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Prognostic significance of Ki-67 in assessing the risk of progression, relapse or metastasis in pheochromocytomas and paragangliomas

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ABSTRACT

Introduction: Since the Fourth edition of the WHO classification, PPGLs have been recognized for their metastatic potential, though no clear features can accurately predict this behavior. The prognostic value of Ki-67 in assessing the risk of progression, relapse, or metastasis in PPGLs remains debated.

Methods: This cohort study included 501 patients diagnosed with PPGLs at the First Hospital of Jilin University between 2000 and 2022, with clinical data, treatment details, pathological indicators, and germline gene test results collected. Bulk sequencing was performed on formalin-fixed paraffin-embedded (FFPE) primary tumor samples from 87 patients. Progression-free survival (PFS) was analyzed using multivariable Cox regression.

Results: Among the 119 enrolled patients with PPGLs, the average age was 45.7 ± 14.0 years, and the median follow-up time was 46 months. A significant finding was the high expression of *CDK1*, a gene known to be significantly associated with the metastatic risk of PPGLs, in samples with Ki-67 ≥ 3% ($p < 0.0001$). More importantly, patients with PPGLs and a Ki-67 level ≥ 3% had a 3.59-fold higher risk of progression, relapse or metastasis compared to those with Ki-67 < 3% (HR = 4.59, 95% CI: 1.06–11.95), after adjusting for all confounding factors. In the composite model, the addition of Ki-67 enhanced the predictive ability of the combined model of SDHB, primary site, tumor size, and invade neighboring tissue (AUC = 0.888, 95% CI: 0.808–0.967 vs. AUC = 0.874, 95% CI: 0.783–0.965).

Conclusion: A Ki-67 level ≥ 3% is associated with an increased risk of progression, relapse or metastasis in patients with PPGLs.

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Ki-67; paraganglioma and pheochromocyte; progression; relapse; metastasis

1. Introduction

Pheochromocytomas and paragangliomas (PPGLs) are rare neuroendocrine tumors originating from the catecholamine-producing adrenal medulla or extra-adrenal paraganglia, with an annual incidence of only 0.2 to 0.8 per 100,000 individuals [1]. All PPGLs possess the potential for relapse or metastasis, leading to the classification of PPGLs as malignant tumors and cases demonstrating distant metastasis termed as 'metastatic PPGLs' according to the 4th edition of the WHO New Classification of Adrenal Tumors in 2017 [2]. Currently, radical resection is still the primary treatment approach for PPGLs without initial distant metastasis, while the prognosis and assessment of metastasis risk in postoperative PPGLs present significant challenges.

The initial grading system attempt was made by Dr. Lester Thompson, who developed the Pheochromocytoma of the Adrenal gland Scaled Score (PASS) based solely on histological criteria as a tool for differentiating pheochromocytomas with potentially aggressive behavior. These parameters cover the classical features of general malignancies, rather than focusing on the specific features of paragangliomas/pheochromocytomas, and do not include paragangliomas, so the scope of application is limited [3]. Although initially associated only with adrenal tumors, it was subsequently found to have some predictive value for extra-adrenal tumors as well, albeit with considerable interobserver variation. Dr. Kimura et al. published the Grading system for Adrenal Pheochromocytoma and Paraganglioma (GAPP) extending the PASS algorithm,

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which utilized histological criteria, Ki-67 index, and catecholamine type scores. In addition to survival predictions, a high GAPP score helps identify cases at risk of subsequent metastasis. The correlation analysis between GAPP score and cellularity, GAPP score and Ki-67 index showed that cellularity and Ki-67 index significantly affected GAPP score [4]. The improved GAPP method, when combined with succinate dehydrogenase B subunit (*SDHB*) immunohistochemistry results, demonstrated good predictive potential. Additionally, Dr. Pierre proposed the Composite Pheochromocytoma/Paraganglioma Prognostic Score (COPPS), which focused on histological features, paying particular attention to tumor size, *S100* expression, and *SDHB* expression, while not considering Ki-67. Ki-67 is a significant predictive marker in many tumors, especially in epithelial neuroendocrine tumors (NETs) [5,6], but among PPGLs the association of Ki-67 with the risk of progression, relapse or metastasis remains controversial [7,8]. In the 2022 WHO classification, there still remains a concern regarding the prognostic evaluation of Ki-67 in PPGLs [9].

In this retrospective study, we aimed to analyze patients diagnosed with PPGLs at the First Hospital of Jilin University between 2000 and 2022. A comprehensive collection of basic clinical information, treatment details, pathological indicators, and germline gene test results was undertaken, and thorough follow-up was conducted. RNA-sequencing (RNA-seq) were performed on Formalin-fixed, paraffin-embedded (FFPE) tumor samples collected from 87 patients with PPGLs to clarify transcriptome features of PPGLs. By including confounding factors, such as the baseline clinical features, functional symptoms of hormone secretion (such as hypertension and palpitation), histological parameters (including tumor size, vascular and nerve invasion), and the histochemical expression of *SDHB* and *SSTR2*, Cox regression analysis was performed to assess the potential of Ki-67 as a predictor of recurrence and transformation in PPGLs.

2. Materials and methods

2.1. Study design and participants

This cohort study included a total of 501 patient's primary diagnosed with PPGLs by pathology in the First Hospital of Jilin University from 2000 to 2022. Of these, 175 patients were excluded for being older than 80 years, having missing clinical features or Ki-67 data, or having concurrent malignancies. Among the remaining patients, 207 were further excluded due to loss to follow-up, or insufficient data regarding relapse, progression or metastasis. The cohort was followed-up annually, with the most recent follow-up conducted in December 2024. Ultimately, 119 patients were included in the final analysis, consisting of 106 surgical patients and 13 non-surgical patients. The flowchart of patient inclusion is shown in Figure 1.

2.2. Statement

The study was approved by the ethics committee and institutional review boards of the First Hospital of Jilin University. Written informed consent was obtained from the patients included. This study adheres to the Declaration of Helsinki.

