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REVIEW

Pheochromocytomas and paragangliomas: assessment of malignant potential

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Abstract Pheochromocytomas and paragangliomas (PPGLs) are rare catecholamine-secreting tumors which arise from the adrenal glands or sympathetic neuronal tissue. Malignant transformation of these tumors occurs in a significant proportion and may therefore lower overall survival rates. In patients with PPGLs it is impossible to identify malignant disease without the presence of metastatic disease, something which can occur as long as 20 years after initial surgery. Early identification of malignant disease would necessitate a more aggressive treatment approach, something which may result in better disease outcome. We have therefore reviewed possible predictors of malignancy and current developments in order to help clinicians to swiftly assess malignant potential in patients with PPGLs. Currently, there is no absolute marker which can objectively reflect malignant potential. Tumor size is the most reliable predictor and should therefore be used as the baseline characteristic. The combination of various clinical markers (extra-adrenal disease and post-operative hypertension), biochemical markers (high dopamine, high norepinephrine and epinephrine to total catecholamine ratio) and/or histological markers (SNAIL, microRNAs and/or microarray results) can raise or lower the suspicion of malignancy. Furthermore, we discuss how clinical markers may affect biochemical results linked to malignancy, how biochemical results may distinguish hereditary syndromes, the role of imaging in determining malignant potential and tumor detection, and recent results of proposed histological markers.

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Introduction

Pheochromocytomas and paragangliomas (PPGLs) are rare catecholamine-secreting tumors that arise from chromaffin cells of the adrenal medulla (pheochromocytoma) or from sympathetic neuronal tissue in extra-adrenal sites (paraganglioma). Paragangliomas of the head and neck usually arise from parasympathetic tissue but often do not secrete catecholamines, and are therefore usually assessed separately. The prevalence of malignancy in PPGLs is reported to range between 2 and 26% [1, 2]. The most common metastatic sites for chromaffin-cell tumors are local lymph nodes, bone, liver, and lung. The 5-year overall survival of patients without metastases has recently been reported as 89.3% [3], whereas patients with metastatic disease exhibit 5-year overall survival rates ranging from 40 to 72% [4, 5]. Moreover, in patients initially diagnosed with benign disease, the main decrease in survival is due to the diagnosis of metastatic disease [3]. It is therefore of paramount importance to diagnose the malignant potential of these tumors before the appearance of metastases. The ability to predict malignancy in these tumors could lead to a more aggressive surgical and/or medical treatment approach which may increase survival rates. In this review, we will describe and analyze factors which have been investigated to predict malignancy in PPGLs, and, in particular, to update these findings with more recent data. We will explore information regarding patient and tumor characteristics, biochemistry, imaging and histological, and molecular markers, to see if we can now make an earlier and more confident prediction of malignant behavior features.

Patient and tumor characteristics

Studies on the prediction of malignancy have focussed on parameters such as age, post-operative symptoms, and tumor location, and pathological features such as tumor type, size, weight, and microscopic features, especially refining previously suggested diagnostic indicators. Some studies have suggested that malignant PPGLs present at a significantly younger age than benign tumors [6–10], although others have found no such correlation [11–14]. Thus, Feng et al. [11] did not find any age differences between 105 benign and 31 malignant tumors, after excluding patients with familial disease who in general present at a younger age. Rather than mean age comparisons, age cut-offs have also been investigated to determine the probability of malignancy. It has been suggested that thresholds of 30 and 40 years [8, 9] are logical as the peak incidence of PPGLs is in the fourth decade, but to confirm such thresholds larger numbers are needed.

It has been generally accepted and more recently confirmed that extra-adrenal PPGLs exhibit a higher chance of malignancy [6, 10, 11, 15, 16], but there is no correlation with left-, right-, or bilateral presentation [9, 11]. Most commonly, extra-adrenal tumors present along the aorta. Jimenez et al. [6] reported that out of 104 extra-adrenal tumors, those located either infra-diaphragmatic, paraaortic, or in the mediastinum showed metastases in approximately 65–70% of patients, compared to 44, 25, and 36% at bladder, adrenal, or other sites, respectively; these findings are in concordance with previous results [15]. In addition, 7 of the 9 metastatic tumors with succinate dehydrogenase-B (SDH-B) mutations were primarily located at extra-adrenal sites, in agreement with recent data on tumor location and poor survival in patients with SDH-B germline mutations [17–19].

Tumor size has often been shown to predict malignancy when comparing average or mean size of benign and malignant tumors [6, 8–11, 14, 20–22]. A cut-off of 5-cm diameter is often suggested. Patients with tumors larger than 5 cm were found twice as likely to eventually present malignant disease [11]. In contrast, an overall survival analysis using the same number showed a significantly higher, but clinically irrelevant chance of death [6]. Moreover, a 5-cm diameter cut-off leads to a false-negative diagnosis in some 10–25%, and indeed malignancy in PPGLS has been reported in tumors as small as 2.8 cm [8]. The same problem occurs when assessing tumor weight, where significant cut-off values of 80 or 150 grams have been suggested [11, 16, 23]. However, tumor size and weight are clearly closely correlated [8, 11].

