Pathological grading for predicting metastasis in phaeochromocytoma and paraganglioma

Noriko Kimura, Ryoichi Takayanagi, Nae Takizawa, Eiji Itagaki, Takayuki Katabami, Narihiko Kako, Hiromi Rakugi, Yukihiro Ikeda, Akiyo Tanabe, Takeshi Nigawara, Sadayoshi Ito, Itaru Kimura, Mitsuhide Naruse and The Phaeochromocytoma Study Group in Japan

Pathology Division, Department of Clinical Research, National Hospital Organization, Hakodate Hospital, Postal code 041-8512, 18-16 Kawahara, Hakodate, Hokkaido, Japan
1Department of Medicine and Bioregulatory Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan
2Department of Urology and Andrology, Kansai Medical University, Osaka, Japan
3Third Department of Internal Medicine (Diabetes, Endocrine and Metabolism), School of Medicine, Kyorin University, Tokyo, Japan
4Department of Medicine, Metabolism and Endocrinology, School of Medicine, St Marianna University, Kawasaki, Japan
5Department of Urology, Miyagi Cancer Center, Natori, Japan
6Department of Geriatric Medicine and Nephrology, Graduate School of Medicine, Osaka University, Osaka, Japan
7Division of Nephrology, Hypertension and Endocrinology, Department of Medicine, Nihon University Itabashi Hospital, Tokyo, Japan
8Department of Endocrinology and Metabolism, Tokyo Women’s Medical University, Tokyo, Japan
9Department of Endocrinology and Metabolism, School of Medicine, Hiroshi University, Hiroshi, Japan
10Division of Nephrology, Endocrinology and Vascular Medicine, Department of Medicine, School of Medicine, Tohoku University, Sendai, Japan
11Council Member Examination Committee of Social Insurance, Ministry of Health, Labour and Welfare, Tokyo, Japan
12Department of Endocrinology, Metabolism, and Hypertension, National Hospital Organization, Kyoto Medical Center, Kyoto, Japan
(The members of The Phaeochromocytoma Study Group in Japan are listed in the ‘Acknowledgements’ section)

Correspondence should be addressed to N Kimura
Email kimura-path@hnh.hosp.go.jp

Abstract

Phaeochromocytomas (PHEO) and paragangliomas are rare catecholamine-producing tumours. Although 10–30% of these tumours metastasise, histopathological criteria to discriminate malignant from benign tumours have not been established; therefore, reliable histopathological markers predicting metastasis are urgently required. A total of 163 tumours, including 40 metastatic tumours, collected by the Phaeochromocytoma Study Group in Japan (PHEO-J) were analysed using a system called grading system for adrenal phaeochromocytoma and paraganglioma (GAPP). The tumours were scored based on GAPP criteria as follows: histological pattern, cellularity, comedo-type necrosis, capsular/vascular invasion, Ki67 labelling index and catecholamine type. All tumours were scored from 0 to 10 points and were graded as one of the three types: well-differentiated (WD, 0–2 points), moderately differentiated (MD, 3–6 points) and poorly differentiated (PD, 7–10 points). GAPP scores of the non-metastatic and metastatic groups were 2.08 ± 0.17 and 5.33 ± 0.43 (mean ± S.E.M., P < 0.001) respectively. There was a significant negative correlation between the GAPP score and the interval until metastasis (r = −0.438, P < 0.01). The mean number of years until metastasis after the initial operation was 5.5 ± 2.6 years. The study

Key Words
- phaeochromocytoma
- paraganglioma
- histopathological diagnosis
- succinate dehydrogenase gene subunit B
- immunohistochemistry
- metastasis
- survival

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included 111 WD, 35 MD and 17 PD types. The five-year survival of these groups was 100, 66.8 and 22.4% respectively. In addition, negative immunoreactivity for succinate dehydrogenase gene subunit B (SDHB) was observed in 13 (8%) MD or PD tumours and ten of the 13 (77%) had metastases. Our data indicate that a combination of GAPP classification and SDHB immunohistochemistry might be useful for the prediction of metastasis in these tumours.

