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ORIGINAL ARTICLE

Heparanase-1 and Cyclooxygenase-2: prognostic indicators of malignancy in pheochromocytomas

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Abstract The objective of this article is to evaluate Heparanase-1 and Cyclooxygenase-2 as tissue-based markers of pheochromocytoma prognosis. Ninety-two sporadic pheochromocytoma patients with a minimum of 8-year follow-up post-diagnosis were enrolled. Slides of normal adrenal glands in nephrectomy specimens from 20 patients with benign renal tumors were as control. Heparanase-1 and Cyclooxygenase-2 expression as well as microvessel density were examined using immunohistochemistry in tissues from these patients. Positive staining for Heparanase-1 was observed in 23.68% of the benign and 77.78% of the malignant cases, whereas none of the normal adrenal controls showed positive staining. Similarly, Cyclooxygenase-2 staining was seen in 23.68% of the benign versus 83.33% of the malignant cases, and none of the normal controls appeared positive for Cyclooxygenase-2. Using both HPA-1 and Cox-2 combined, the positive predictive value of malignancy was significantly increased to 0.72, compared to about 0.45 by their own. Malignant cases showed higher microvessel density compared to benign tumors and normal controls (36.41, 21.43, and 13.36%, respectively). Heparanase-1 and Cyclooxygenase-2 may contribute to the invasive characteristics of malignant pheochromocytomas. Heparanase-1 and Cyclooxygenase-2 combined is better than their own to be used as a marker to distinguish malignant from benign pheochromocytoma.

Keywords Pheochromocytoma · Heparanase-1 · Cyclooxygenase-2 · Microvessel density · Immunohistochemistry

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Introduction

Pheochromocytomas are catecholamine-secreting tumors deriving from the chromaffin cells of the adrenal medulla or the paraganglion system. They are relatively uncommon but potentially life-threatening. It is commonly accepted that distinguishing between benign and malignant pheochromocytomas is extremely difficult, and the diagnosis of malignant pheochromocytoma can only be determined by the presence of recurrence or metastatic disease at a site where chromaffin tissues do not normally exist. The reported incidence of malignant pheochromocytoma ranges from 10 to 26% [1]. Pheochromocytomas metastasize via hematogenous or lymphatic pathways, and the most common metastatic sites are lymph nodes, bone, lung, and liver [2]. It has been reported that a malignant diagnosis is more likely if the primary tumor is located extra-adrenally and possesses histological properties of coarse nodularity,

confluent tumor necrosis, and the absence of hyaline globules [3]. However, there are no histological features which definitively distinguish malignant from benign tumors, follow-up of patients is therefore extremely important in management of the disease, and all patients require long-term and close follow-up.

Extracellular matrix (ECM) degradation and angiogenesis are critical steps in the growth, migration, and invasion of tumor cells. ECM degradation allows tumor cells to penetrate the tissue barrier and become metastatic, while angiogenesis promote the process of sprouting and branching of new vessels from the pre-existing capillaries and post-capillary venules. Heparanase-1 (HPA-1) is an endo-β-D-glucuronidase that specifically degrades heparan sulfate proteoglycans, a chief component of the ECM [4]. Cyclooxygenase (Cox) is the rate-limiting enzyme in prostanoid biosynthesis, and two isoforms, Cox-1 and Cox-2 have been identified. Human HPA-1 and Cox-2 were found to be upregulated in various types of primary tumors, correlating in some cases with increased tumor invasiveness and vascularity and with poor prospective survival.

However, expressions of HPA-1 and Cox-2 in pheochromocytomas and their relationship with tumor angiogenesis have not been clarified until recently. In this study, we investigated the expression of HPA-1 and Cox-2 in normal adrenal gland and in our large collection of benign and malignant pheochromocytomas.

Materials and methods

Patients

Following approval from institutional ethics review board, the complete medical records of 92 sporadic pheochromocytoma patients with a minimum of 8-year follow-up post-diagnosis were reviewed from our database. These patients underwent adrenalectomy at Ruijin hospital between October 1986 and August 2001. Syndromic or familial cases were not included in this study. Standard demographic data, information on presenting signs and symptoms, results of routine laboratory tests, and preoperative radiographic imaging studies were obtained by reviewing these records. Computed tomography (CT) scan was performed in all patients, and radiotracer-labeled meta-iodobenzyl-guanidine (^{131}I-MIBG) scan was used to confirm the diagnosis of pheochromocytoma. The follow-up data were obtained from patient medical records.

