > 20 genes, including SDHXs, RET, VHL, NF1, TMEM127, MAX, and others. Such genetic alterations are mainly involved in the pathogenesis of pseudohypoxia, Wnt, and kinase signaling, and other intracellular signaling cascades. In addition, recurrent somatic mutations are frequently detected and overlapped with the presence of genetic alterations associated with hereditary diseases. In addition, therapeutic strategies specifically targeting such genetic abnormalities have been proposed, but they are not clinically applicable at this
INTRODUCTION

Pheochromocytomas (PHEOs)/paragangliomas (PGLs) or PPGLs are not only oncological diseases due to their invasive or metastatic properties, but also life-threatening endocrinological disorders associated with medical therapy resistant hypertension due to catecholamine excess (1–4). Differentiation between “PHEOs” and “PGLs” is defined based on the sites of the primary lesion as follows; PHEOs are derived from chromaffin cells in the adrenal medulla, and PGLs from sympathetic or parasympathetic paraganglion cells located in extra-adrenal tissues (5).

Distant metastasis is detected in 5%–20% of PHEOs, and relatively higher in PGLs, ranging from 15% to 35% (6–9). The five-year survival rate of metastatic disease has been reported to be approximately 50% or less (10–12). However, it is difficult to predict metastatic potential based on histopathological findings alone, and none of the previously proposed histopathological scoring systems can reach the levels of accurate metastasis prediction. Therefore, all PPGLs were proposed to have malignant potential according to the WHO classification in 2017, because of the absence of hallmark diagnostic markers (5).

In contrast, recent developments in molecular analysis have clarified the genetic landscape or characteristics of PPGLs, which could reflect the risks of metastatic potential (1–4, 6). The results of those studies revealed a higher incidence of genetic abnormalities associated with hereditary diseases, spanning more than 20 relevant genes in > 40% of all cases (1–4, 6). Among the genes above, the presence of SDHX mutations is reported to increase the risks of developing aggressive disease behavior by altering intracellular metabolism, especially the tricarboxylic acid (TCA) cycle (4, 13–17).

In this review, we therefore summarized the previously proposed histopathological/clinicopathological scoring systems, including their limitations for predicting the metastatic potential of the disease, and pitfalls when interpreting the findings. In addition, the clinical significance of recently reported genetic abnormalities and genotype-phenotype associations are also summarized.

GENETIC ABNORMALITIES IN PPGLS

PPGLs were previously called "10%-diseases" associated with hereditary disorders. However, recent developments in genetic analysis using next-generation sequencing and large-scale integrated analysis by The Cancer Genome Atlas (TCGA) database has identified a much larger number of relevant genetic abnormalities (6, 18). The prevalence of PPGLs associated with hereditary diseases involves approximately 40% of all patients (6). Pathogenic variants with genetic alterations in relevant genes are generally exclusive to each other, but it is also true that somatically mutated driver genes are involved in further development of PPGLs in a minor population with germline mutations (6), which is considered unique to this tumor. In addition, comprehensive genetic analysis by Fishbein et al. further demonstrated that 27% of PPGLs have germline mutations, 39% somatic mutations (with 5%–10% overlap with germline mutations), 7% gene fusions, and 89% copy number alterations (6). PPGLs are sub-classified into three different groups, according to their genotype-related pathophysiology (4, 6, 19–21). The most prevalent subtype is the “pseudohypoxia type”, with genetic alterations in SDHX families, FH, VHL, and EPAS1 (13–17, 22–27). The second is the “Wnt-signal type” associated with somatic alterations in genes involved in Wnt-signaling pathways, including CSDE1 mutation and MAML3 gene translocation (6, 28). The third is the “kinase signal type” with genetic alterations involving RET, NFI, MAX, and TMEM127, and which is frequently associated with MEN2 (multiple endocrine neoplasia type 2) gene abnormalities (4, 6, 29–35). In addition, a fourth group was also recently proposed as a cortical admixture subtype, although the detailed features involved have remained uncertain compared to the three major subtypes indicated above (6). Therefore, in this paper, individual genotypes and their pathophysiological characteristics are briefly reviewed. Previously reported genetic alterations associated with PPGLs are also summarized in Table 1.