**Introduction**

Phaeochromocytomas (PHEO) of the adrenal gland and sympathetic paragangliomas (PGL) are catecholamine-producing tumours. Although 10–30% of these tumours metastasise, histopathological criteria that discriminate malignant from benign tumours have not been established and only the presence of metastasis is considered evidence of malignancy in the current WHO definition (Thompson et al. 2004). Although most cases of PHEO/PGL are surgically curable, malignant PHEO/PGL are intractable diseases that require an early diagnosis and effective treatment. We organised a task force group for PHEO/PGL in Japan, the Phaeochromocytoma Study Group in Japan (PHEO-J) that was composed of endocrinologists, urologists, endocrine surgeons, radiologists, molecular biologists and pathologists and was supported by the Ministry of Health, Labour, and Welfare. The aim of the study was to survey PHEO/PGL in Japan. Based on the results of PHEO-J in 2012, the estimated total number of patients with PHEO/PGL was 2920, including 320 patients with metastasis. The proportions of patients with malignant, multiple, extra-adrenal and familial PHEO/PGL were 11, 12.7, 17.3 and 10% respectively (Naruse & PHEO-J Study Group 2011). Among patients with malignant PHEO/PGL, 36.8% were initially diagnosed as benign and 59.6% showed absence of metastasis at initial operation (Naruse & PHEO-J Study Group 2011). The results indicated the difficulties associated with differential diagnosis of malignant from benign disease in the absence of distant metastases and strongly emphasised the need to establish reliable histopathological criteria for predicting metastasis.

Many studies have attempted to discriminate benign and malignant PHEO/PGL. Of these, some molecular biomarkers such as the Ki67 labelling index (LI; Nagura et al. 1999, Elder et al. 2003), loss of cell adhesion molecules such as CD44 and human telomerase reverse transcriptase expression (van der Harst et al. 2000, Elder et al. 2003, August et al. 2004) have been proposed as useful markers for the detection of high-grade malignancy. However, these markers failed to detect low- and moderate-grade malignant PHEO/PGL. Although the presence of only one indicator does not allow a definite diagnosis of malignancy in PHEO/PGL, certain pathological features, such as size and site (O’Riordain et al. 1996), local extension, angio-invasion, mitotic index/proliferative activity using Ki67 LI, irregular zellballen pattern, and presence of confluent (comedo-type) tumour necrosis (Zelinka et al. 2011), have been used in previous studies (Kimura & Sasano 1990, Linnoila et al. 1990, Unger et al. 1991). Such features have been combined in a scoring system named the Phaeochromocytoma of the Adrenal Gland Scaled Score (PASS) for use in diagnosis (Thompson 2002). PASS was the first scoring system for diagnosis of adrenal PHEO. However, the reproducibility and clinical significance of PASS have not been established (Wu et al. 2009), probably because the PASS classification contains too many histological parameters that cover classical features of general malignancy rather than focusing on specific features of PHEO/PGL. In general, for endocrine tumours, the biological behaviour of the tumours usually reflects the differentiation of hormone-producing functions. Cell maturation and cell proliferation in endocrine tumours usually oppose each other; poorly differentiated (PD) tumours grow rapidly and cause poor prognosis whereas well-differentiated (WD) tumours grow slowly and metastasise later. The distinction between PD and WD tumours is important for the determination of a patient’s prognosis. Kimura et al. (2005) presented a grading system for PHEO/PGL based on the concept that norepinephrine-producing tumours are less differentiated than epinephrine-producing tumours. However, this study was a report from a single institute and a multi-centre study is required. Here, we examined the materials gathered from a nationwide survey by PHEO-J using the previously described grading system, which we have named the grading system for adrenal phaeochromocytoma and paraganglioma (GAPP) to determine whether GAPP has clinical applications.
Recently, there has been rapid progress in the molecular analysis of PHEO/PGL and around 16 genes responsible for PHEO/PGL have been discovered: NF1, von Hippel–Lindau disease (VHL), RET, SDHC, SDHD, SDHB, SDHAF2, SDHA, TMEM127, MAX, IHD1, KIF2, HRAS, HIF2, PHD2 and FH (Gimenez-Roqueplo & Tischler 2012, King & Pacak 2013, Castro-Vega et al. 2014). Of these, mutations in the succinate dehydrogenase genes (SDHA, SDHB, SDHC and SDHD) are responsible for a large percentage of hereditary PHEO/PGL syndrome (HPPS) cases, and it has been reported that an extra-adrenal site, recurrence and malignancy are strongly associated with the SDHB mutation (Gimenez-Roqueplo et al. 2003, Neumann et al. 2004). In this study, SDHB gene analysis was not available because of ethical considerations; however, immunohistochemical studies of SDHB were carried out using central pathological analysis. We attempted to clarify the features of metastatic and non-metastatic tumours using GAPP classification and SDHB immunohistochemistry to facilitate the differential diagnosis of malignant from benign disease in PHEO/PGL.