Tissue samples

Paraffin-embedded specimens from 94 pheochromocytomas collected from surgical specimens from 92 patients were evaluated. Slides of normal adrenal glands were from 20 patients undergoing nephrectomy for a benign renal tumor. All histopathologic slides were reviewed by two endocrine pathologist blinded to the clinical data independently. There was no discrepancy between the two pathologists in classifying the tumors. A pheochromocytoma was considered malignant based on the consensus: (1) presence of widespread local invasion at time of surgery, (2) history of recurrence, and (3) metastasis occurs to sites normally devoid of chromaffin tissue. Based on these criteria, 18 pheochromocytomas were classified as malignant and 76 were considered benign.

Immunohistochemistry staining

Paraffin-embedded tissue were dewaxed in xylene and rehydrated by serial concentrations of ethanol and then treated with 3% H_2O_2 to refrain endogenous peroxidase. They were then heated in a microwave at 750 W for 15 min to repair the tissue antigen. After had cooled to room temperature, the sections were then blocked with 10% normal goat serum for 10 min. This was followed by a PBS wash and incubation with polyclonal rabbit anti-human HPA-1 antibody (GenScript, USA) diluted to 1:100 for 12 h at 4°C. The slides were then incubated by anti-rabbit EnVisionTM kit (DAKO, USA) for 30 min at 37°C. After a PBS wash, the sections were developed in diaminobenzidine substrate. The sections were then counterstained in hematoxylin for 2 min and then dehydrated in ethanol and xylene before being mounted. The staining results of pancreatic carcinoma tissue sections which HPA-1 positive had already known were regarded as positive control, PBS replacing first antibody was as negative control.

Sections were re-prepared by EnVision immunohistochemical staining. Cox-2 was polyclonal rabbit anti-human antibody (Santa Cruz, USA), diluted to 1:200. CD34 was monoclonal mouse anti-human antibody (Novocastra, USA), diluted to 1:200. The staining results of colon carcinoma tissue sections which Cox-2 or CD34 positive had already known were regarded as positive control, PBS replacing first antibody was as negative control.

Evaluation of immunohistochemical results

Positive HPA-1 staining was characterized by the presence of purple-brown granules located diffusely in the cytoplasm of the tumor cells. Lack of any obvious purple-brown or brown-red pigmentation was considered negative [5]. Positive Cox-2 expression appeared as brown intracytoplasmic granules inside tumor cells [6]. Under high power microscope (400×), five visual fields were randomized selected in each section, and 200 cells were

counted in each high power field. The stains were scored according to the percentage: 0, <5%; 1, 5–29%; 2, 30–50%; and 3, >50% of the cells staining, and intensity: 0, no staining; 1, weak; 2, moderate; and 3, strong staining. Positive or negative expression was made according to the combination of these two variables. Total score >3 was regarded as positive, and ≤3 as negative. Microvessel density (MVD) is regarded as golden standard in evaluating tumor angiogenesis and is evaluated according to the method mentioned by Weider [7]; high vascular density area was selected under low power objective and counted the numbers of vascular stained by CD34 in three visual fields under high power microscope (400×), and average value was regarded as the MVD value of the tumor.

Statistical analysis

Analyses were performed using the SPSS statistical software. The χ^2 test was used to compare HPA-1 and Cox-2 expressions in benign, malignant pheochromocytomas, and normal adrenal medulla tissues, and the mean primary tumor size in each group was also examined. MVD result was expressed as Mean ± SD, and Wilcoxon combined test was used to compare MVD expression in benign, malignant pheochromocytomas, and normal adrenal medulla tissues. Spearman rank correlation analysis was used to determine the relationship between HPA-1, Cox-2 expression, and MVD. The level of statistical significance was chosen as $P < 0.05$.