2.3. Data collection

The clinical data collected for this study encompassed various variables, including gender, age, primary tumor

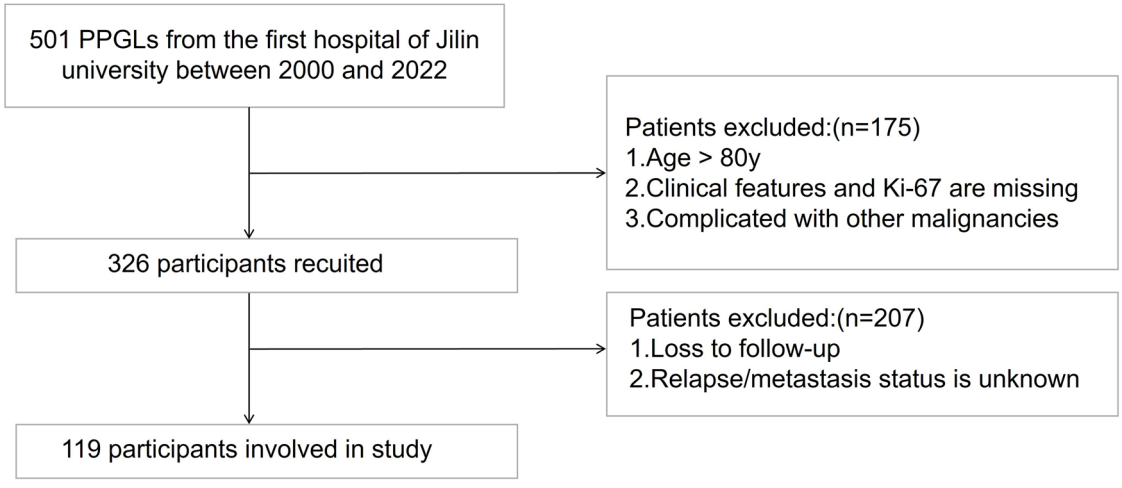

Figure 1. Flow diagram of the screening and enrollment of study participants.

site, functional status (such as hypertension and palpitation), germline gene test results, tumor size, histological criteria, and details of the surgical procedures performed. The study investigated locoregional relapse and the recurrence of metastasis, including *in situ* recurrence, bone metastasis, lymph node metastasis, lung metastasis, liver metastasis, and other forms of metastasis. Functional tumors are defined by elevated levels of catecholamines, including adrenaline, noradrenaline, and/or dopamine, along with their metabolites, and are typically associated with hormone-related symptoms such as hypertension, headache, episodic sweating, and palpitations. Family history is defined as the presence of first-degree relatives (parents, siblings, or children) or second-degree relatives (grandparents, aunts, uncles, or grandchildren) with PPGLs [10]. Germline gene testing was performed in patients with a high suspicion of pathogenic mutations and a positive family history of PPGLs [11].

2.4. Immunohistochemical staining (IHC)

The staining and scoring of Ki-67, SDHB, SSTR2, and CD8a were carried out by the Department of Pathology at the First Hospital of Jilin University. *SDHB* expression in pathological tissues was considered positive. The scoring system for *SSTR2* followed the scoring criteria used for *HER2*. All specimens analyzed in this study included 106 surgical specimens and 13 tissue biopsy specimens. The scoring categories for *SSTR2* were defined as follows: negative (non-responsive or < 10% tumor cell membrane staining), 1+ (≥10% of tumor cells with faint or barely visible membrane staining), 2+ (≥10% of tumor cells with weak to moderate basal lateral membrane, lateral membrane, or complete membrane staining), and 3+ (strong staining ≥ 10% of tumor cells with basal lateral membrane, lateral membrane, or complete membrane staining). The Ki-67 index calculation was done artificially and was done and reviewed by two different senior pathologists. All immunohistochemically labelled cells with positive nuclear staining in tissue sections were counted, and the number of stained nuclei was then expressed as a percentage of immunoreactive cells (the index), with 1,000 tumour cells counted in the highest labelled areas (hotspots) [12]. To assess the infiltration of $CD8^+$ lymphocytes, the percentage of $CD8^+$ lymphocytes was calculated against the total amount of lymphocytes. The levels of lymphocytic infiltration of CD8 were determined as follows: (a) no positive cells or few dispersed positive cells; (b) infiltration of less than 25% of the stromal area or sparsely dispersed positive cells across the entire core area; (c) infiltration of 25% to 49% of the stromal area; (d) infiltration of 50% or greater of the stromal area. The a-c infiltration levels were considered low, and the d level was considered high [13].

2.5. RNA extraction, sequencing and processing of sequencing data

The extraction and sequencing of RNA, as well as the processing of sequencing data, were previously conducted and are described in detail elsewhere. In brief, RNA extraction and sequencing were performed on Formalin-fixed, paraffin-embedded (FFPE) primary tumor samples collected from 87 patients diagnosed with PPGLs. The RNA extraction was carried out using a QIA Symphony SP instrument (QIAGEN GmbH, Hilden, Germany) and the QIA symphony RNA extraction kit (cat. no: 931636, QIAGEN GmbH, Hilden, Germany), following the manufacturer's protocol. The quality and quantity of the extracted RNA were assessed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). The average RNA integrity number (RIN) was 2.1. The RNA sequencing procedure was conducted by Beijing Genomics Institute Genomics (BGI Genomics) https://www.genomics.cn/about.html. The HiSeq 2500 instrument from Illumina was employed for sequencing the prepared libraries. Immune cell deconvolution of the RNA-seq data was performed with xCell performs [14,15].

2.6. Outcome

The primary endpoint of interest in this study was the occurrence of locoregional relapse, progression or metastasis to other sites, such as the liver, bone, lungs, etc., following the pathological diagnosis of PPGLs. The outcome measured in the study was progression-free survival (PFS), defined as the time from diagnosis to relapse, progression, metastasis, or the end of follow-up in December 2024. For surgical patients, PFS was defined as the time to locoregional relapse or the development of new postoperative metastases. For nonsurgical patients, it was defined as the occurrence of new metastases or disease progression. During this period, data on survival status and relevant information were collected from the electronic medical record system or obtained through follow-up telephone interviews.

2.7. Statistical analysis

Descriptive analysis was conducted for all participants. Continuous variables were presented as mean ± standard

deviation (SD) or median (interquartile range, IQR), while categorical variables were reported as frequencies or percentages (n, %). The Free Statistics software was utilized to analyze the differences between continuous and categorical variables using independent t-tests and chi-square tests, respectively. Missing data for categorical variables were indicated as 'unknown' in each table, while no missing data were observed for continuous variables.

Hazard ratios (HR) and their corresponding 95% confidence intervals were calculated. A significance level of $p < 0.05$ was considered statistically significant. Univariate analysis was performed on all variables that could potentially influence PFS in the study. PFS-related factors were identified within the entire study population. A Kaplan-Meier curve was generated to illustrate the survival probabilities over the follow-up period, stratified by the Ki-67 index. Multivariate analysis was conducted to adjust for covariates such as age and sex, aiming to further evaluate the correlation between the Ki-67 index and progression, relapse or metastasis of PPGLs. Subgroup analysis was employed to assess whether this correlation remained consistent within each subgroup.