“Pseudohypoxia Type”

“Pseudohypoxia type” is the most prevalent phenotype in PPGLs, and the great majority of genetic abnormalities involving this phenotype have been detected in genes involved in the TCA cycle, including SDHX family, FH, VHL, EPAS1, SLC25A11, and others (13–17, 22–27). Chromaffin cells are physiologically involved in oxidative metabolism status, with abundant aerobic respiration by mitochondria synthesizing ATP by activating the TCA cycle. However, genetic alterations in genes encoding catalyzing enzymes involved in the TCA cycle, such as succinate dehydrogenase, are known to result in loss of their physiological functions. These altered genes subsequently promote anaerobic metabolism by tumor cells, shifting ATP resources from the TCA cycle into the system of metabolic glycolysis (62–64). These alterations in intracellular metabolism eventually result in degradation of chromatin remodeling, reactive oxygen species production, and DNA methylation (62–66). These intracellular changes also enable tumor cells to efficiently synthesize ATP, although the amounts of ATP synthesized from glycolysis per reaction does not reach...
### TABLE 1 | Previously identified mutated driver genes associated with PPGLs.

| Type | Gene | Coding Protein | Chromosome location | Germiline or Somatic | Predominant tumor site | Contribution to metastatic potential | Associated hereditary diseases | Reference |
|------|------|----------------|---------------------|----------------------|------------------------|---------------------------------------|---------------------------------|-----------|
| 1    | SDHA | Succinate Dehydrogenase Complex Flavoprotein Subunit A | 5p15.33 | Germine | PGL>PHEO | Low | Familial PGL type 5 | (6, 13), |
| 1    | SDHB | Succinate Dehydrogenase Complex Iron Sulfur Subunit B | 1p36.13 | Germine | PGL>PHEO | Intermediate | Familial PGL type 4 | (14) |
| 1    | SDHC | Succinate Dehydrogenase Complex Subunit C | 1q23.3 | Germine | PGL>>PHEO | Very low | Familial PGL type 3 | (15) |
| 1    | SDHD | Succinate Dehydrogenase Complex Subunit D | 11q23.1 | Germine | PGL>PHEO | Low | Familial PGL type 1 | (6, 16) |
| 1    | SDHAF2 | Succinate Dehydrogenase Complex Assembly Factor 2 | 11q12.2 | Germine | PGL>>PHEO | Very Low | Familial PGL type 2 | (17, 36) |
| 1    | FH   | Fumarate Hydratase | 1q43 | Germine | PHEO, PGL | Low | FH-deficient HLRCC (Hereditary leiomyomatosis and renal cell carcinoma) | (37) |
| 1    | VHL  | Von Hippel-Lindau Tumor Suppressor | 3p25.3 | Germine | PHEO>PGL | Low-Intermediate | Von-Hippel-Lindau disease | (6, 25), |