**Subjects and methods**

**Patients and tissues**

A total of 994 patients from 178 institutes registered online. For ethical reasons, the registration was limited to adult patients. All data on patients were sent from each institute via a registration form. There were two types of forms: clinical registration and data for pathological analysis. Briefly, a clinical registration form was composed of date of informed consent, patient birthday, year, age, sex and present state (alive or dead), which were entered at the first registration. If there was no problem with registration, then the second step of registration started. Clinical information was composed of i) year and day of initial diagnosis, ii) family history, iii) clinical diagnosis at present, adrenal, extra-adrenal or unknown and also benign or malignant or unknown for each tumour, iv) location of tumours, v) number of tumours, single or multiple or unknown and vi) metastasis: absent, present or unknown. This survey research was carried out three times with 6-month intervals. The last time of follow-up was the time of the last registration. Metastasis was confirmed by imaging such as computed tomography (CT), magnetic resonance imaging (MRI) or iodine-131-meta-iodobenzylguanidine (MIBG) scintiscan, catecholamine analysis and operation if possible.

For pathological analysis, patient clinical information such as i) clinical diagnosis at the time of registration, ii) age and sex, iii) year of initial operation, iv) location of tumours, v) tumour size, vi) number of tumours, vii) metastasis: absence or presence (lymph node, bone, lung, liver and others, including what location and how many), viii) year and date of metastasis and ix) catecholamine data: plasma/urine, adrenalin, noradrenalin and dopamine were supplied.

Among these registrations, tumour specimens from 163 patients were voluntarily submitted for pathological analysis, including 123 without metastasis and 40 with metastasis. The mean age of patients was 50.7 ± 15.5 years (range: 21–80 years). The duration of follow-up ranged from 1 to 33 years with mean follow-up duration of 6.61 ± 0.74 (P < 0.01, Grubbs–Smirnov examination). Twelve patients died of multiple metastases. The locations of the primary tumours were as follows: 127 in the adrenal glands, including 13 that occurred bilaterally, and 36 in extra-adrenal regions (28 in the retroperitoneum and eight in the urinary bladder).

The surgically removed tumours were fixed in 10% formalin and embedded in paraffin. Each institute prepared ten unstained glass slides with 3-μm thick tissue sections of the representative tumour tissues. These slides were sent to the National Hospital Organisation Hakodate Hospital for central pathological analysis. All pathological materials were associated with patients’ clinical data, photographs of gross and cut findings of the tumours and original pathological diagnoses from each institute to provide information such as vascular or capsular invasion or histological variation that might be lacking in the section sent to Hakodate Hospital due to sampling errors.

**Pathological analysis**

All sections were subjected to haematoxylin and eosin staining, elastica-Masson trichome staining to confirm vascular invasion and immunostaining for chromogranin A to confirm PHEO/PGL, Ki67 for proliferating cells, S100 protein for sustentacular cells, D2-40 to confirm lymph vessel invasion and SDHB for HPPS. Immunostaining for phenylethanolamine N-methyltransferase (PNMT) was performed in cases of epinephrine-producing extra-adrenal PGL. Immunohistochemical procedures were performed using an automated immunostainer (Benchmark, Ventana, Tucson, AZ, USA) according to the manufacturer’s protocol. The primary antibodies and final dilutions were as follows: chromogranin A (monoclonal, 1:200; Dako, Carpinteria, CA, USA), Ki67 (MIB1, monoclonal, prediluted; Novocastra, Burlingame, CA, USA), S100 (polyclonal, prediluted; Novocastra), D2–40 (monoclonal, prediluted;
Nichirei Bioscience, Tokyo, Japan), SDHB (FL-280, polyclonal, 1:50, Santa Cruz Biotechnology, Inc. and HPA002868, polyclonal, 1:50, Sigma–Aldrich, Inc.) and PNMT (polyclonal, 1:500, Bioclone, Marrickville, New South Wales, Australia). The appropriate positive and negative controls were included in parallel.