3. Results

3.1. Baseline characteristics of the study participants

This retrospective study ultimately included a total of 119 participants, of whom 55 (55/119) were women and 64 (64/119) were male, with a mean age of 45.7 ± 14.0 years. The median follow-up period was 46.0 (33.0–162.0) months. Among the 119 patients, a total of 30 patients (25.2%) had relapses or metastases, including 10 cases of locoregional relapse, 13 cases of bone metastasis, 12 cases of lymph node metastasis, 7 cases of lung metastasis, and 4 cases of liver metastasis.

3.2. Association of Ki-67 with recurrence and/or metastasis of PPGLs

The univariate analysis initially examined the risk factors associated with progression, relapse or metastasis of PPGLs and reported HR and 95% CI (Supplementary Table 1). The analysis revealed several factors significantly associated with progression, relapse or metastasis, including the primary location of the tumor (adrenal primary), hereditary nature of the tumor, tumor size, invasion of neighboring tissues, Ki-67 index, *SDHB* expression, local compression, and surgical intervention (all with $p < 0.05$). By calculating the sensitivity and specificity of different Ki-67 levels for prognosis assessment in PPGLs patients, we plotted the ROC curve and calculated the area under the curve (AUC = 0.837). The cut-off values for Ki-67 were determined by maximizing the Youden index (sensitivity plus specificity minus one), which suggested a threshold of 2.25%. At this threshold, the model achieved a sensitivity of 0.71 and a specificity of 0.84. Considering clinical practicality and significance, and referencing previous literature, we selected < 3% and ≥ 3% as the cut-off value of Ki-67, at which the AUC was 0.793 [16–18].

Taking 3% as the cut-off value of Ki-67, the subjects were divided into two groups: patients with Ki-67 < 3% ($n = 85$), of which 40 were female (40/85) and 45 were male (45/85), with an average age of 47.6 ± 12.6 years, and patients with Ki-67 ≥ 3% ($n = 34$), including 15 females (15/34) and 19 males (19/34), with a mean age of 41.2 ± 16.4 years. Other covariates include sex, age, hereditary, functional status, primary location, surgery, SDHB, SSTR2, invade neighboring tissues etc. No significant differences in sex, functional status, locoregional relapse, liver metastases, and tumor size were observed between the two groups (all $p > 0.05$) (Table 1). Notably, patients with a Ki-67 ≥ 3% had a 2.55-fold higher risk of progression, relapse or metastasis compared to those with a Ki-67 < 3% (HR = 3.55, 95% CI: 1.62–7.55). The Kaplan-Meier curve (Figure 2) demonstrated 4.59 that patients with a Ki-67 index ≥ 3% had a significantly higher risk of relapse and lower survival probability ($p = 0.0005$).

To determine whether Ki-67 is an independent prognostic factor for the risk of PPGLs progression, relapse or metastasis, we included various parameters that potentially affect PPGLs recurrence/metastasis, such as tumor size and *SDHB* mutation [19], as covariates in a multivariate analysis. The results indicated that Ki-67 ≥ 3% still increased the risk of progression, relapse or metastasis in patients with PPGLs and had a 3.59-fold increased risk of progression, relapse or metastasis than patients with Ki-67 < 3% in PPGLs (HR = 4.59, 95% CI: 1.06–11.95) after adjusting for all covariates (Table 2).

To detect the association between the Ki-67 and the risk of progression, relapse or metastasis in patients with PPGLs in different subgroups, the analysis and interaction analyses were stratified according to confounding factors (age, primary site, hereditary, *SDHB*). No significant interactions were observed in the subgroups (all interactions had a P-value greater than 0.05), indicating that Ki-67 ≥ 3% in all subgroups increased the risk of progression, relapse or metastasis in patients with PPGLs (Figure 3).

To improve the assessment of progression, relapse or metastasis in patients with PPGLs, we expanded

Table 1. Characteristics of patients.

Variables	Total ($n = 119$)	Ki-67 < 3% ($n = 85$)	Ki-67 ≥ 3% ($n = 34$)	*p*-value
Gender, *n* (%)				0.771
Female	55 (46.2)	40 (47.1)	15 (44.1)	
Male	64 (53.8)	45 (52.9)	19 (55.9)	
Age, Mean ± SD	45.7 ± 14.0	47.6 ± 12.6	41.2 ± 16.4	0.025
Primary site, *n* (%)				< 0.001
Adrenal	61 (51.3)	53 (62.4)	8 (23.5)	
Peritoneal and retroperitoneal	38 (31.9)	26 (30.6)	12 (35.3)	
Carotid body aneurysm	6 (5.0)	2 (2.4)	4 (11.8)	
Pelvis and bladder	7 (5.9)	3 (3.5)	4 (11.8)	
Mediastinum and pericarium	4 (3.4)	1 (1.2)	3 (8.8)	
Pancreas	3 (2.5)	0 (0)	3 (8.8)	
Adrenal primary, *n* (%)				< 0.001
No	58 (48.7)	32 (37.6)	26 (76.5)	
Yes	61 (51.3)	53 (62.4)	8 (23.5)	
Functional, *n* (%)				0.071
No	65 (54.6)	42 (49.4)	23 (67.6)	
Yes	54 (45.4)	43 (50.6)	11 (32.4)	
Hereditary, *n* (%)				0.039
No	70 (58.8)	56 (65.9)	14 (41.2)	
Yes	27 (22.7)	15 (17.6)	12 (35.3)	
Unknown	22 (18.5)	14 (16.5)	8 (23.5)	
Invade neighboring tissues, *n* (%)				< 0.001
No	92 (77.3)	73 (85.9)	19 (55.9)	
Surrounding fibrous tissue	14 (11.8)	8 (9.4)	6 (17.6)	
Adjacent organs	13 (10.9)	4 (4.7)	9 (26.5)	
SDHB status, *n* (%)				< 0.001
Negative	23 (19.3)	10 (11.8)	13 (38.2)	
positive	88 (73.9)	72 (84.7)	16 (47.1)	
Unknown	8 (6.7)	3 (3.5)	5 (14.7)	
SSTR2 expression, *n* (%)				0.02
Negative	26 (21.8)	24 (28.2)	2 (5.9)	
1+	12 (10.1)	9 (10.6)	3 (8.8)	
2+	36 (30.3)	24 (28.2)	12 (35.3)	
3+	39 (32.8)	26 (30.6)	13 (38.2)	
Unknown	6 (5.0)	2 (2.4)	4 (11.8)	
Hypertension, *n* (%)				0.202
No	59 (49.6)	39 (45.9)	20 (58.8)	
Yes	60 (50.4)	46 (54.1)	14 (41.2)	
Palpitation, *n* (%)				0.352
No	99 (83.2)	69 (81.2)	30 (88.2)	
Yes	20 (16.8)	16 (18.8)	4 (11.8)	
Local compression, *n* (%)				0.011
No	72 (60.5)	58 (68.2)	14 (41.2)	
Yes	42 (35.3)	24 (28.2)	18 (52.9)	
Unknown	5 (4.2)	3 (3.5)	2 (5.9)	
Relapse or metastasis, *n* (%)				< 0.001
No	89 (74.8)	75 (88.2)	14 (41.2)	
Yes	30 (25.2)	10 (11.8)	20 (58.8)	
Locoregional relapse, *n* (%)				0.146
No	109 (91.6)	80 (94.1)	29 (85.3)	
Yes	10 (8.4)	5 (5.9)	5 (14.7)	
Bone metastases, *n* (%)				< 0.001
No	106 (89.1)	83 (97.6)	23 (67.6)	
Yes	13 (10.9)	2 (2.4)	11 (32.4)	
Lymph node metastases, *n* (%)				0.004
No	107 (89.9)	81 (95.3)	26 (76.5)	
Yes	12 (10.1)	4 (4.7)	8 (23.5)	
Lung metastases, *n* (%)				0.002
No	112 (94.1)	84 (98.8)	28 (82.4)	
Yes	7 (5.9)	1 (1.2)	6 (17.6)	
Liver metastases, *n* (%)				0.322
No	115 (96.6)	83 (97.6)	32 (94.1)	
Yes	4 (3.4)	2 (2.4)	2 (5.9)	
Tumor Size, Median (IQR)	5.0 (3.8, 7.5)	5.0 (3.7, 7.0)	6.0 (4.0, 9.0)	0.295
Surgery, *n*(%)				0.002
No	13(10.9)	4(4.7)	9(26.5)	
Yes	106(89.1)	81(95.3)	25(73.5)	