| 1    | EPAS1 | Endothelial PAS Domain Protein 1 (HIF2A) | 2p21 | Germine, Somatic | PHEO, PGL | Low-Intermediate | Pacak-Zhuang syndrome | (6, 26, 27) |
| 1    | EGLN1 | Egl-9 Family Hypoxia Inducible Factor 1 | 1q42.2 | Germine | PHEO, PGL | Not characterized | Polycythemia | (6, 38) |
| 1    | EGLN2 | Egl-9 Family Hypoxia Inducible Factor 2 | 19q13.2 | Germine | PHEO | Not characterized | Polycythemia | (38) |
| 1    | MDH2 | Malate Dehydrogenase 2 | 7q11.23 | Germine | PHEO, PGL | Not characterized | Not characterized | (23, 39), |
| 1    | SLC25A11 | Solute Carrier Family 25 Member 11 | 17p13.2 | Germine | PGL | Not-Intermediate | Low-Intermediate | (40) |
| 1    | DLST | Dihydrolipoamide S-Succinylttransferase | 14q24.3 | Germine | PHEO, PGL | Not characterized | Not characterized | (41) |
| 1    | DNMT3A | DNA Methyltransferase 3 | 2p23.3 | Germine, Somatic | PHEO, PGL | Not characterized | Multiple Myeloid Leukemia (AML) Acute Myeloid Leukemia (AML) | (42) |
| 1    | G0T2 | Glutamic-Oxaloacetic Transaminase 2 Alpha | 16q21 | Germine, Somatic | PHEO, PGL | Not characterized | Not characterized | (44) |
| 2    | CSDE1 | Cold Shock Domain Containing E1 | 1p13.2 | Somatic | PHEO, PGL | Not characterized | --- | (6) |
| 2    | MAML3 | Mastermind Like Transcriptional Coactivator 3 | 4q31.1 | Somatic, Transfusion | PHEO, PGL | Low-Intermediate | --- | (6, 28), |
| 3    | KIF1B | Kinesin Family Member 1B | 1p36.22 | Germine | PHEO? | Not characterized | --- | (45) |
| 3    | RET | Proto-Oncogene Tyrosine-Protein Kinase Receptor Ret | 10q11.21 | Germine, Somatic | PHEO>>PGL | Low | --- | (6, 29–31), |
| 3    | NF1 | Neurofibromin 1 | 17q11.2 | Germine, Somatic | PHEO>PGL | Low | Nuerofibromatosis type 1 | (6, 29–32), |
| 3    | MAX | MYC Associated Factor X | 14q23.3 | Germine | PHEO>PGL | Low | Familial PCC | (6, 34, 35), |
| 3    | TMEM127 | Transmembrane Protein 127 | 2q11.2 | Germine | PHEO>PGL | Low | Familial PCC | (6, 33), |
| 3    | HRAS | GTPase Hras | 11p15.5 | Somatic | PHEO? | Not characterized | --- | (6) |
| 3    | BRAF | Serine/Threonine-Protein Kinase B-Raf | 7q23 | Somatic | PHEO, PGL | Not characterized | --- | (6) |
| Others | MEN1 | Menin 1 | 11q13.1 | Germine | PHEO, PGL | Not characterized | Multiple endocrine neoplasia type 1 | (46) |
| Somatic | IRP1 | Iron Regulatory Protein 1 | 9p21.1 | Somatic | PHEO, PGL | Not characterized | --- | (47) |
| Somatic | SETD2 | Histone-Lysine N-Methyltransferase SETD2 | 3p21.31 | Somatic | PHEO, PGL | Low | --- | (6, 18, 48), |
| Somatic | FGFR1 | Fibroblast Growth Factor Receptor 1 | 8p11.23 | Somatic | PHEO, PGL | Not characterized | --- | (6, 49), |