For pathological analysis, we scored histological features based on the GAPP classification, which consisted of the following parameters: histological pattern, cellularity, presence or absence of comedo-type necrosis, vascular or capsular invasion, Ki67 LI (%) and catecholamine types produced by the tumours. More specifically, the histological pattern was classified into three types: zellballen, irregular zellballen and pseudorosette. The zellballen pattern is a specific pattern of the paraganglion system composed of nests of catecholamine-producing cells, sustentacular cells and capillaries surrounding these cells. The irregular zellballen pattern is a mixture of small and large irregular tumour cell nests in which the size of the larger nests is at least ten times that of the smaller nests. The pseudorosette pattern is characterised by centrally located, delicate vessels surrounded by tumour cells with cytoplasmic processes around them and corresponds to the pseudopapillary pattern in intrathoracic paravertebral PGL described by Lack (2007). Irregular zellballen pattern or pseudorosette pattern is counted even if only focal. It is not necessary for these patterns to be diffuse. A diffuse growth pattern was not included in the score because, in the authors’ experience, it is most common in adrenergic PHEO and usually innocent.

The scores of the histological patterns were 0, 1 and 1 for the zellballen, irregular zellballen and pseudorosette patterns respectively. If the pseudorosette pattern was observed even focally, it was counted as a score of 1. Cell number within a square (cellularity) was counted under

**Figure 1**

Representative images of histological features. (1) Histological pattern. (1, A) Regular zellballen pattern, (1, B) regular zellballen pattern, (1, C) large irregular zellballen pattern and (1, D) pseudorosette pattern (× 100). (2) Cellularity. The grid in the circle on the left corner shows the area used to count cellularity. These images were taken under the same magnification (×400). (3) Comedo-type necrosis. Arrows indicate foci of coagulation necrosis (×200). (4) Ki67 labelling index (×200). Arrow indicates Ki67-positive nuclei of tumour cells.
high-power magnification (×400) using a 10-mm micrometer (Nikon S-6, Ver.YS1; Nikon, Tokyo, Japan) on an eyepiece. Two fields of the highest cellularity were used for counting to assess cellularity. Scores of 0, 1 or 2 were assigned for number of tumour nuclei <150, 150–250 or more than 250 respectively. The presence of comedo-type necrosis scored 2 points. Comedo-type necrosis was typically centrally located necrosis of a highly cellular nest. PHEO/PGL occasionally showed coagulation necrosis or scar formation in the tumour due to a sudden drop in blood pressure; such degenerative changes were not counted as comedo-type necrosis. For Ki67 LI, two of the most highly labelled areas (hot fields) were photographed at ×200 magnification and were counted using a digital image analyser (Lumina Vision, Mitani Corp., Tokyo, Japan) and scored as 0, 1 and 2 for <1, 1–3 and >3% respectively. The number of cells counted was different case by case depending on cellularity; however, usually it was from 500 to 2000. Representative images are shown in Fig. 1. Catecholamine type was provided by the clinical data. If the plasma or urinary epinephrine levels were abnormally high with or without elevated dopamine levels, the type was determined to be norepinephrine and scored 1 point. The non-functioning type scored 0 points. Scores of these parameters (0–2 points for each) were summed for a total number of points with a maximum of 10 points (Table 1). The individual GAPP point was given based on histological analysis of the tumours. Based on our experience in the field, 2 points were given to findings suggestive of malignancy, 1 point was given to the findings suggestive of possible malignancy and 0 points were given to the findings suggestive of rare malignancy. The total points were then classified into three differentiation types: WD (0–2 points), moderately differentiated (MD, 3–6 points) and PD (7–10 points). This grading system is summarised in Table 2.

For assessment of the pathological grading, sections were thoroughly examined twice, at the initial analysis and 6 months after the first observation, to avoid observational errors.