Abbreviations: *SDHB*, succinate dehydrogenase complex iron sulfur subunit B; *SSTR2*, Somatostatin Receptor 2.

upon Ki-67 by incorporating factors previously reported to have a significant impact on PPGL progression, relapse or metastasis, including SDHB status [20,21], primary tumor site [22], tumor size [20,21,23], and histological criteria (invasion of neighboring tissues, adipose tissue, vascular or capsular). This was achieved by plotting ROC curves and calculating the AUC. The results indicated that Ki-67 (AUC = 0.842, 95%

CI: 0.757–0.925) outperformed SDHB (AUC = 0.752, 95% CI: 0.625–0.879) in the prediction model alone. In the composite model, the addition of Ki-67 enhanced the predictive ability of the combined model of SDHB, primary site, tumor size, and invade neighboring tissue (Model 2, AUC = 0.888, 95% CI: 0.808–0.967 VS Model 1, AUC =0.874, 95% CI: 0.783-0.965) (Figure 4).

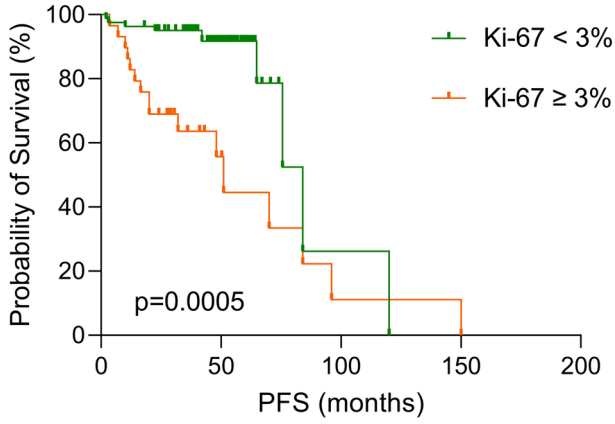

Figure 2. Kaplan–Meier survival curves for progression-free survival in patients with PPGLs.

3.3. High Ki-67 is positively related with mesenchymal and immunosuppressive microenvironment transcriptional subtypes

To further elucidate the relationship between the tumour the biological characteristics and immune microenvironment of PPGLs with varying Ki-67 levels and their association with progression, relapse or metastasis, this study performed RNA-seq analysis on FFPE samples obtained from 87 patients. The analysis revealed no differences in neuroendocrine associated genes (*NE*), such as Synaptophysin (*SYP*), Chromogranin A (*CHGA*) and *SSTR2* were observed in cases with Ki-67 < 3% and Ki-67 ≥ 3% [24–28]. However, a significant finding was the high expression of *CDK1*, a gene known to be significantly associated with the

Table 2. The cox regression for Ki-67 on the progression, recurrence and metastasis of PPGLs.

	Crude Model		Model2		Model3		Model4	
Variable	HR(95%CI)	*P*-value	HR(95%CI)	*P*-value	HR(95%CI)	*P*-value	HR(95%CI)	*P*-value
Ki-67 < 3%	1(Ref)		1(Ref)		1(Ref)		1(Ref)	
Ki-67 ≥ 3%	3.55 (1.62 ~ 7.75)	0.005	3.55 (1.62 ~ 7.75)	0.005	5.45 (1.43 ~ 20.86)	0.013	4.59 (1.06 ~ 11.95)	0.041

Note: Model1: Crude Model; Model2: Adjusts for gender + age; Model3: Adjusts for Model1 + primary site + functional status + tumor size + invade neighboring tissues + *SDHB* + *SSTR2* subtype; Model4: Adjusts for Model2 + surgery + local compression + hypertension + palpitation.
Abbreviations: *SDHB*, succinate dehydrogenase complex iron sulfur subunit B; *SSTR2*, Somatostatin Receptor 2.