Some have indicated that patient sex may influence the chance of malignancy but this seems unlikely [16, 24, 25]. By contrast, persistent hypertension post-operatively increases the risk of malignancy (HR 5.3; $P \leq 0.01$) as it may represent occult metastases [11, 23]. At present, one can summarise that tumor size, the presence of extra-adrenal disease and the presence of post-operative hypertension seem to be the only clinical factors which help predict the risk of malignancy in PPGLs. However, while the presence of multiple clinical factors is a good indication for a more aggressive treatment approach, by themselves these factors are insufficient to actually diagnose malignancy.

Biochemistry

Our increasing knowledge of the pathways of catecholamine synthesis and metabolism has enabled us to diagnose PPGLs with greater certainty. By expanding this knowledge to reveal discriminative predictive properties, it was suggested that examination of blood and urine metabolites might also indicate the risk of malignancy. This is particularly the case in recent studies on the now numerous patients with genetic syndromes. The pathways of catecholamine synthesis, starting from tyrosine and ending with epinephrine (EPI), and the different metabolic products, are shown in Fig. 1. Over the years different substances have been used to diagnose PPGLs including dopamine (DA), 3-methoxytyramine, norepinephrine (NE) and epinephrine, and their metabolites normetanephrine (NM) and metanephrine (MN), measured in both blood and urine. More recently, in addition to these substances, other markers such as aromatic L-amino acid decarboxylase (ALAAD), DA β-hydroxylase, and vanillylmandelic acid (VMA) have been investigated to determine putative predictive properties for malignancy.

Chromogranin A (ChrA) is a soluble protein which is co-stored and co-secreted with catecholamines from vesicles in the adrenal medulla and sympathetic nerve endings during exocytosis. The expected positive correlation between ChrA and the size of PPGLs has been shown in the past [26, 27], and some data have also correlated markedly elevated ChrA with malignancy [28, 29]. However, when investigating 39 tumors, van der Harst et al. [30] did not find an association between ChrA and malignancy: 80% (8/10) of malignant tumors (median ChrA 151 U/l; range 17.9–864) and 69% (20/29) of the benign tumors (median ChrA 146 U/l; range 13–2,500) presented with elevated ChrA levels. However, they did show positive correlations between ChrA levels and tumor volume ($r = 0.34$; $P = 0.04$) and especially weight ($r = 0.67$; $P < 0.01$).

Tumors that predominantly or exclusively produce DA are rare. Even so, strong evidence suggests that high levels of DA are predictive of malignancy as this may represent

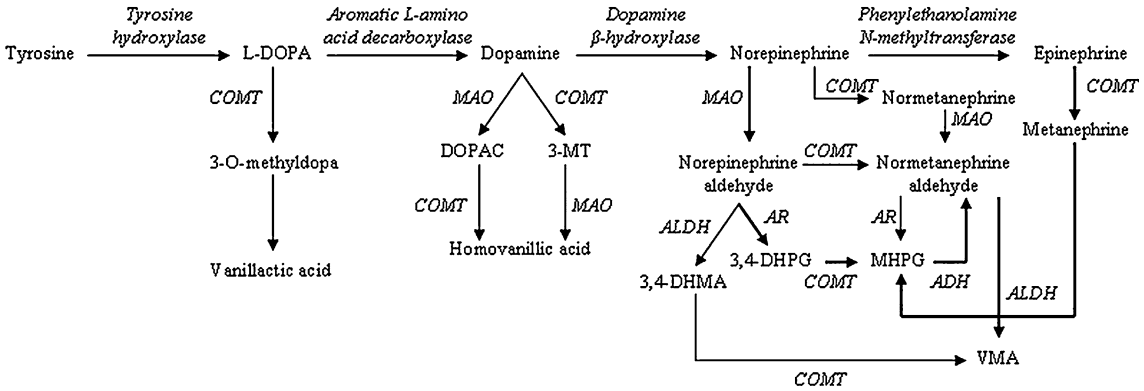

Fig. 1 Catecholamine synthesis and metabolism pathways. *Abbreviations*: *3-MT* 3-methoxytyramine, *3,4-DHMA* 3,4-dihydroxymandelic acid, *3,4-DHPG* 3,4-dihydroxyphenylglycol, *ADH* alcohol dehydrogenase, *ALDH* aldehyde dehydrogenase, *AR* aldehyde reductase, *COMT* catechol-*O*-methyltransferase, *DOPAC* 3,4-dihydroxyphenylacetic acid, *MHPG* 3-methoxy-4-hydroxyphenylglycol, *MAO* monoamine oxidase and *VMA* vanillylmandelic acid

'premature' catecholamine secretion [23, 30–33]. A recent report by Zelinka et al. [9] combined DA-secreting tumors with non-secretory tumors but found no difference between benign and malignant tumors. The probable reason for this may be the low number of patients with SDH-B mutations in their patient population ($N = 2$); such tumors are associated with both higher percentage of malignancy and DA-secreting/silent tumors [34–38]. The group of malignant DA-secreting/silent tumors did, however, show a significantly decreased survival in this study, exhibiting a 5-year survival of around 50% [9].