(Continued)
phenotype.

CSDE1 and transfusion of MAML3 distant metastasis or recurrence, especially in cases involving PPGLs, but the presence of this particular type of genetic

known germline mutations, including VHL, NF1, and RET (6). These CSDE1 genetic alterations result in loss-of-function (6). These CSDE1 gene fusions are reported to be

dysregulated translation initiation, apoptosis, RNA stability, and differentiation/development of neuronal tissue (71, 72). The functional roles of mutated variants of CSDE1 were also previously validated by microarray analysis using mouse embryonic stem cells (73, 74).

PPGLs with MAML3 gene fusions are reported to be associated with a higher prevalence of metastatic diseases, frequently in conjunction with SDH loss (6, 28). Comprehensive genetic analysis revealed that the UBTF-MAML3 fusion gene activates Wnt-Shh signaling, but only a small number of studies have investigated the clinical significance of this chimeric fusion gene (6, 28). Therefore, the detailed underlying mechanisms, as well as their prevalence, have not been thoroughly studied, and further investigations are warranted.

“Kinase Signal Type”
The “kinase signal type” is associated with systemic hereditary diseases such as MEN2A/2B (RET mutation) and neurofibromatosis type 1 (NF1 mutation) (29–32). Familial PHEOs with TMEM127 or TMEM117 mutations are also categorized into this subtype (33–35). Among them, the gain-of-function caused by RET gene mutation has been studied in the most detail. RET encodes a transmembrane receptor tyrosine kinase involved in the development of the neural crest. RET mutations detected in MEN2A are reported to cause homodimerization, which subsequently activates PI3K-akt, ras, p38-mapk, and jun N-terminal kinase pathways in a ligand-independent manner, promoting abnormal cell proliferation (75–77). Recently, somatic mutations detected involving FGFR1, NF1, BRAF, HRAS, and others have also been reported to contribute to the activation of the relevant pathways indicated above (6). However, the underlying

| Type | Gene | Coding Protein | Chromosome location | Germline or Somatic | Predominant tumor site | Contribution to metastatic potential | Associated hereditary diseases | Reference |
|------|------|----------------|---------------------|-------------------|-----------------------|-------------------------------------|-------------------------------|-----------|
| Somatic | MET | Hepatocyte Growth Factor Receptor | 7q31.2 | Somatic | PHEO, PGL | Not characterized | —— | (50) |
| Somatic | TPS3 | Cellular Tumor Antigen P53 | 17p13.1 | Somatic, Germline | PHEO, PGL | Not characterized | Li-Fraumeni Syndrome | (6) |
| Somatic | ARNT | Aryl Hydrocarbon Receptor Nuclear Translocator | 1q21.3 | Somatic | PGL | Not characterized | —— | (6) |
| Somatic | MYOSB | Myosin VB | 18q21.1 | Somatic | PHEO, PGL | Not characterized | —— | (51, 52) |
| Somatic | MYCN | N-Myc Proto-Oncogene Protein | 2p24.3 | Somatic | PHEO, PGL | Not characterized | —— | (51) |
| Somatic | VCL | Vinculin | 10q22.2 | Somatic | PHEO, PGL | Not characterized | —— | (51) |
| Somatic | KMT2D | Histone-Lysine N-Methyltransferase 2D | 12q13.12 | Somatic | PHEO, PGL | Not characterized | —— | (53) |
| Somatic | TERT | Telomerase Reverse Transcriptase | 5p15.33 | Somatic | PHEO, PGL | Low-Intermediate | —— | (54–57) |
| Somatic | ATRX | Transcriptional regulator ATRX | Xq21.1 | Somatic | PHEO, PGL | Low-Intermediate | —— | (6, 36, 58–60) |
| Somatic | IDH1 | Isocitrate Dehydrogenase (NADP(+)) 1 | 2q34 | Somatic | PHEO, PGL | Not characterized | —— | (5) |
| Somatic | IDH2 | Isocitrate Dehydrogenase (NADP(+)) 2 | 15q26.1 | Somatic | PHEO, PGL | Not characterized | —— | (51) |
| Somatic | H3F3A | H3 Histone Family Member 3A | 1q42.12 | Somatic | PHEO, PGL | Not characterized | —— | (50) |

Type 1: Pseudo-hypoxia type, Type 2: Wnt signal type, Type 3: Kinase signal type.

In addition to more than 20 genes with germline mutations, recently detected genes with somatic variants are also summarized in this table. Some genes with somatic variants were classified into three previously known types if the detailed function of the mutated genes was clarified.
mechanisms involving the kinase signaling pathway remain unknown, especially whether these pathways possibly interact with the downstream pathways of other subtypes.

Others (Somatic Abnormalities)
With the exception of three major subgroups, multiple somatic genetic abnormalities have been reported, involving IRP1 (47), SETD2 (6, 18, 48), FGFR1 (6, 49), MET (50), TP53 (6), ARNT (6), MYOSB (51, 52), MYCN (51), VCL (51), KMT2D (53), TERT (54–57), ATRX (6, 57–59), IDH1 (6, 58), IDH2 (36), and H3F3A (50). However, it is also true that majority of newly reported somatic gene abnormalities are detected in only a minor proportion of patients with PPGLs. Among these somatic gene abnormalities, aberrant telomere maintenance mechanism (TMM), which is caused by TERT (telomerase reverse transcriptase) structural rearrangement, genetic abnormalities, and ATRX mutations, has been reported to be associated with poor clinical outcomes in patients (54–57). Structural rearrangement of TERT has also been reported to result in its over-expression as a result of the placement of enhancers proximal to the TERT promoter (56). The presence of somatic mutations detected in the TERT promoter is not necessarily concordant with TERT overexpression, but a specific hot-spot, C228T, is reported to be associated with adverse clinical outcomes in patients (57, 78). However, its cross-interaction with SDHX-related pseudohypoxic pathways cannot be ruled out.