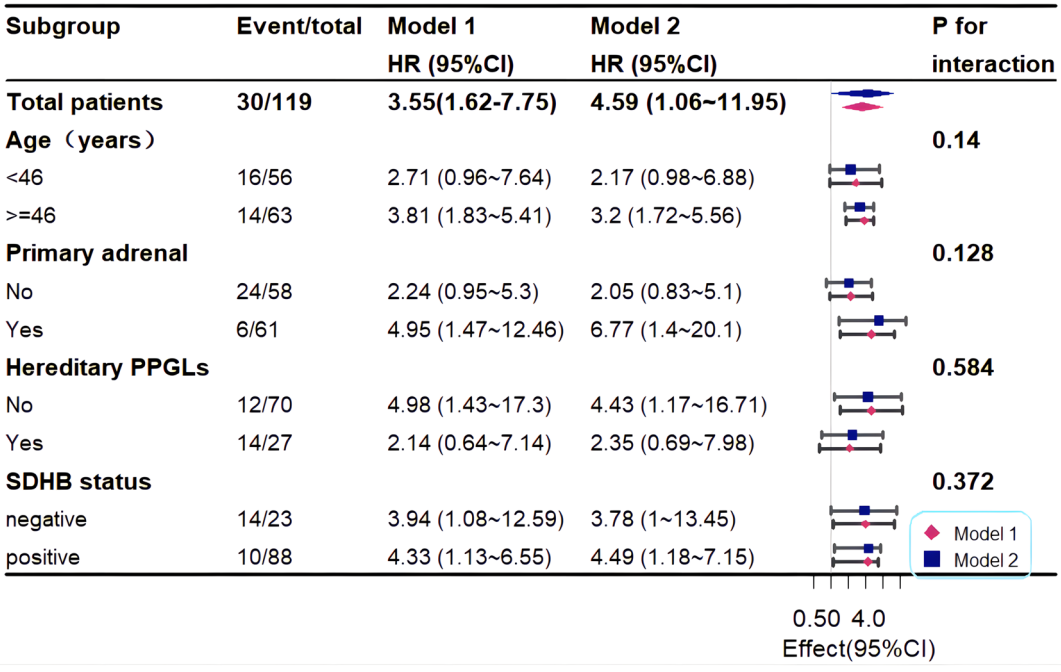

Figure 3. The association between the Ki-67 and the risk of progression, relapse or metastasis in patients with PPGLs in different subgroups.
Note: Model1: Crude model for patients with Ki-67 ≥ 3%, Model2: Patients with Ki-67 ≥ 3% stratified according to all confounding factors.
Abbreviations: *SDHB*, succinate dehydrogenase complex iron sulfur subunit B.

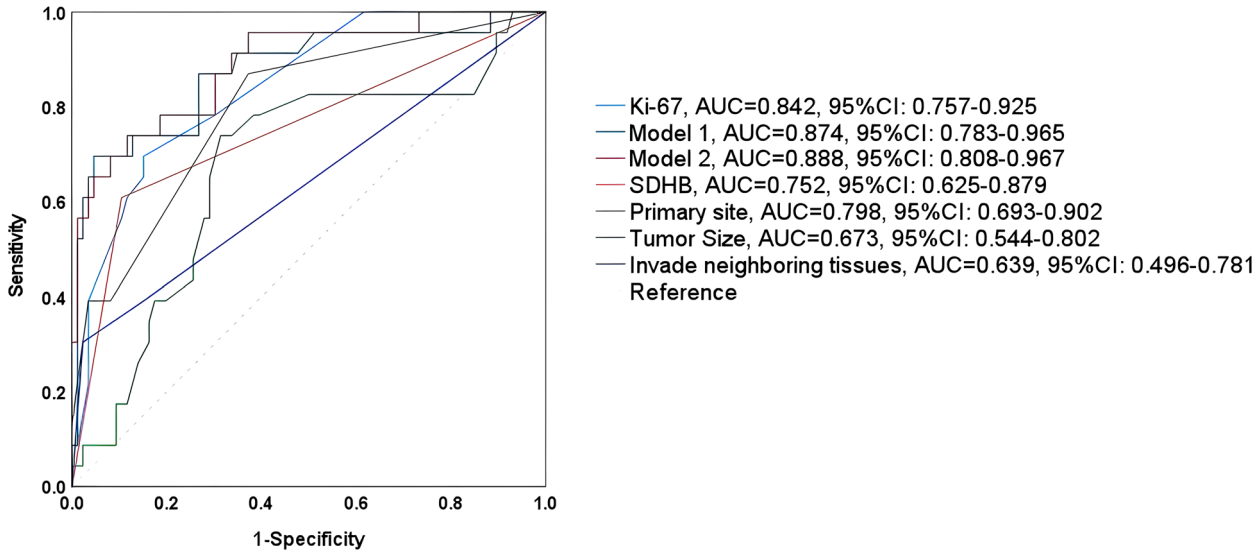

Figure 4. ROC curves for different parameters to assess progression, relapse or metastasis.
Model1: SDHB + primary site + tumour size + Invade neighboring tissues.
Model2: Model1 + Ki-67.

metastatic risk of PPGLs, in samples with Ki-67 ≥ 3% ($p < 0.0001$) [29]. Additionally, molecular features of epithelial-mesenchymal transition (*EMT*), including mesenchymal markers *VIM*, *ZEB1*, and *SNAI2* tended to be higher in samples with Ki-67 ≥ 3%, suggesting a potential link between high Ki-67 expression and increased metastasis potential (Figure 5A) [30–33]. Moreover, among the 415 genes ($p < 0.01$) that exhibited significant differences between samples with Ki-67 < 3% ($n = 73$, Ki-67-L) and those with Ki-67 ≥ 3% ($n = 14$, Ki-67-H) (Figure 5B), we observed that the highly expressed genes in the Ki-67-H group were primarily enriched in cell cycle, sprouting angiogenesis, and cell migration-related pathways (Figure 5C). On the other hand, the highly expressed genes in the Ki-67-L group were mainly enriched in steroid and glucocorticoid metabolism pathways (Figure 5D).

In our subsequent analysis of the immune microenvironment, we made noteworthy observations. The Ki-67-H group showed lower infiltration of T and NK cells, myeloid dendritic cells (mDCs), and Th1 cells compared to the Ki-67-L group. Conversely, there was a significant increase in the infiltration of hematopoietic stem cells (HSCs) in the Ki-67-H group (Figure 6A-B). It is worth mentioning that HSCs have been reported to exhibit an immunosuppressive effect [34]. These findings suggest that the Ki-67-H group is more likely to exhibit characteristics of a 'cold' tumor, characterized by reduced immune cell infiltration and an increased presence of HSCs. To further validate this characteristic, we performed IHC of tumor-infiltrating $CD8^+$ T cells and NK cells (CD56 positive immune cells). The staining results supported our observations, indicating lower levels of these immune cells in the tumor microenvironment of the Ki-67-H group compared to the Ki-67-L group (Figure 6C). To fully reflect the differences in their biological behaviors, we supplemented the analysis of the differences in *EMT* scores, proliferation scores, and neuroendocrine phenotype scores between the two groups. The results showed that the *EMT* score and proliferation score were higher in the high Ki-67 group than in the low expression group (Figure 7A). In the characterization of the microenvironment, we found that the low Ki-67 group had active lipid metabolism and a lower degree of inflammation, while the high Ki-67 group had higher energy metabolism compared to the low expression group, which may support the proliferation and growth of tumor cells (Figure 7B).