The lyase enzyme ALAAD is involved in the early stages of the catecholamine producing pathway. Because of its association with recurrence in neuroblastomas, van der Harst et al. collected data regarding ALAAD levels in PPGLs. They found elevations in 6/11 malignant compared to 3/35 benign tumors, with respective median levels of 61 pg/ml (15.6–330.8) and 30 pg/ml (12–236) [30]. The metabolite VMA is produced in the later stages of catecholamine metabolism, and some studies have noted a significant difference in VMA excretion between benign and malignant tumors [11, 22, 23]. However, VMA is elevated in both benign and malignant, is not elevated in EPI-secreting PPGLs [39], and was found to correlate with both NE ($r = 0.64$; $P \leq 0.01$) and DA ($r = 0.67$; $P \leq 0.01$), but not with EPI [30]. Thus, it seems more to reflect the amount of NE which is already measurable.

A significant difference in the elevation of urinary and/or plasma NE between benign and malignant PPGLs has been often reported [9–11, 30]. The lack of locally produced corticosteroids due to the anatomical location of metastatic PPGLs may down regulate phenylethanolamine *N*-methyltransferase (PNMT): thus, higher NE levels might be expected in metastatic disease. Nevertheless, not all reports have shown higher NE levels in malignant PPGLs [23, 40].

As for EPI, reports which show significant differences in malignant PPGLs are sporadic [11], as most reports do not find significant differences [9, 10, 23, 30, 40, 41]. Even so, EPI-positive malignant tumors have been associated with significantly better overall survival [9].

While there is uncertainty as to whether EPI or NE can predict malignancy, two reports have claimed that the ratio of EPI/EPI + NE can be useful, and to date this claim has not been contradicted [10, 30]. Both of these studies also found size (defined as diameter measured post-operatively) to be either predictive of malignancy (malignant 90.9 mm ± 31.1 ($N = 11$) vs. benign 55.7 mm ± 28.6 ($N = 118$); $P \leq 0.01$) [10], or to show a better positive correlation with NE than with EPI (NE $r = 0.42$; $P \leq 0.01$ vs. EPI $r = 0.22$; $P = 0.05$) [30]. This suggests that the ratio of EPI/EPI + NE may rather predict diameter/size than the actual malignancy *per se*. Furthermore, the quantity and production of EPI and NE are PNMT-dependent. Therefore, PNMT itself and also the amount/presence of corticosteroids may particularly affect this ratio. In addition, the levels of corticosteroids, the availability of PNMT and the variable tumor production and secretion of catecholamines will determine the ration of MN to NM. It is, however, worth noting that post-operative DA levels >100 pg/ml, NE levels >1,900 pg/ml, ALAAD levels >56 mU/l and a ratio of EPI/total catecholamines <11% are all associated with significantly decreased metastasis-free survival [30].

Recent findings have shown different levels of biochemical entities according to the various hereditary mutations which are known to cause PPGLs. Patients with multiple endocrine neoplasia 2 and neurofibromatosis 1 mutations have been found to have an 'adrenergic' phenotype, whereas Von Hippel–Lindau (VHL), SDH-B, and SDH-D rarely show increased EPI/MN. If they do, the levels are proportionally smaller than the elevations in NE

[37, 42, 43]. Furthermore, mutations especially of SDH-B have been found to exhibit a 'noradrenergic' and 'dopaminergic' phenotype [36, 37]. Eisenhofer et al. [37] reported on 173 patients with proven familial disease and found that the combined measurement of NE, EPI, and DA has a high predictive value to distinguish those with RET mutations from those with VHL and SDH-B/SDH-D. Using such plasma and urine measurements, the percentages correctly classified were 64–100%, as shown in Table 1.

Table 1 Discriminant analysis for classification of patients according to neurochemical profile

Test or test combination	Percent correctly classified	
	MEN 2 and NF1 vs. VHL, SDHB, and SDHD (%)	VHL vs. SDHB and SDHD (%)
Plasma *O*-methylated metabolites		
NMN	47	60
MN	97	50
MTY	53	78
MN and NMN	99	59
MN and MTY	99	79
NMN and MTY	54	78
MNM and MN and MTY	100	78
Plasma catecholamines		
NE	55	59
EPI	81	46
DA	47	61
EPI and NE	84	60
EPI and DA	82	59
NE and DA	53	65
NE and EPI and DA	85	70
Urine metanephrines		
NMN	50	66
MN	98	57
NMN and MN	98	64
Urine catecholamines		
NE	57	60
EPI	92	62
DA	61	59
EPI and NE	94	61
EPI and DA	93	62
NE and DA	67	62
NE and EPI and DA	94	69