CHALLENGES OF PREDICTIVE CLINICOPATHOLOGICAL/HISTOPATHOLOGICAL SCORING SYSTEMS FOR MALIGNANT BEHAVIOR/METASTASIS IN PPGLS

Histopathological risk stratification, or discerning malignancy, in PPGL patients is very challenging and is generally considered one of the most difficult differential diagnoses in the field of surgical pathology. Several histopathological scoring systems have been proposed, including PASS and GAPP scores, but it is also true that those above could by no means precisely predict the clinical outcome and/or the degree of aggressive clinical behavior in PPGL patients (5, 79–81). As a basis for these two established representative histological scoring systems, several combined scoring systems with genetic abnormalities and immunohistochemical findings have also been recently proposed, such as M-GAPP (Modified-GAPP) score (82), ASES (Age, Size, Extra-adrenal location, and Secretary type) score (83) and COPPs (Composite Pheochromocytoma/paraganglioma Prognostic score) (84). However, further investigations are needed to clarify the practical value of such systems in discerning the clinical behavior of patient tumors.

Therefore, in this section, previously proposed histopathological/clinicopathological scoring systems and the recent validation studies of these systems were covered to clarify the usefulness and limitations of histopathological findings to predict the clinical behavior of tumors, as well as the potential pitfalls involving interpretation of such findings with high inter-/intra-observer variation by both pathologists and clinicians.

PASS (Pheochromocytoma of the Adrenal Gland Scale Score)
PASS was the first histopathological scoring system proposed by the group of Armed Forces Institute of Pathology led by Thompson in 2002, and this system was composed of twelve findings based on histological features as follows (summarized in Figure 1A): 1) large cell nests or diffuse growth of >10%, 2) central or confluent tumor necrosis, 3) high cellularity, 4) cell mononoty, 5) tumor cell spindling (even if focal), 6) mitotic figures >3 figures/10 high power fields, 7) atypical mitotic figure(s), 8) extension into adipose tissue, 9) vascular invasion, 10) capsular invasion, 11) profound nuclear polymorphism, 12) and nuclear hyperchromasia (79). Tumors with 4 points or more were proposed to be associated with a high prevalence of distant metastasis, and those with less than 4 points considered as benign (never metastatic) (79). Of particular note, the use of PASS in extra-adrenal PGLs was limited because this particular scoring system was designed only for PHEOs, and included those criteria only applicable to intra-adrenal tumors such as extension into adipose tissue (81).

After the proposal of PASS, several validation studies were reported in the literature (82, 85–87). The presence of relatively high inter-/intra-observer variation has been reported in the confirmatory studies indicated above. Among those 12 histological features above, the presence of capsular and vascular invasion, extension into adipose tissue, and atypical mitosis could reach relatively high inter-observer concordance in > 80% of the examined cases (88). However, the histological features of high cellularity, profound nuclear polymorphism, and nuclear hyperchromasia resulted in low inter- and intra-observer concordance in their interpretation, even among pathologists with sufficient experience and knowledge in this field (88). Furthermore, it is also pivotal to note that the gradients of scoring points of individual histological features did not necessarily match the degree of inter-/intra-observer variation (88). Scoring systems based only on morphological or histological findings could become more subjective and, therefore, some studies employing combined PASS and genetic abnormality, as well as immunohistochemistry, have been proposed in recent years in order to overcome potential disadvantages or pitfalls of the system, as described above.