4. Discussion

Since the Fourth edition of the World Health Organization (WHO) classification, it has been recognized that PPGLs can exhibit metastatic potential, and there are no clear-cut features that can accurately predict their metastatic behavior. Here, in this retrospective cohort study, we made significant findings regarding the association of the Ki-67 index with various factors. We observed that a high Ki-67 index was associated with elevated expression of metastasis-related genes, including *CDK1*, *VIM*, *ZEB1*, and *SNAI2*. Additionally, we found reduced infiltration of immune cells and an increased presence of hematopoietic stem cells (HSCs) in the tumor microenvironment of patients with high Ki-67 index. More importantly, our study demonstrated that patients with a Ki-67 index ≥

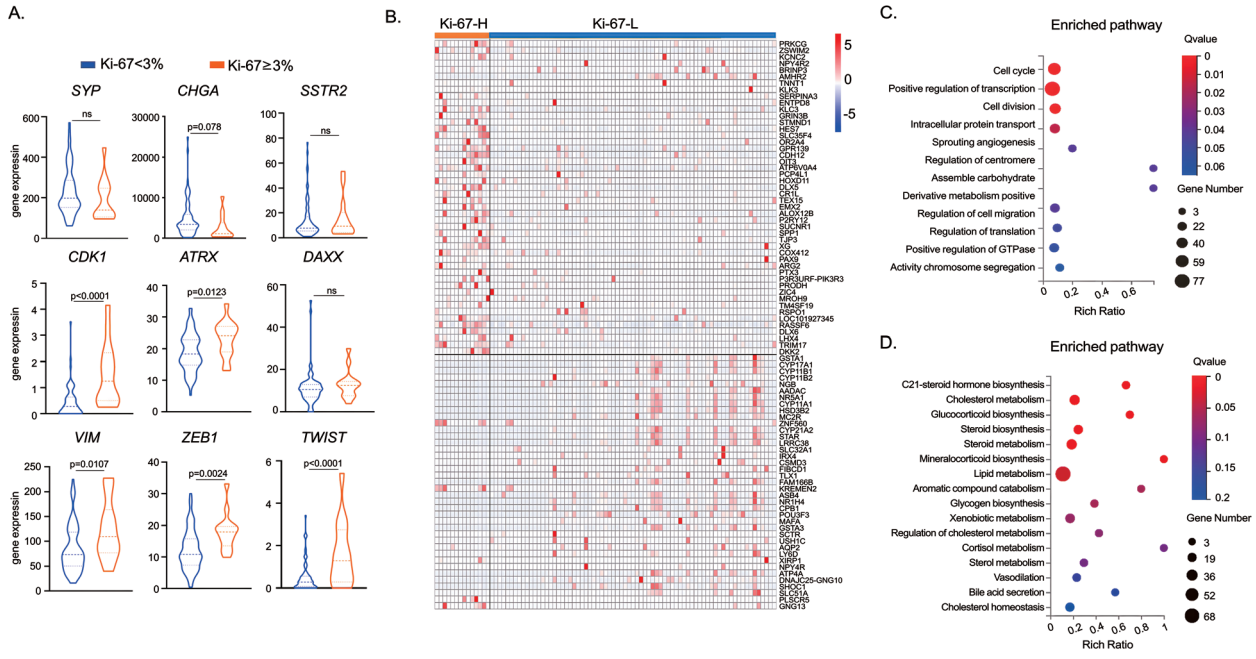

Figure 5. Transcriptome sequencing analysis of PPGLs based on Ki-67.
Note: Ki-67-H: Ki-67 ≥ 3%, Ki-67-L: Ki-67 < 3%.
(A) Expression of neuroendocrine associated genes and epithelial-mesenchymal transition (*EMT*) markers in patients with PPGLs.
(B) Heat map showing 415 genes with significant differences between Ki-67 ≥ 3% and Ki-67 < 3%.
Fig.5C-5D: Bubble diagram illustrating biobehavioral gene enrichment pathways in two groups.

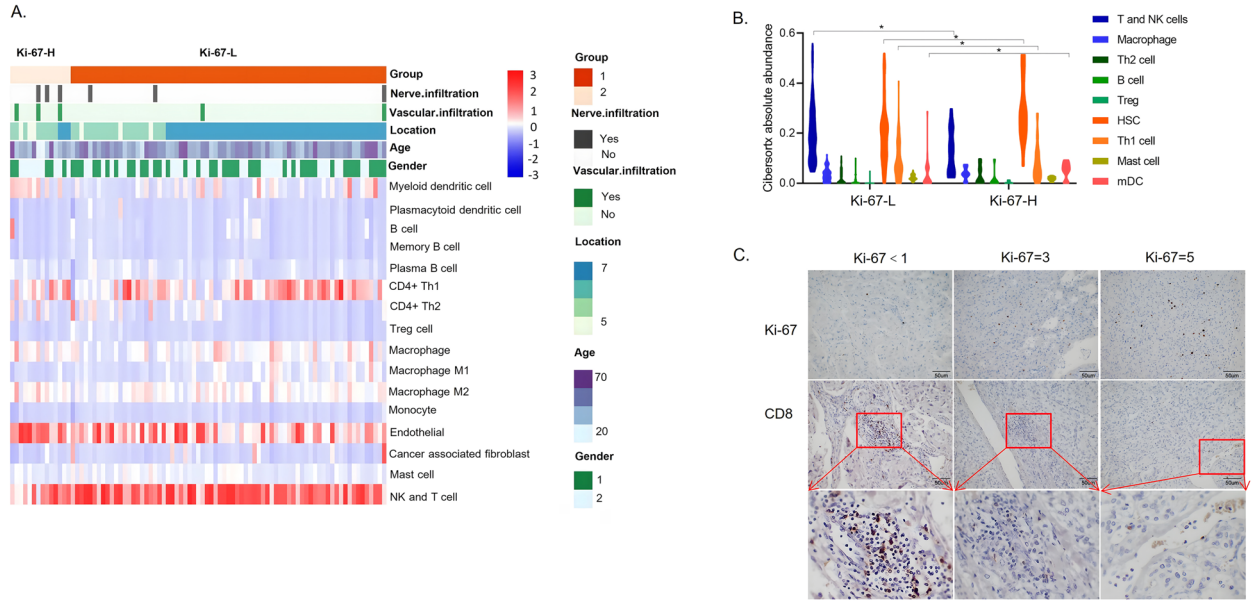

Figure 6. Analysis of the immune microenvironment in PPGL patients.
Note: Ki-67-H: Ki-67 ≥ 3%, Ki-67-L: Ki-67 < 3%.
(A) Heat map depicting cellular components of the immune microenvironment in patients with PPGLs.
(B) Comparison of cellular fractions between patients with high and low Ki-67. Figure 6C: Immunohistochemical plots showing $CD8^+$ T cells and Ki-67 expression at different Ki-67 indexes.
Abbreviations: mDCs, myeloid dendritic cells; HSCs, hematopoietic stem cells.