Adapted from Eisenhofer et al. [37]. Reproduced by permission

MEN 2, multiple endocrine neoplasia type 2, *NF1* neurofibromatosis type 1, *VHL* von Hippel–Lindau disease, *SDHB/SDHD* succinate dehydrogenase B/D, *NMN* normetanephrine, *MN* metanephrine, *MTY* methoxytyramine, *NE* norepinephrine, *EPI* epinephrine, *DA* dopamine

Nevertheless, the question as to whether these biochemical findings only reflect the effect of (anatomical) tumor characteristics, or whether they truly predict tumor malignancy, remains unclear. Future studies should incorporate statistical solutions to answer this question. Catecholamine/size ratios and multivariate logistic regression models seem very useful for the distinguishing between size, weight, and biochemical characteristics [11, 23]. Furthermore, most published data do not separate syndromic PPGLs from sporadic PPGLs. Recently, other mutations, such as SDH complex assembly factor 2, flavoprotein SDH complex subunit A (fp) (SDHA), and transmembrane protein 127, have been found to associate with PPGLs. It would appear that biochemical parameters as such are unable to precisely predict malignancy in PPGLs, although they may be useful pointers.

Imaging

Biochemical diagnosis is typically followed by imaging studies because the precise localization of PPGLs is important to determine the correct therapeutic approach. While anatomic location is performed using commonly used imaging modalities such as CT and MRI, PPGLs can be visualized very well via their specific cellular and intracellular characteristics using newer techniques such as [$^{123/131}$I]-metaiodobenzylguanidine (MIBG) scintigraphy, 6-[^{18}F]fluoro-L-3,4-dihydroxyphenyl-alanine (DOPA) positron emission tomography (PET), 6-[^{18}F]fluorodopamine (FDA) PET, 2-[^{18}F]fluoro-2-deoxy-D-glucose (FDG) PET, somatostatin analogs, diffusion-weighted MRI (DWI-MRI) and the combination of PET/CT. There is evidence that a malignant adrenal pheochromocytoma is more likely to appear as a heterogeneous mass on an MRI scan, but the positive predictive value of this is low [42]. Currently, anatomic imaging presents only limited possibilities regarding the prediction of malignancy in PPGLs, but it will aid in the detection of extra-adrenal tumors or metastases. Future imaging prospects such as the detection of apoptosis, oxidative stress, and angiogenesis [43] might allow imaging to play a bigger predictive role.

The established functional imaging modality to detect extra-renal or metastatic PPGLs is [$^{123/131}$I]-MIBG scintigraphy. This type of scan shows reasonable sensitivity, especially for benign PPGLs, and has the advantage of automatically indicating if [^{131}I]-MIBG treatment is possible. However, poor uptake in extra-adrenal tumors and suboptimal or false-negative results in metastatic tumors remains problematic [44–49]. Thus, various forms of PET imaging have been used in attempt to increase tumor detection. However, low availability of PET-imaging compounds and their cost has rendered them less attractive

to clinicians. Of 17 patients presenting with non-metastatic adrenal and extra-adrenal tumors, [^{18}F]-DOPA PET was found to have 100% specificity and sensitivity in one report [50]. However, in malignant and SDH-B-related tumors, [^{18}F]-DOPA PET performs poorly and for such tumors either [^{18}F]-FDA PET or [^{18}F]-FDG-PET is recommended [48, 51–54].

In patients who are suspected to harbor malignant PPGLs, labeled somatostatin analogs such as [^{111}In]-pentetreotide, [^{68}Ga]-DOTATOC and [^{68}Ga]-DOTANOC have shown some promising results, and may present complementary information to [$^{123/131}$I]-MIBG scanning. Somatostatin analogs may especially be sensitive for metastatic and DA-secreting tumors [55, 56]. The disadvantage to the use of modalities which use the NE transporter or somatostatin receptors is that they can become less sensitive if the tumor de-differentiates [44, 49, 57]. This problem does not occur when using whole body DWI-MRI to look for extra-adrenal disease. A study by Takano et al. compared DWI-MRI with [^{123}I]-MIBG scintigraphy and [^{18}F]-FDG-PET, and identified a total of 130 foci. Of these, DWI-MRI, PET-FDG, and MIBG detected 89.2, 80.3, and 68.6%, respectively. The combination of PET-FDG and MIBG found 102/130 foci whereas the combination of DWI-MRI and MIBG found all 130 foci. Furthermore, DWI and PET-FDG showed metastatic lesions in all 11 patients compared to 9 patients with MIBG, and DWI-MRI presented the best results in detecting common metastases in the lymph node; for other tissue specific results see Fig. 2 [58].

In conclusion, the role of imaging in predicting malignancy in PPGLs is very limited. However, on-going technical advances and the development of new and better evidence on existing methods are bound to lead to a bigger contribution in the future.