GAPP Score (Grading of Adrenal Pheochromocytoma and Paraganglioma) and M-GAPP (Modified GAPP)
The GAPP score was proposed by Kimura et al. in 2014 and required not only morphological findings, but also clinically proven catecholamine-producing types and proliferative ability of tumor cells by Ki-67 (MIB-1) labeling index (LI), in contrast to PASS, which could be performed only on hematoxylin-eosin
FIGURE 1 | Previously proposed histopathological/clinicopathological scoring system. (A) PASS (Pheochromocytoma of the adrenal gland scale score). (B) GAPP (Grading of adrenal pheochromocytoma and paraganglioma). (C) M-GAPP (Modified GAPP). (D) ASES (Age, Size, Extra-adrenal location and Secretory type) score. (E) COPPs (Composite Pheochromocytoma/paraganglioma Prognostic score).
stained tissue slides. This GAPP scoring system classified PPGLs into three different grades: well- (0-2 points), moderately (3-6 points), and poorly differentiated (7-10 points) PPGLs (80). The details of this scoring system are summarized in Figure 1B. The five-year survival rates of these three groups are 100% (well-differentiated), 66.8% (moderately differentiated), and 22.4% (poorly differentiated) (80). GAPP has been used in some diagnostic pathology laboratories, but several limitations or pitfalls have been raised regarding its clinical utility (4, 5, 81). In particular, MEN2A-associated PPGLs are over-diagnosed by both PASS and GAPP in predicting the potential malignant behavior of tumors (85). MEN2A-associated PPGLs rarely metastasize, although large cell nests or diffuse growth patterns (MEN2A-associated: 77% vs. benign: 30%, malignant: 90%) and increased Ki-67 LI of > 3% (MEN2A-associated: 31% vs. sporadic: 11%) are frequently detected in such cases, which result in high scores (85). In addition, the original GAPP system did not include finding regarding SDHX status (80). Therefore, Koh et al. subsequently proposed a modified GAPP score, modifying the gradient of the scoring points, and added the findings of SDHB immunohistochemistry (82). The details of M-GAPP are summarized in Figure 1C. The sensitivity of GAPP and M-GAPP is relatively high, while their specificity only reaches 50%–60% in terms of predicting distant metastasis in PPGL patients (82). The area under the curve (AUC) of these scoring systems resulted in 0.822 for M-GAPP, 0.728 for GAPP, and 0.753 for PASS (82), and there were no differences among the predictive values for patients. Therefore, other clinicopathological factors such as tumor size or patient age should be considered when determining the malignant potential of PPGLs. Further improvements in histopathological evaluation are warranted to more precisely predict the malignant potential of tumors.

**ASSES (Age, Size, Extra-Adrenal Location, and Secretory Type) Score**

ASSES (Age, Size, Extra-adrenal location and Secretory type) scoring was recently proposed by Cho et al. in 2018 (83). They performed a retrospective analysis using a relatively large number of cases, including 333 PPGLs (83). In contrast to other histopathological predictive models, ASES is entirely composed of only 4 clinical parameters (Figure 1D). The AUC to predict malignant behavior is reported to be 0.735 (88), and the practical advantages of using this scoring system includes no requirement for surgical specimens, which could apply this scoring system to all PPGLs, regardless of clinical stage (83). However, the sensitivity and specificity of these histology-based scoring systems remain unknown.

**COPPs (Composite Pheochromocytoma/Paraganglioma Prognostic Score)**

COPPs (Composite Pheochromocytoma/paraganglioma Prognostic score) was recently proposed by Pierre et al. in 2019, integrating morphological features and immunohistochemical findings of S-100 and SDHB (84). They examined a total of 147 PPGLs and performed multivariate analysis, including incorporation of the morphological features listed in PASS, immunohistochemical findings of S-100, Ki-67, and MCM6, clinicopathological factors (tumor size, age, and hypertension) and genotype (84). Finally, COPPs were defined according to the following criteria: three clinicopathological parameters (tumor size > 7 cm, necrosis, and vascular invasion), loss of S-100 immunoreactivity (loss of intervening sustentacular cells), and loss of SDHB immunoreactivity (suggesting SDHB mutation) (84) (Figure 1E). When compared with previously proposed scoring systems, COPPs could provide a high AUC to predict potential metastasis in patients (sensitivity: 100%, specificity: 94.7%) (84). However, prospective validation studies involving COPPs have not been reported, and not all of the parameters proposed in this scoring system are readily available in clinical practice. Thus, COPPs could not reach the levels suitable for practical usage in current clinical settings and awaits validation.