### Table 1  GAPP parameters and scoring point

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Points scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histological pattern</td>
<td></td>
</tr>
<tr>
<td>Zellballen</td>
<td>0</td>
</tr>
<tr>
<td>Large and irregular cell nest</td>
<td>1</td>
</tr>
<tr>
<td>Pseudorosette (even focal)</td>
<td>1</td>
</tr>
<tr>
<td>Cellularity</td>
<td></td>
</tr>
<tr>
<td>Low (&lt;150 cells/U)</td>
<td>0</td>
</tr>
<tr>
<td>Moderate (150–250 cells/U)</td>
<td>1</td>
</tr>
<tr>
<td>High (more than 250 cells/U)</td>
<td>2</td>
</tr>
<tr>
<td>Comedo necrosis</td>
<td></td>
</tr>
<tr>
<td>Absence</td>
<td>0</td>
</tr>
<tr>
<td>Presence</td>
<td>2</td>
</tr>
<tr>
<td>Vascular or capsular invasion</td>
<td></td>
</tr>
<tr>
<td>Absence</td>
<td>0</td>
</tr>
<tr>
<td>Presence</td>
<td>1</td>
</tr>
<tr>
<td>Ki67 labelling index (%)</td>
<td></td>
</tr>
<tr>
<td>&lt;1</td>
<td>0</td>
</tr>
<tr>
<td>1–3</td>
<td>1</td>
</tr>
<tr>
<td>&gt;3</td>
<td>2</td>
</tr>
<tr>
<td>Catecholamine type</td>
<td></td>
</tr>
<tr>
<td>Epinephrine type (E or E+NE)</td>
<td>0</td>
</tr>
<tr>
<td>Norepinephrine type (NE or NE+DA)</td>
<td>1</td>
</tr>
<tr>
<td>Non-functioning type</td>
<td>0</td>
</tr>
<tr>
<td>Total maximum score</td>
<td>10</td>
</tr>
</tbody>
</table>

U, number of tumour cells in a square of a 10 mm micrometer observed under high power magnification (×400); E, epinephrine; NE, norepinephrine; DA, dopamine.

### Table 2  GAPP score and histological grade

<table>
<thead>
<tr>
<th>GAPP score</th>
<th>Histological grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–2</td>
<td>Well-differentiated type</td>
</tr>
<tr>
<td>3–6</td>
<td>Moderately differentiated type</td>
</tr>
<tr>
<td>7–10</td>
<td>Poorly differentiated type</td>
</tr>
</tbody>
</table>

The ztTEST was applied for comparison of tumour size and GAPP score between metastatic and non-metastatic tumours. The correlation between the GAPP score and interval between the initial operation and the time of initial metastasis was analysed using Pearson’s simple linear regression and correlation. The correlation between the GAPP score and cellularity, GAPP score and Ki67 LI, and Ki67 LI and cellularity was analysed using Pearson’s simple linear regression and correlation. Survival of the WD, MD and PD types was compared using the Kaplan–Meier method and log rank significance test. All the individual parameters of GAPP and the corresponding P values in predicting metastasis were examined by multivariate analysis by Pearson’s simple linear regression among six groups. Results were considered significant for a P value <0.05.

Statistical analyses were performed using StatMate IV Software (Takahashi Y, ATMS, Tokyo, Japan, 2009).
Ethics

The study was approved by the Institutional Ethics Committee of the National Hospital Organisation, Kyoto Medical Centre (responsible for the PHEO-J study) and the National Hospital Organisation, Hakodate Hospital (responsible for the histopathological analysis). All tissue specimens and clinical information were collected after the material was anonymised at each institute.

Results

Clinical features

The mean follow-up after the initial operation was 3.01 ± 0.36 years in the non-metastatic group (range: 0–20 years) and 4.97 ± 1.03 years in the metastatic group (range: 0–33 years). The mean tumour size was 5.1 ± 0.3 cm in the non-metastatic group (range: 1.1–20.0 cm) and 8.7 ± 0.7 cm in the metastatic group (range: 3.0–16.5 cm). The metastatic tumours were larger than the non-metastatic tumours ($P < 0.001$). Catecholamine types produced by the tumours were as follows: epinephrine type in 78 cases, norepinephrine type in 79 cases and non-functioning type in six cases. Metastases were observed in 11 (14.1%) tumours of the epinephrine type compared with 28 (35.4%) tumours of the norepinephrine type; thus metastases were predominant in norepinephrine-type tumours. There were six non-functioning-type tumours and one of these (16.7%) metastasised. Locations of the metastatic tumours were the adrenal gland in 24 cases (60%) and extra-adrenal regions in 16 (40%). The malignancy rate was 18.9% (24 of 126) in the adrenal gland and 44.4% (16 of 36) in extra-adrenal regions (Table 3).