Results

Tumor characteristics and clinical features of pheochromocytoma patients were summarized in Table 1. Of the pheochromocytomas, 17 (18.48%) of the 92 patients were diagnosed as malignant according to the criteria mentioned above. There were 43 males and 49 females with 9 men and 8 women who were diagnosed as clinically malignant pheochromocytoma. Thirty-five patients had a right adrenal neoplasm, 31 had a left adrenal tumor, while 2 patients had bilateral pheochromocytomas. One of the two patients had clinically benign pheochromocytoma, while another developed a clinically malignant tumor. The mean primary tumor largest diameter in benign and malignant group was 5.2 ± 2.1 and 8.5 ± 3.5 cm, respectively ($P < 0.001$). The mean age at time of diagnosis was 43.05 years for the benign group and 45.06 years for the malignant group. Extra-adrenal pheochromocytoma was found in 16 patients in benign group (21.33%) and 8 in malignant group (47.05%). Three patients were classified as malignant pheochromocytoma at the time of surgery due to invasion into inferior vena cava or ipsilateral kidney. Four patients were considered as malignant because of local recurrence and all of them received surgical resection. Seven cases were diagnosed as malignancy because the presence of peri-nephric, peri-aortic, or neck lymph node metastases during follow-up period, and four of them underwent operation. Three patients had multiple metastasis including lung, liver, bone, neck lymph node, and peri-aortic lymph node. Mean time to first recurrence or development of metastases in 14 patients was 70.2 months, range from 28 to 179 months. Eight of these 14 patients underwent surgical treatment. Heparanase-1 and Cyclooxygenase-2 staining were performed in these eight patients, and similar results were observed in each patient in comparison with their primary immunohistochemical results. In order to relief symptoms, combination therapy with ^{131}I-MIBG and chemotherapy was performed in other six patients who were unsuitable for surgical therapy.

The HPA-1 and Cox-2 staining data are summarized in Table 2. Positive staining for HPA-1 was observed in 23.68% (18/76) of the benign and 77.78% (14/18) of the malignant cases, whereas none of the normal adrenal controls showed positive staining. The difference of HPA-1 expression between benign and malignant pheochromocytomas was statistically significant ($P < 0.05$). For positive HPA-1 staining alone, the positive predictive

Table 1 Tumor characteristics and clinical features of pheochromocytoma patients

Clinical characteristic	Benign		Malignant	
	Adrenal	Extra	Adrenal	Extra
Total number of tumors/patients	60/59	16/16	10/9	8/8
Gender				
Male	27	7	5	4
Female	32	9	4	4
Tumor location				
Left adrenal	31		4	
Right adrenal	27		4	
Bilateral	1		1	
Primary tumor size				
<4 cm	8	1	1	0
4.1–7.9 cm	41	12	2	3
>8 cm	10	3	7	5
Mean diameter (cm)	5.2 ± 2.1 cm		8.5 ± 3.5 ($P < 0.001$)	
Age at presentation (year) mean (range)	43.05 (11–77)		45.06 (19–75)	
Follow-up (months) mean (range)	162.51 (97–264)		92.2 (11–220)	
Mean time to first recurrence/development of metastases (months)			70.2 (28–179) $n = 14$	

value of malignancy was 0.44. Cox-2 staining was seen in 23.68% (18/76) of the benign versus 83.33% (15/18) of the malignant cases, and none of the normal controls appeared positive for Cox-2. The difference of Cox-2 expression between benign and malignant pheochromocytomas was statistically significant ($P < 0.05$). For positive Cox-2 staining alone, the positive predictive value of malignancy was 0.45. Thirteen out of 18 malignant pheochromocytomas were stained positive for both HPA-1 and Cox-2, while only 5 out of 76 benign tumors were positive. Two of malignant pheochromocytomas (11.11%) stained negative by both HPA-1 and Cox-2, compared to 59.21% of benign tumors ($n = 45$). Using both HPA-1 and Cox-2 combined (where a test was considered positive if HPA-1 and Cox-2 were both positive in one case), the positive predictive value of malignancy was significantly increased to 0.72, compared to 0.44 by HPA-1 or 0.45 by Cox-2 alone.

Expression of MVD was highest in malignant pheochromocytomas (36.41%), the next in benign pheochromocytomas (21.43%), and the last in normal adrenal medulla tissues (13.36%). Statistically significance was observed between each group. HPA-1 or Cox-2 expression in benign and malignant pheochromocytomas was correlated with MVD (Figs. 1, 2, 3, 4, 5, and 6).