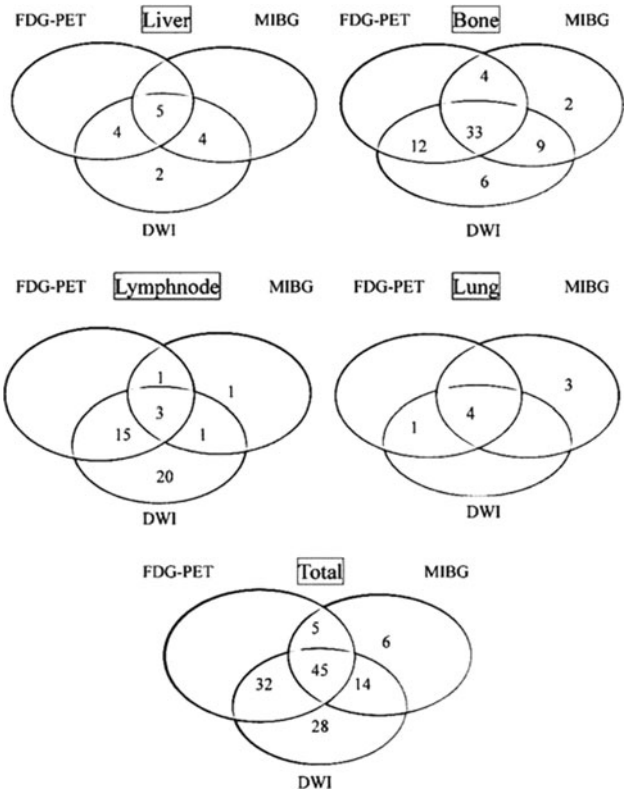

Fig. 2 Number of metastatic lesions depicted by 2-[^{18}F]-fluoro-2-deoxy-D-glucose positron emission tomography (FDG-PET), ^{123}I-metaiodobenzylguanidine scintigraphy (MIBG), and DWI-MRI. Adapted from Takano et al. [58]. Reproduced by permission

Histology

Many types of cancer exhibit discriminative histological features. Unfortunately, this is rarely the case for PPGLs. In order to determine malignancy a wide variety of microscopic, histological and immunohistochemical features have been assessed and incorporated into three multifactorial scoring scales: Linnoila et al. [16] for PPGLs, the Pheochromocytoma of the Adrenal gland Scaled Score (PASS) for sympathetic paragangliomas [12], and a scaling score by Kimura et al. [7] for PPGLs. These scaling scores provide a reasonable indication of malignancy but do not possess enough certainty to provide conformation. Linnoila et al. used a logistic regression analysis of 16 non-histological and histological parameters to show that 4 of them were predictive of malignancy (extra-adrenal location, coarse nodularity of the primary tumor, confluent tumor necrosis and an absence of hyaline globules). However, their model based on these markers showed that only approximately 70% of tumors could be classified correctly. The cut-off point for the PASS scoring scale has also been questioned ($\geq$4 vs. $\geq$6) [8], and significant intra- and inter-observer variations have been reported [59]. The scaling system by Kimura et al., which divides tumors into three groups (well-, moderately- and poorly differentiated tumors), showed a correlation with both metastatic potential and patient survival. Even so, since 13% of the 'well differentiated group' also showed metastatic disease its discrimination is not adequate enough for common use.

In the past, a wide variety of microscopic and (immuno) histological markers have been found to distinguish benign from malignant PPGLs, a selection of which is shown in Table 2. Because of this large number, we will discuss the more recent reference points and focus on new techniques such as gene expression profiling and (micro)RNA arrays.

In order to identify molecular markers for malignancy in PPGLs, several gene expression profiling studies have been performed [60–63]. These studies have identified sets of genes known to encode for various proteins which may be used as markers for malignancy. Interestingly, over 80% of these genes were down-regulated, which has led to the