Histology

All tumours were scored from 0 to 10 points and graded accordingly as WD, MD and PD types. There were 111 WD, 35 MD and 17 PD tumours. Metastasis was observed in 40 tumours: four WD, 21 MD and 15 PD. The rate of metastasis in each group was 3.6% for WD, 60% for MD and 88.2% for PD. Although most metastatic tumours were PD and MD, four of the WD tumours also metastasised. Of these four WD tumours, three PHEOs had remarkable invasion into the capsular or central vein of the adrenal gland, and one of these three cases had fat infiltration adjacent to the tumour as well as vascular invasion. The remaining PHEO was associated with NF1, and the patient had multiple bone metastases 4 years after the initial operation for adrenal PHEO. The submitted metastatic bone tumour specimen was not satisfactory for pathological examination due to its small amount tissue with necrosis. Although levels of urinary catecholamine metabolites were slightly elevated, it was uncertain whether the metastatic tumours were PHEO or another kind of tumour associated with NF1 because the primary tumour had no necrosis.

Correlation between GAPP score and metastasis

GAPP scores of the non-metastatic ($n = 123$) and metastatic ($n = 40$) groups were $2.08 ± 0.17$ and $5.33 ± 0.43$ (mean ± s.e.m.) respectively. There was a significant difference between the non-metastatic and metastatic groups ($P < 0.001$; Fig. 2). The accuracy of tumour grading of WD based on a score of 0–2 points, MD for a score 3–6 points and PD for a score 7–10 points was confirmed by these data.

Correlation between GAPP score and time until metastasis after the initial operation

The interval from the initial operation to the time of initial metastasis was compared for 40 metastatic tumours. The mean number of years until metastasis was 5.5 ± 2.6. There was a significant negative correlation between the GAPP score and the interval until metastasis ($r = −0.438$, $P < 0.01$); the higher the GAPP score, the shorter the interval until metastasis (Fig. 3).

Five-year survival, Kaplan–Meier survival curves and GAPP grading

Five-year survival of the groups was 100% for WD, 66.8 ± 0.2% for MD and 22.4 ± 0.1% for PD tumours. The correlation between GAPP score and survival is shown by the Kaplan–Meier survival curves in Fig. 4. There were significant differences in survival rate examined by log rank test between the WD and MD groups ($P < 0.001$ and $P = 9.74×10^{−15}$), the WD and PD groups

<table>
<thead>
<tr>
<th>Catecholamine types</th>
<th>Number of patients</th>
<th>Number of metastasis</th>
<th>Ratio of metastasis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epinephrine</td>
<td>78</td>
<td>11</td>
<td>14.0</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>79</td>
<td>28</td>
<td>35.4</td>
</tr>
<tr>
<td>Adrenal</td>
<td>49</td>
<td>13</td>
<td>26.5</td>
</tr>
<tr>
<td>Extra-adrenal</td>
<td>30</td>
<td>15</td>
<td>50.0</td>
</tr>
<tr>
<td>Non-functioning (extra-adrenal)</td>
<td>6</td>
<td>1</td>
<td>16.7</td>
</tr>
<tr>
<td>Total</td>
<td>163</td>
<td>40</td>
<td>24.5</td>
</tr>
</tbody>
</table>
Correlation between GAPP score and cellularity, GAPP score and Ki67 LI and Ki67 and cellularity

The range of cellularity was from 30 to 370/unit (average \( \pm \) S.E.M.: 173 \( \pm \) 89.6), and these data reflect pleomorphic morphology of PHEO/PGL. The Ki67 LI was 1.55 \( \pm \) 0.21 (average \( \pm \) S.E.M., range: 0–15) in the non-metastatic group and 7.29 \( \pm \) 1.30 (average \( \pm \) S.E.M., range: 0–40) in the metastatic group. The Ki67 LI was significantly different between the metastatic and non-metastatic groups \((P<0.001)\) and \(P=3.99057 \times 10^{-11}\). The highest Ki67 LI was observed in the retroperitoneal PGL and had multiple metastases in bones, lungs and liver at 5 months after initial operation.