3% had an increased risk of progression, relapse or metastasis compared to those with a Ki-67 index < 3% in PPGLs. This conclusion remained consistent in subgroup analyses, highlighting the evaluative significance of Ki-67 in predicting recurrent metastasis of PPGLs.

The PASS scoring system includes several morphological parameters, such as high cellularity, mitotic figures > 3/10 high-power fields (HPF), and vascular invasion; however, the Ki-67 index was not included. The accuracy rate of metastatic prediction results (PASS

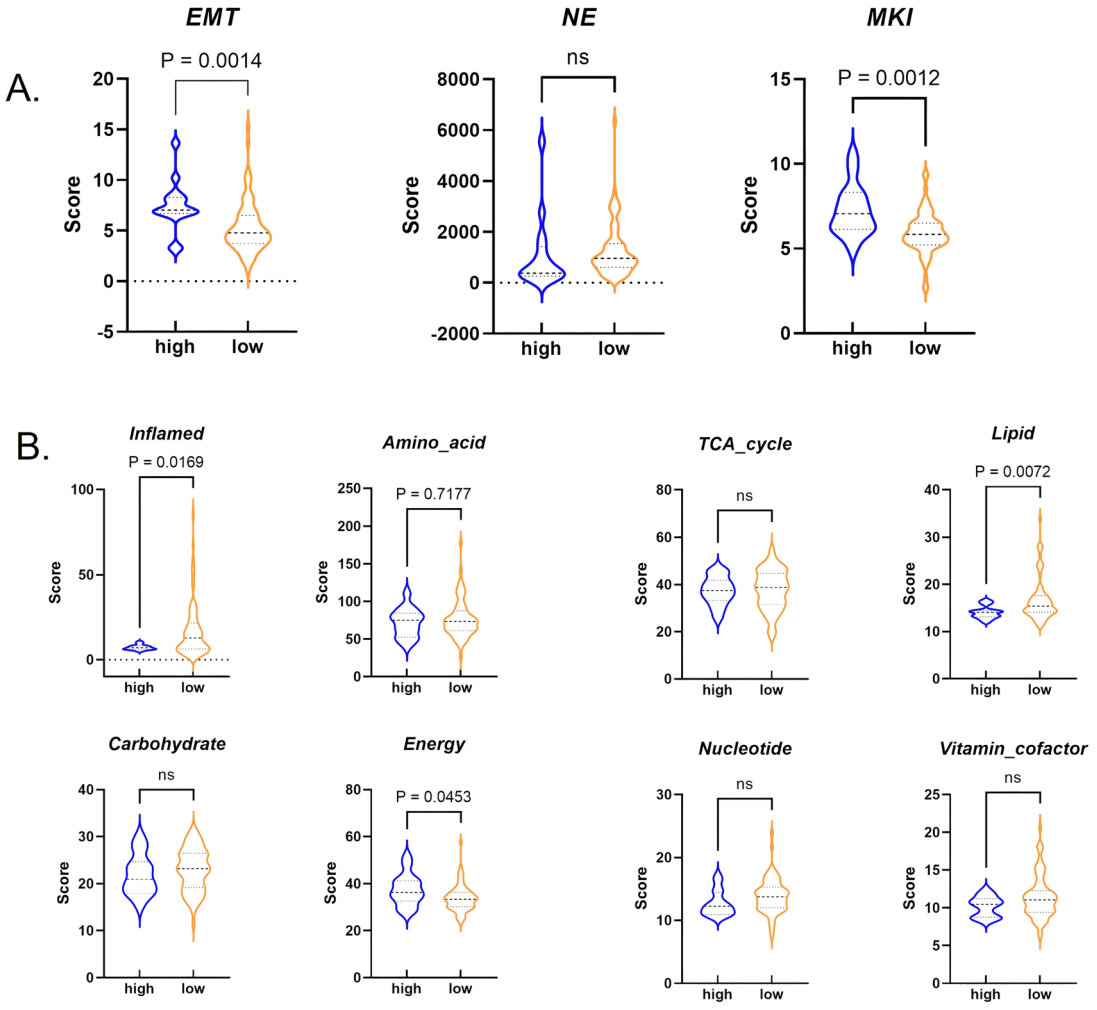

Figure 7. Analysis of biological behaviour scores in PPGL patients.
Note: high: Ki-67 ≥ 3%, low: Ki-67 < 3%.
(A) The *EMT* score and proliferation score were higher in the high Ki-67 group than in the low expression group.
(B) Comparison of immune microenvironment scores between the two groups.
Abbreviations: *EMT*, epithelial-mesenchymal transition. *MKI*, Marker of Proliferation Ki-67. *NE*, Neuroendocrine. TAC-cycle, Tricarboxylic Acid Cycle.

≥ 4 points) is 31% [35]. Additionally, the substantial subjectivity of morphological assessments conducted by diagnosing physicians, along with the exclusion of paragangliomas, significantly limits its clinical applicability. The GAPP scoring system has augmented the morphological parameters by adding the Ki-67 index and catecholamine types, allowing for the assessment of metastatic risk in moderately differentiated and poorly differentiated PPGLs at rates of 60% and 88%, respectively. However, similar to the PASS score, despite including multiple composite parameters, the subjective nature of most morphological parameters undermines reproducibility, making widespread adoption difficult. The Ki-67 index serves as a critical objective marker in routine pathological diagnosis and has extensive clinical applicability. However, even though it has been considered an important prognostic indicator for various malignant tumors, there is still no consensus on its prognostic value in PPGLs. Therefore, this study will focus on the significance of the Ki-67 index as a solitary prognostic factor, in conjunction with patient clinical characteristics, family history, and *SDHB* mutations, to determine whether Ki-67 can act as an independent prognostic factor for PPGLs and establish its cutoff value. Based on this, integrating multi-parameter models with defined prognostic significance, such as *SDHB* mutations, is expected to help develop a more objective and widely applicable predictive scoring system.