Table 2 Previously investigated markers of malignancy in PPGLs

Marker	Predictive potential[a]	Sample[b]	*P*	Reference
Mean MIB-1 (%)	0.2% (0 ± 2.5%) vs. 3.0% (0 ± 12%)	93/36	S	van der Harst et al. [13]
	1.28 (±1.172) vs. 10.81 (±8.784)	28/9	S	Boltze et al. [24]
HSP-90 score	4.50 (±1.380) vs. 10.22 (±2.710)	28/9	S	
Telomerase	0% vs. 100%	28/9	S	
Necrosis	23% vs. 44%	97/34	S	van der Harst et al. [13]
Vasoinvasion	29% vs. 43%	96/32	NS	
Capsular ingrowth S100 positive sustentacular cells	No positivity: 6% vs. 42%	69/26	S	
	Sporadic: 26% vs. 19%			
	Moderate: 51% vs. 39%			
	Abundant: 16% vs. 0%			
Ki-67	Sensitivity 38% vs. 100% specificity	21/13	S	Elder et al. [85]
	40% vs. 5%	40/5	NS	Strong et al. [8]
Mean VEGF	1.4 (±0.43) vs. 3.1 (±0.24)	10/6	S	Favier et al. [86]
Mean VEGFR 2	2.1 (±0.27) vs. 3.1 (±0.27)	9/6		
Heparanase-1	37% vs. 100%	19/10		Quiros et al. [87]
	PPV: 0.44 and NPV: 0.94	76/18	S	Zhu et al. [14]
COX-2	PPV: 0.45 and NPV: 0.95			
N-Cadherin	44% vs. 100%	27/8	S	Khorram-Manesh et al. [88]
Cyclin D1	70% vs. 60%	40/5	NS	Strong et al. [8]
P53	2.5% vs. 0%			
P27	2.5% vs. 0%			
Bcl-2	7.5% vs. 0%			
P21	10% vs. 20%			
mdm2	10% vs. 0%			
ERBB-2	+++ 29% vs. 14%[c] vs. 0%	25/7[c]/7	S	Yuan et al. [89]
	++ 57% vs. 29%[c] vs. 28%			
	+ 14% vs. 0%[c] vs. 20%			
	− 0% vs. 57%[c] vs. 52%			
Tumor suppressor genes: combination of: nm23-H, TIMP-4, CRSP-3, E-cadherin	Sensitivity 100% vs. 80% specificity[d]	15/10	S	Ohta et al. [90]
Stathmin	++ 12.5% vs. 0%	19/10	–	Bjorklund et al. [91]
	+ 25% vs. 16%			
	± 54% vs. 37%			
	− 8.5% vs. 47%			

S significant, *NS* nonS, *HSP-90* heat shock protein-90, *VEGF* vascular endothelial growth factor, *VEGFR 2* VEGF receptor 2, *HIF 2-α* hypoxia-inducible factor 2-α, *COX-2* cyclooxygenase-2, *Rb* retinoblastoma, *mdm2* mouse double minute 2, *ERBB-2* erythroblastic leukemia viral oncogene homolog 2, *nm23-H* non-metastatic clone 23 gene, *TIMP-4* tissue inhibitor of metalloproteinase-4, *CRSP-3* cofactor required for Sp1 transcriptional activation, subunit 3, *PPV* positive predictive value, *NPV* negative predictive value

[a] Predictive potential showed as positivity in benign versus malignant samples, +SD

[b] Tumors shown as the sample size of benign/malignant tumors per marker

[c] Extra-adrenal site

[d] Calculated using a threshold according to median malignant value

hypothesis that malignant PPGLs are less differentiated and may arise from immature neuroendocrine precursor cells, this being supported by other findings [63–65]. In view of the large number of genes which are differentially expressed, Waldmann et al. looked at the overlap of the two largest studies. The authors compared their list of genes to that of Brouwers et al. [61, 63] and noted 35.9% (704/1,962) of up-regulated genes and 32% (886/2,691) of down-regulated genes to be in common. Furthermore, combining both analyses, they found a total of 39 genes that were significantly expressed, 18 of which were found significantly expressed in both separate analyses.

Interestingly, the vast majority of the overlapping down-regulated genes were cation binders or played a role in the cell cycle. The comparison of these studies is shown in Fig. 3.

The zinc-finger transcription factor SNAIL has been described as regulating the expression of genes which enable metastatic spread and tumorigenesis [66–68]. Hayry et al. [66] found SNAIL expression to be undetectable in 18/32 non-metastatic tumors compared to none of the metastatic tumors. Specimens exhibiting low or moderate SNAIL expression were seen in 13/32 non-metastatic samples and in 8/18 metastatic samples. The group which presented with a high SNAIL expression profile consisted of 10 metastatic and 1 non-metastatic tumor samples. With a cut-off value at >70% immunoreactive cell nuclei, metastatic disease was detected with a sensitivity and specificity of 50 and 97%. Waldmann et al. [67] found 17% of the benign tumors to express SNAIL compared to 58% positive expression in the malignant group. Furthermore, they also showed that *Twist*, also a transcriptional repressor of E-cadherin which has been found to be over-expressed in many cancer types, was expressed in 57% of benign and 92% of malignant tumors. The combination of SNAIL and *Twist* expression predicted malignancy with a sensitivity and specificity of 66 and 93%, respectively [67].

Interestingly, Waldmann et al. observed that SNAIL expression was mostly restricted to peripheral areas representing the invasive front, or the site where malignant cells invaded bordering non-malignant tissue. This has also been described in adrenocortical carcinomas, malignant parathyroid neoplasia, and neuroendocrine tumors [69–71]. Moreover, both *Twist* and SNAIL are considered to play a pivotal role in tumor progression and spread by promoting the down-regulation of E-cadherin and inducing the epithelial-to-mesenchymal transition [72]. Taken together, SNAIL alone or in combination with *Twist* seems to be a valuable determinant in predicting malignancy in PPGLs. While more data are needed to confirm the results, SNAIL and *Twist* expression seems to be a sensitive and especially specific marker which may be able to be put to good use in malignancy determining scaling score systems.