Correlation coefficient between GAPP score and cellularity was 0.629 \((P<0.001)\), between GAPP score and Ki67 LI was 0.617 \((P<0.001)\), and between Ki67 LI and cellularity was 0.463 \((P<0.001)\). This means that both cellularity and Ki67 LI significantly influenced GAPP scores. There was also a correlation between Ki67 LI and cellularity.

Multivariate analysis among GAPP parameters and metastasis revealed that all six parameters were significant for predicting metastasis. Catecholamine phenotype was \(P<0.05\), and all the other five parameters were \(P<0.001\) (Table 4).

**GAPP scoring for bilateral PHEO/PGL**

There were 13 patients with bilateral PHEO. The mean GAPP score of these tumours was 1.77 \( \pm \) 0.31. There was no significant difference in GAPP score between bilateral PHEO and non-metastatic PHEO.

**SDHB immunohistochemistry and tumour metastasis**

SDHB negativity by immunohistochemical staining was observed in 13 (8%) tumours: eight MD and five PD. Of these 13 tumours, 10 (77%) had metastases. None of the WD tumours were negative for SDHB (Table 5).

**Discussion**

The mean follow-up in the non-metastatic group and metastatic group after the initial operation was 3.01 \( \pm \) 0.36 and 4.97 \( \pm \) 1.03 years respectively and it was valid to compare the histology and prognosis between these two groups. Tumours of malignant PHEO/PGL were larger than those of non-metastatic tumours, as previously described (O’Riordain et al. 1996, Chrisoulidou et al. 2007). Our data also indicated that metastases were more common in norepinephrine-producing tumours than in epinephrine-producing tumours. PHEO/PGL tumours may exhibit different biochemical phenotypes because extra-adrenal tumours secrete predominantly norepinephrine whereas adrenal tumours secrete mainly epinephrine (van der Harst et al. 2002). In this study 38.6% (49 of 127) of the adrenal tumours were norepinephrine type. The metastatic rate of the adrenal tumours was 14.1% for the epinephrine type and 26.5% for the norepinephrine type. All except one case of PGL in this study produced norepinephrine. Norepinephrine-producing tumours lack PNMT (the enzyme that converts norepinephrine to epinephrine) and are considered to be less differentiated than epinephrine-producing tumours based on catecholamine synthesis. Dopamine hypersecretion was considered a feature of immaturity and a marker for malignant PCC/PGL (van der Harst et al. 2002).
Eisenhofer et al. (2012) reported that the plasma level of methoxytyramine, the O-methylated metabolite of dopamine, is 4.7-fold higher in patients with metastases than in those without, suggesting its use as a potential biomarker.

Our study also included six non-functioning-type tumours with one (16.7%) case of metastasis. The mechanisms underlying non-functioning PHEO/PGL are not fully understood; however, a deficiency of catecholamine synthesising enzymes was reported (Kimura et al. 1992). In this study, the metastatic rate in the non-functioning type of tumour was similar to that of epinephrine-producing tumours. GAPP is composed of six parameters; those parameters have previously been examined for significance for differentiating between benign and malignant pheochromocytomas. Ki67, which is a marker for proliferating cells, has been of particular interest and examined previously (Nagura et al. which is a marker for proliferating cells, has been of particular interest and examined previously (Nagura et al. 2009; Elder et al. 2003). Ki67 LI range is very wide in PHEO/PGL and it is generally accepted that PHEO/PGL with high Ki67 LI highly metastasise and have malignant course. However, it has been well known that PHEO/PGL with no or very low Ki67 LI sometimes metastasise. That was the reason why Ki67 LI has not been accepted as a single indicator for malignant PHEO/PGL. This study revealed that Ki67 LI in the metastatic group is significantly higher than that in the non-metastatic group. However, the range of Ki67 LI was broad and there was overlap between the two groups. Thus, Ki67 LI should be retained as one of the parameters. This study first revealed that cellularity is a very important parameter, more important more than Ki67 LI.