**PRACTICAL IMMUNOHISTOCHEMICAL (IHC) PPGL MARKERS**

In addition to the above previously proposed clinicopathological scoring systems, several immunohistochemical (IHC) markers have also been reported in the literature to be able to differentiate metastatic from non-metastatic PPGLs. In this paper, the practical usefulness of IHC and its limitations and pitfalls in daily clinical settings are summarized.

**Conventional Markers**

SDHB IHC has been employed to detect SDHB gene mutations with relatively high concordance (sensitivity: 100% [95% CI: 87%–100%], specificity: 84% [95% CI: 60%–97%]) as demonstrated by the total absence of immunoreactivity, with positive immunoreactivity in endothelial cells as a positive IHC control (89). However, it is pivotal to note that interpretation of SDHB IHC is sometimes difficult because of the presence of false-negative findings, caused by various pre-analytical factors such as inappropriate fixation, which results in various staining patterns, including potential false-negative findings (89, 90). In particular, patterns of SDHB immunoreactivity with a complete absence, or weak but diffuse dot-like cytoplasmic staining patterns were detected in SDHB-mutated PPGLs (90). Therefore, confirmatory genetic analysis is practically mandatory for cases with equivocal immunoreactivity.

Both S-100 and Ki-67 are well-known and widely used markers for evaluation of the malignant potential of PPGLs (80, 81, 84). S-100 is generally immunolocalized in sustentacular cells surrounding tumor cells (91). Absence or attenuation of S-100 immunoreactivity in endothelial cells is considered to re-flect diffuse growth patterns that deviate from the structure of Zellballen, possibly resulting in the aggressive clinical behavior of tumors (84, 91). However, detailed characterization of sustentacular cells remains to be conducted.
commercially available (94). Antibodies against all four enzymes used for IHC are treating relatively large volumes of patients with PPGLs because they incorporate IHC analysis of these four catecholamine-synthesizing enzymes, histologically representing confluent necrosis, diffuse growth, nuclear polymorphism, and tumor cell spindling (94). Therefore, it is considered worthwhile to incorporate IHC analysis of these four catecholamine-producing enzymes into routine clinical practice in institutions treating relatively large volumes of patients with PPGLs because antibodies against all four enzymes used for IHC are commercially available (94).

**Catecholamine-Synthesizing Enzymes**

In addition to broadly used IHC markers, analyses of hormonal activities and IHC analysis of catecholamine-synthesizing enzymes such as tyrosine hydroxylase (TH), dopamine beta hydroxylase (DBH), dopa decarboxylase (DDC) and phenylethanolamine N-methyltransferase (PNMT) have also been reported in the literature. The expression profiles of these enzymes do not only characterize the secretory phenotypes of norepinephrine or epinephrine, but also reflect differentiation of the tumor cells in PPGLs (80, 92). PNMT catalyzes the final step of catecholamine biosynthesis from norepinephrine into epinephrine. Of particular interest, pseudohypoxic PPGLs are generally negative for PNMT, and have silent clinical and hormonal phenotypes, which could delay therapeutic intervention in such patients (93). Fukaya et al. reported that lower DDC immunoreactivity was detected in poorly differentiated PPGLs, histologically representing confluent necrosis, diffuse growth, nuclear polymorphism, and tumor cell spindling (94). Therefore, it is considered worthwhile to incorporate IHC analysis of these four catecholamine-producing enzymes into routine clinical practice in institutions treating relatively large volumes of patients with PPGLs because antibodies against all four enzymes used for IHC are commercially available (94).