Elevated expression of hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) are associated with higher tumor aggressiveness and worse outcome in many cancers. Both sporadic and VHL or SDH-*x*-related PPGLs may present pseudo-hypoxic phenotypes which may lead to uncontrolled expression of the antisense transcript of hypoxia-inducible factor-1 (aHIF) and cluster specific genes such as VEGF, aquaporin 3, cytochrome b561, p57Kip2, slit homolog 3 and SDH-C [73–75]. Because aHIF inhibits the translation of HIF-1α mRNA (messenger RNA) during sustained hypoxia, over-expression of aHIF is a sign of sustained hypoxia [76]. Furthermore, aHIF per se has been associated with aggressive tumor behavior and increased metastatic potential in breast cancer [77]. In order to asses these findings in PPGLs, Span

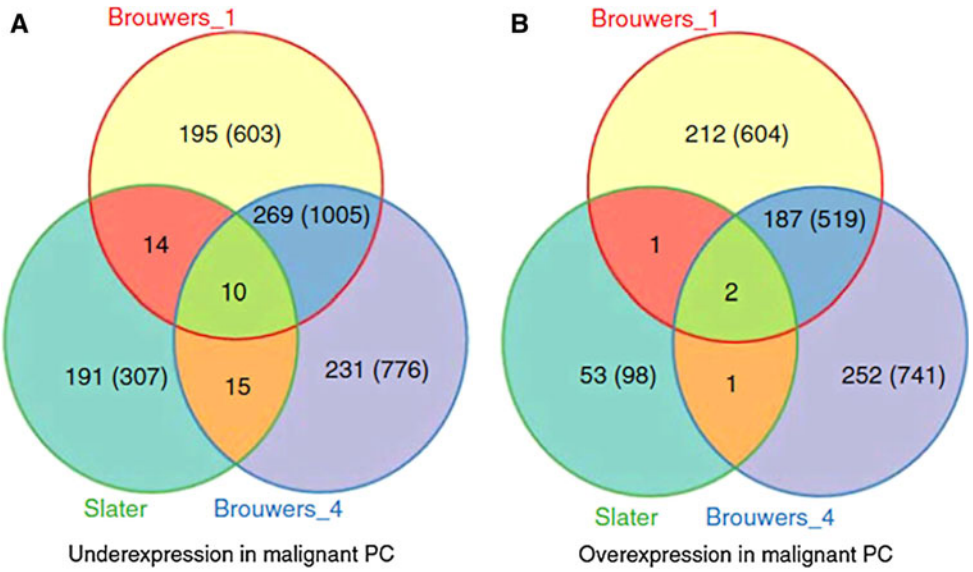

Fig. 3 Venn diagram with the overlap between the microarray of Waldmann et al. [63] and the NIH dataset of Brouwers et al. [61]. On the *left diagram* underexpressed genes in malignant PPGLs, on the *right* overexpressed genes in malignant PPGLs are shown. Brouwers 1 and 4 represent all malignant tumors [1] and all primary malignant tumors [4]. Slater represents data of this study. Colors (*yellow*, *purple*, and *turquoise*) are assigned to genes, which are underexpressed or overexpressed without an intersection between the three groups (Slater, Brouwers_1 and 4). Overlapping genes are indicated by the colors *orange*, *green*, *red*, and *blue*. The *numbers* of genes which are assessed by both chips (Slater/Brouwers) are shown without parentheses. If genes were only on the array of Brouwers et al., number is given in *parentheses*. Adapted from Waldmann et al. [63]. Society for Endocrinology© (2011). Reproduced by permission

et al. [78] performed quantitative real-time poly chain reaction (PCR) on the described markers ($N = 87$, 7 metastases; median follow-up 7.04 years). Their results showed that aHIF and VEGF were over-expressed in tumors with a pseudo-hypoxic signature due to SDH-*x* and VHL mutations, whereas slit homolog 3, p57Kip2, cytochrome b561, and SDH-C were found to be over-expressed in non-metastatic tumors. Furthermore, they reported a negative correlation with metastasis-free survival of both aHIF and VEGF over-expression, but a positive correlation with high SDH-C expression levels was found. However, the highly variable expression of aHIF makes its use as a malignancy marker doubtful, and therefore the authors conclude that larger cohorts with longer follow-up are needed to confirm the metastatic potential of PPGLs with over-expressed aHIF.