Histological classification using GAPP revealed that non-metastatic tumours could be distinguished from metastatic tumours with few exceptions. More than 80% of PD and 60% of MD tumours metastasised, compared with fewer than 4% of WD tumours. Thus, WD tumours might be considered virtually benign, whereas MD and PD tumours are likely to be malignant. However, if vascular/capsular invasion is evident, even WD tumours may metastasise. Thus, all PHEO/PGLs should be treated as malignant tumours among which WD tumours are low grade, MD tumours are intermediate grade and PD tumours are highly malignant. Furthermore, Kaplan–Meier survival curves showed that patients with WD tumours had 100% survival whereas patients with MD and PD tumours showed progressively worse survival. This indicates that if WD tumours do metastasise, they grow slowly and the patients have a long survival time after surgery. Thus, it is very important to distinguish WD tumours from MD and PD tumours, and patients with MD or PD tumours should be carefully followed for a long time. For assessment of GAPP score, we observed twice at intervals, and occasionally, there was little difference between first- and second-look grading. If there were minor differences in GAPP scores, the grading was not influenced because there were ranges in grading: scores of 3–6 points are classified as MD and scores of 7–10 points are classified as PD. Such small differences in the GAPP score at second look were within the range of grading.

As Gimenez-Roqueplo et al. (2003) first reported that an extra-adrenal site, recurrence and malignancy were strongly associated with SDHB mutations and suggested that the presence of SDHB mutants should be considered a high-risk factor for malignancy or recurrence, genotype-phenotype correlations in patients with SDHB-associated PHEO/PGL have been closely studied in cases of malignant PHEO/PGL (Timmers et al. 2007). A malignant PGL was documented in 37.5% of SDHB carriers, 3.1% of SDHD carriers and none of the SDHC mutation carriers (Burnichon et al. 2009). van Nederveen et al. (2009) reported loss of SDHB protein immunoreactivity in tumours with HPPS with a sensitivity of 100% and a specificity of 84%. Therefore, by routinely performing SDHB immunohistochemistry, the malignant potential of PCC/PGL associated with HPPS could be assessed with a high degree of reliability. In this study, SDHB-negative immunoreactivity was observed in 13 (8%)

### Table 4  Individual GAPP parameters and the corresponding \( P \) values in predicting metastasis by multivariate analysis

<table>
<thead>
<tr>
<th>GAPP parameters</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histological pattern</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>Cellularity</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>Comedo necrosis</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>Vascular or capsular invasion</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>Ki67 labelling index</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>Catecholamine type</td>
<td>(&lt;0.05)</td>
</tr>
</tbody>
</table>
tumours, all of which were MD and PD types, and ten of the 13 (77%) had metastases. These data confirm previous reports that the SDHB mutation is a high-risk factor for malignancy or recurrence (Gimenez-Roqueplo et al. 2003, Neumann et al. 2004). Although immunohistochemistry is a useful tool, it is also necessary to confirm gene mutations to avoid false-negative or false-positive results because assessment of immunoreactivity of SDHB is sometimes difficult, especially in cases of weak immunoreactivity (van Nederveen et al. 2009).

Regarding bilateral PCC/PGL associated with multiple endocrine neoplasia type 2 and VHL, the GAPP score was low, and there were no significant differences between bilateral and non-metastatic PCC/PGL.

In conclusion, we have demonstrated the ability of GAPP classification to differentiate low-grade malignancies from moderate to high-grade malignancies with different rates of metastasis. Combined use of GAPP and SDHB immunohistochemistry might be useful for the prediction of tumour metastasis and patient prognosis. The concordance rate and reproducibility of diagnosis by GAPP should be validated in further studies.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
N Kimura performed histological analysis using GAPP and SDHB immunohistochemistry and wrote the manuscript. R Takayanagi, N Takizawa, E Itagaki, T Katabami, N Kakoi, H Rakugi, Y Ikeda, A Tanabe, T Nigawara and S Ito submitted materials and clinical data for histopathological analysis. I Kimura performed statistical analysis. M Naruse organised the project and reviewed the manuscript.

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