**Newly Proposed Markers**

In addition to the classical markers above, several relatively unique IHC markers have recently been proposed for predicting the presence of distant metastasis in PPGLs. Deng et al. reported lower immunoreactivity of Snail, Galectin-3, and IGF-1R in benign PHEOs without local invasion and distant metastasis, based on a study of 226 PPGL cases (95). Leijon et al. immunolocalized SSTR (somatostatin receptor) family as a potential prognostic factor or a therapeutic target, and reported that 71.4% (10/14) of cases of metastasized PPGLs abundantly expressed SSTR2 (96). Among them, different immunoprofiles were detected between metastasized PGLs and PHEOs (PGLs: 100% (9/9 cases), PHEOs: 20% (1/5 cases). In contrast, SSTR4 and SSTR5 were IHC-negative in the majority of the cases examined, and both SSTR1 and SSTR3 were divergent and independent of SDHX deficiency, as well as the presence of metastases (96). However, the usefulness of somatostatin analogs in the treatment of patients with PPGLs has not been established, and the clinicopathological value of SSTR IHC should be validated by further studies. Surrogate markers associated with tumor immune microenvironmental factors have been studied recently, especially PD-1/PD-L1 in PPGLs (97, 98). Guo et al. examined PD-L1 immunoreactivity in 77 PPGL cases using an anti-PD-L1 antibody (clone EI3N) and reported that 59.74% (46/77 cases) of PPGLs were IHC-positive for PD-L1, with high co-efficiency of Ki-67 LI, as well as the presence of hypertension (97). On the other hand, Pinato et al. examined 100 PPGL cases using the same anti-PD-L1 antibody (clone EI3N) and anti-PD-L2 antibody (polyclonal) (98). They reported that PD-L1 was IHC-positive in 18% (18/100 cases) and PD-L2 in 16% (16/100 cases) of PPGLs, respectively (98). Of particular interest, PD-L2 immunoreactivity in tumor cells was significantly correlated with overall survival of patients in their study (98). The presence of PD-L1 immunoreactivity in tumor cells could potentially indicate the utility of immune-checkpoint inhibitors, but standardization of histopathological evaluation of such markers, as well as unification of IHC antibody clones, are mandatory before various immune checkpoint inhibitors can be used therapeutically in PPGLs. In addition, few studies have reported histopathological surrogate markers of the tumor-immune microenvironment in PGLs, and the clinical therapeutic efficacy of immune-checkpoint inhibitors remains unknown.

In summary, with a possible exception of SDHB, IHC-based analysis was less predictive than genetic analysis and past clinical history of the relevant hereditary diseases, and none of the above could be an independent predictive marker or a therapeutic target molecule. Therefore, future clinical trials as well as investigations of novel therapeutic targets are warranted in PPGLs.

**SUMMARY**

Recent advances in genetic and molecular characterization have classified PPGLs into subgroups based on their genotype-related pathophysiology. These genetic abnormalities are frequently detected in approximately 40% of PPGLs, far more than proposed over the past decades. Among them, SDHX mutations are the most frequently detected, resulting in pseudohypoxic status of tumor cells and which correlate with patient clinical outcomes, especially in detecting metastatic potential. Several histopathological and clinicopathological scoring systems have been proposed, but it is still challenging for diagnostic pathologists to predict malignant behavior based on histopathological findings of resected specimens alone, in contrast to other tumors such as adenocortical neoplasms. Therefore, comprehensive scoring systems, combined with histopathological findings, genotyping, IHC, hormonal activities (metabolic phenotypes), the sites of involvement, and other clinical parameters have recently been proposed in the literature. However, none of the scoring systems reported could reach the necessary levels of practical usage or incorporation into clinical guidelines with high accuracy. In addition, no surrogate markers of specific therapy in patients with PPGL have been identified. Further investigations are required to clarify detailed pathophysiology of PPGLs, as well as more precise patient risk stratification.
AUTHOR CONTRIBUTIONS
All authors contributed to the article and approved the submitted version.

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Yamazaki et al. Updates of PPGL Pathology

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.