Discussion

Malignant pheochromocytoma has a poor prognosis, and the 5-year survival rate is about 44%, while the 5-year survival rate of surgical-treated benign pheochromocytomas is over

Table 2 HPA-1 and Cox-2 staining data

	Benign ($n = 76$)	Malignant ($n = 18$)	PPV	NPV
HPA-1			0.44	0.94
Stain (+)	18	14		
Stain (−)	58	4		
Cox-2			0.45	0.95
Stain (+)	18	15		
Stain (−)	58	3		
HPA-1 stain (+) and Cox-2 stain (+)	5	13	0.72	0.91
HPA-1 stain (+) and Cox-2 stain (−)	13	1		
HPA-1 stain (−) and Cox-2 stain (+)	13	2		
HPA-1 stain (−) and Cox-2 stain (−)	45	2		

PPV positive predictive value, *NPV* negative predictive value

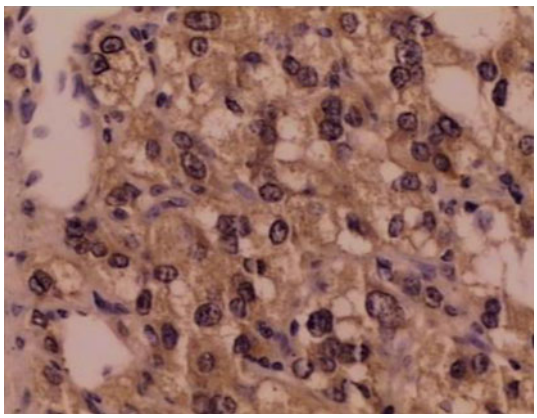

Fig. 1 The strongly positive expression of HPA-1 in malignant pheochromocytoma (×200)

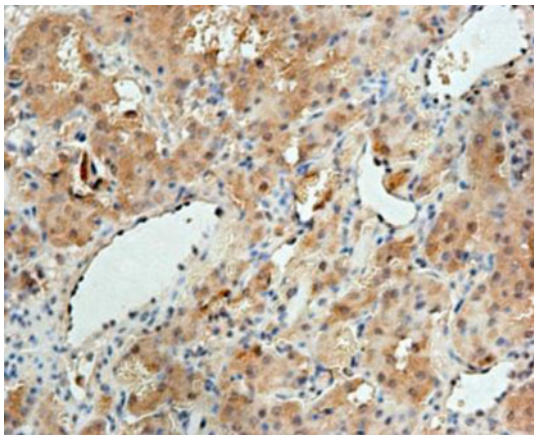

Fig. 2 The strongly positive expression of Cox-2 in malignant pheochromocytoma (×200)

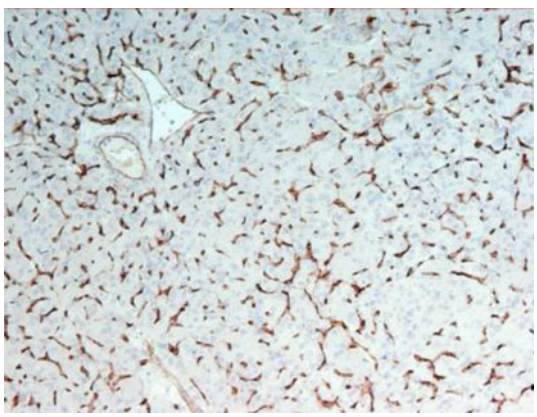

Fig. 3 The high expression of CD34 IHC staining MVD in malignant pheochromocytoma (×100)

95% [8]. However, the pathologic distinction between clinically benign and malignant pheochromocytoma can be virtually impossible to make. The diagnosis of malignant

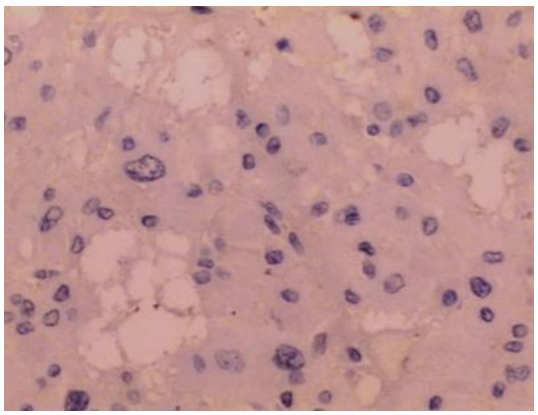

Fig. 4 The negative expression of HPA-1 in benign pheochromocytoma (×200)