The peptidylglycine α-amidating monooxygenase (PAM) enzyme, which is responsible for the stability and activity of pituitary adenylate cyclase-activating polypeptide, neuropeptide Y and adrenomedullin (AM), has shown differential expression between malignant and benign pheochromocytomas in the past [62]. These proteins may influence tumor behavior as they exert proliferative and/or anti-apoptotic effects. Specifically, the proposed actions of the AM peptide include vasodilatation, increased diuresis and urinary Na^+ secretion [79]. Furthermore, it can exert local pro-proliferative and anti-apoptotic actions on several cell types, and it is involved in tumoral neoangiogenesis [80, 81]. When looking at these trophic-amidated peptides and their receptors, Thouennon et al. [82] showed that the AM receptor RDC1 exhibited a 4-fold higher expression in malignant compared to benign PPGLs. They then showed that AM can increase the number of tumor cells in primary cultures and that it exerts a pro-survival effect in rat pheochromocytoma cells. After reducing the receptor expression in PC12 cells by 80%, they showed a decrease in the number of cells after 2 days. It would be interesting to see the further therapeutic development and the discriminative characteristics of both PAM and RDC1 regarding malignancy in PPGLs in larger studies.

MicroRNAs are small, single-stranded non-protein-coding RNA fragments which can negatively regulate protein expression via either cleavage or translational repression of mRNA. Recently, many (endocrine-related) cancers have been shown to exhibit altered microRNA expression between tumoral and normal tissue, and also between benign and malignant tumors, but no reports on their expression in PPGLs existed until a very recent study. Meyer-Rochow et al. [83] investigated 12 malignant and 12 benign tumors, and compared these with 5 normal adrenal medulla samples via microarray expression profiling. They found a total of 18 microRNAs to be differentially expressed between benign and malignant samples. Unsupervised hierarchical clustering correctly divided all but one of the benign, and all but two of the malignant samples into two different groups. They then validated the results of three microRNAs (miR-15a, miR-16, and miR483-5p), which were reported to be involved in the pathogenesis of other cancers via real-time quantitative PCR in their own cohort of 24 tumors and in an external cohort of 20 samples (10 benign, 10 malignant). In the external cohort, miR-15a and miR-16 expression was significantly reduced in malignant compared to benign tumors ($P < 0.01$ and $P = 0.03$). Furthermore, increased expression of miR-483-5p was found in the malignant tumors although this was not significant ($P = 0.2$). Nonetheless, when they included both cohorts miR-483-5p did show significantly increased expression in the malignant tumors, and also between malignant tumors and the adrenal medulla (both $P < 0.01$).

Interestingly, the genomic location or miR-483-5p suggests a high probability of co-expression with insulin-like growth factor-2 (IGF-2), which has also been observed in adrenocortical carcinomas [84]. Measurement of IGF-2 mRNA showed a significant increase of IGF-2 in malignant compared to benign tumors ($P < 0.01$). This was supported by a significant positive Spearman correlation coefficient of $+0.72$ ($P < 0.01$). Furthermore, immunohistochemical staining for the IGF-2 protein of 15 benign and 10 malignant tissue samples showed that strong staining was present in 80% of the malignant compared to only 27% in the benign tumors. After choosing certain quantitative cut-off points for miR-15a and IGF-2, a sensitivity of 80% and specificity of 100% was found. Immunohistochemical staining of IGF-2 alone provided a sensitivity and specificity of 80 and 73%. Thus, these data suggest that the use of microRNAs in general, together with mRNA expression and immunohistochemical staining of IGF-2, may be valuable markers for determining malignancy in PPGLs. Further investigations in larger cohorts are necessary to confirm these promising results.

Conclusions

In conclusion, there are considerable data on many different proposed markers for malignancy in PPGLs. Currently; the most reliable predictors are still the size and location of the tumor. It seems that most of the proposed biochemical examination factors, such as high NE levels, high ChrA levels and a low EPI/EPI + NE ratio, also reflect these characteristics. Increased production of DA is uncommon; however, when present it does seem to predict a worse degree of tumor dedifferentiation compared to NE-producing tumors. Anatomical imaging has only a small contribution as it is mainly useful in localizing the tumor

rather than identifying malignancy as such. Nevertheless, new and promising technological advancements may expand the predictive role of imaging. Various histological markers have been shown to possess discriminative properties. However, these results await validation in larger trials with longer follow-up periods as PPGLs may only exhibit their malignant potential after many years.

The development of a gold standard which will allow physicians to be more certain as to whether or not a pheochromocytoma or paraganglioma is malignant and/or will cause metastases is very important. The best, and probable quickest, way to achieve this will be via a systematic approach which uses a combination of clinical, biochemical, imaging, and histological factors. Multiple known mutations have been found to present various differences and it will be important to discriminate familial from sporadic disease accordingly.

Clinically, the assessment of malignant potential in any case of confirmed PPGLs should consist of a carefully weighted combination of all the markers we have discussed. However, as of now, no marker is 100% predictive and therefore we suggest that the principal baseline characteristic should still be size and other markers may be added to either raise or lower the suspicion of malignant disease accordingly. Specific situations such as a high NE/ChrA/DA without a large primary tumor or radiographic distant disease and postoperative hypertension should determine further investigation, as should any particular germline mutation.

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