In previous studies, high Ki-67 positivity rates were often associated with malignancy, although the sensitivity of Ki-67 for detecting malignant PPGLs was found to be low. Falhammar H et al. argued that Ki-67 has limited value in predicting the metastatic development of individual patients due to a significant number of false-positive and false-negative results [2,36]. Currently,

as research progresses, an increasing number of studies, particularly those based on the GAPP scoring system, have embodied the value of Ki-67 in PPGLs. Although there is no WHO grading system specifically applied to PPGLs, Ki-67 is considered a continuous variable that is associated with the risk of progression, recurrence or metastasis [37–39]. It has been introduced as a core element in PPGLs reporting templates. However, the variation in positive cut-off values for Ki-67 among different authors limits its utility as the sole criterion for predicting malignant behavior. In our study, we observed that a Ki-67 value ≥ 3% was associated with a 3.59-fold increased risk of progression, relapse or metastasis compared to patients with a Ki-67 value < 3% in PPGLs, even after adjusting for all confounding factors. Consistent with our findings, a recent comparison of the PASS and GAPP systems found that a Ki-67 labeling index of 1% to 3% was significantly correlated with decreased recurrence-free survival [40]. These findings suggest that a Ki-67 value of 3% may serve as a useful cut-off for assessing the clinical risk of progression, relapse or metastasis in PPGLs.

Succinate dehydrogenase (*SDH*), also known as mitochondrial respiratory complex II, consists of *SDHA*, *SDHB*, *SDHC*, and *SDHD* subunits. Loss-of-function mutations in *SDHx* can result in significant activation of hypoxia signaling pathways, mainly due to the accumulation of succinic acid and abnormal electron transport [41]. It has been widely reported that decreased *SDHB* expression is associated with a higher risk of developing metastatic PPGLs [42–45]. To investigate the independent role of Ki-67 in assessing the risk of progression, relapse or metastasis in PPGLs patients, the influence of SDHB status was taken into consideration as a subgroup analysis. The findings revealed that regardless of the presence or absence of *SDHB* deletion, Ki-67 was positively correlated with recurrent metastasis of PPGLs. This observation further confirms the clinical significance of Ki-67 in the evaluation of progression, relapse or metastasis in PPGLs patients.

This study has several limitations that should be acknowledged. Firstly, the sample size was relatively small, with only 119 patients included for statistical analysis after excluding ineligible patients from the initial pool of 501 patients. The study was conducted at a single center, specifically the First Hospital of Jilin University, which may limit the generalizability of the results and their representativeness to the broader population. Secondly, limited disease awareness among some patients, along with insufficient patient education during clinical care, has contributed to a high rate of loss to follow-up. Given the extended follow-up period of over 10 years, this could introduce recall bias and impact the accuracy of recorded progression-free survival (PFS) data. To mitigate these limitations, we implemented structured annual follow-ups for all enrolled patients beginning in 2022. Thirdly, the follow-up duration in this study may be insufficient. A long-term follow-up study of PPGLs reported a median time to relapse of approximately 6 years after PPGL resection [18]. Our study cohort includes not only patients who underwent surgical resection but also 13 patients who did not, which may contribute to a shorter median PFS in this cohort. Although 33 patients experienced recurrence, disease progression, or metastasis during the current follow-up period of 33 to 162 months, which allows for statistically significant survival analyses and provides insights into the risk of early recurrence or metastasis, the fact that the metastasis of PPGLs can occur up to 30 years after diagnosis highlights the need for extended follow-up. We will continue monitoring this cohort to further investigate the impact of Ki-67 on the long-term risk of progression, relapse or metastasis in PPGLs. High-risk patients, such as younger patients and those with genetic disorders, large tumors, and/or paragangliomas, should receive lifelong annual follow-ups [46]. Lastly, the occurrence of hypertension and palpitations was recorded and treated as independent covariates in this study is another limitation that needs to be acknowledged. Among the 63 participants with palpitations and/or hypertension, 17 had both hypertension and palpitations, 43 had hypertension alone, and 3 had palpitations alone, suggesting that the majority of patients did not experience both conditions simultaneously. Given that co-occurrence is observed in clinical practice, the collinearity between these two variables could potentially influence the statistical results, we conducted a covariance diagnosis for hypertension and palpitations, yielding a VIF (Variance Inflation Factor) value of 1.107. Multicollinearity is present when the VIF is higher than 5 to 10 or the condition indices are higher than 10 to 30 [47]. This indicates that the covariance between hypertension and palpitations is weak, and the model analysis remains reliable. In our study, there is no significant differences were observed between groups categorized by Ki-67 expression levels (≥ 3% vs. < 3%), and in the Cox regression analyses, the model was adjusted for both hypertension and palpitations simultaneously.

5. Conclusion

A Ki-67 level of ≥ 3% has been found to be significantly associated with an elevated risk of progression, relapse or metastasis in patients diagnosed with PPGLs. These findings provide important insights into the prognostic value of Ki-67 and underscore its potential as a valuable marker for risk assessment in PPGLs. By incorporating Ki-67 assessment into clinical practice, healthcare professionals can gain better prognostic

information and make more informed decisions regarding the management and treatment of PPGLs patients.

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Author contributions

Zilan Luo and Lingyu Li drafted the initial version of the manuscript, and all authors participated in its review and editing. Additionally, Zilan Luo collaborated with Xu Yan on designing the methodology, and Zilan Luo was involved in patient follow-up, while Xu Yan prepared and reviewed pathological specimens required for the study. Yang Liu provided technical support for the necessary software in the research. Fengrui Nan handled the organization of experimental data. Yuhong Lei and Yuan Ren collected patient information and, together with Zilan Luo, were responsible for patient follow-up. Lingyu Li secured funding for the research project.

All authors have approved the current version and will be fully responsible for the content of the manuscripts.

Disclosure statement

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.

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Data availability statement

The patient information collected in this study was derived from electronic medical records and telephone follow-ups. The samples for RNA sequencing were obtained from Formalin-fixed, paraffin-embedded (FFPE) primary tumor samples. Data cannot be shared due to ethical, privacy, or security concerns. The data will be made available upon reasonable request. Please contact the corresponding author.

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