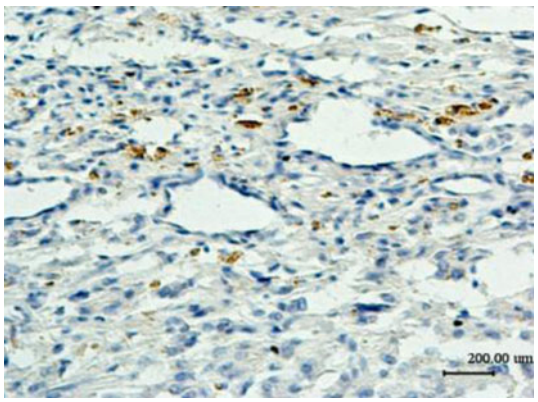

Fig. 5 The negative expression of Cox-2 in benign pheochromocytoma (×200)

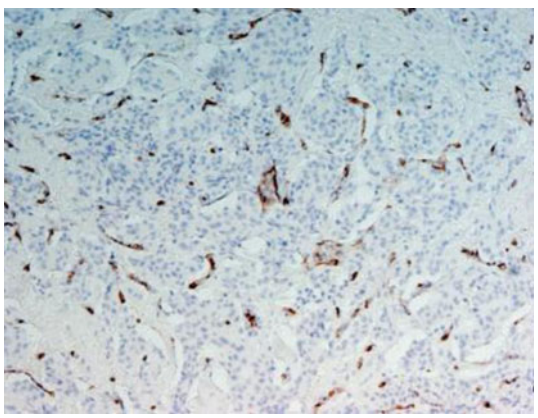

Fig. 6 CD34 IHC staining MVD in benign pheochromocytoma was moderately positive (×100)

pheochromocytoma can only be determined by the presence of recurrence or metastatic disease at a site where chromaffin cells do not normally exist. In this study, we evaluated HPA-1, Cox-2, and MVD to determine their usefulness as prognostic tools in grading pheochromocytomas. The pheochromocytomas in these series had an equal gender distribution similar to what has been previously reported. Seventeen of the 92 cases (18.48%) were classified as malignancy, and this incidence is slightly higher than the classically reported 10%. Extra-adrenal pheochromocytoma was found in 8 of the 17 malignant neoplasms (47.05%) while present in only 16 of the 75 patients (21.33%) with benign tumors. Bilateral tumors were only found in two cases, which mainly because syndromic or familial cases were not enrolled in the study. The mean size of malignant pheochromocytoma was dramatically larger than that of benign tumor.

The nucleotide sequence of HPA-1 gene has been cloned and identified in mammals including human beings since 1999. Heparanese-1 protein is an endo-β-D-glucuronidase, which degrades heparin sulfate side chains of heparin sulfate proteoglycans in the ECM. HPA-1 plays an important role in tumor invasion and metastasis. Human HPA-1 mRNA and protein expression were found to be upregulated in various types of primary tumors by using in situ hybridization (ISH) or immunohistochemistry (ICH) [9, 10]. HPA-1 overexpression was correlated in some cases with increased tumor invasiveness and vascularity and with poor prospective survival [11, 12].

There has been no further study on HPA-1 expression in adrenal pheochromocytoma since Quiros and his colleagues first published their article in 2002. Quiros et al. [5] evaluated HPA-1 expression by both immunohistochemistry (IHC) and in situ hybridization (ISH). They found that the percentage of HPA-1 expression in malignant and benign pheochromocytomas was 50 and 21%, respectively, by using ISH, while the percentage was 80 and 32% in malignant and benign pheochromocytomas by using ICH. Considering both tests cumulatively, all malignant pheochromocytomas stained positive for HPA-1 by ISH and ICH, while only 37% of the benign tumors were positive for HPA-1 expression. In this study, we found that positive staining for HPA-1 was observed in 23.68% of the benign and 77.78% of the malignant cases, whereas none of the normal adrenal controls showed positive staining. According to our study, the positive predictive value of HPA-1 alone was 0.44. The result was similar to that of the previous study.

Cyclooxygenase is the key enzyme in conversion of arachidonic acid to PGs, and two isoforms, Cox-1 and Cox-2, have been identified. Cox-2 has been associated with carcinogenesis, and it is overexpressed in many human malignancies, such as gastrointestinal tumor, lung cancer, hepatic cancer, pancreatic carcinoma, squamous cell carcinoma of head and neck, and urogenital system carcinoma [13–18].

Cox-2 regulates the processing of generation and development of tumors by various mechanisms such as increasing cell proliferation, inhibiting cell apoptosis, and promoting angiogenesis [19]. There are few researches on Cox-2 expression in adrenal pheochromocytomas. Salmenkivi et al. [20] first examined Cox-2 expression in pheochromocytomas and demonstrated Cox-2 play a role in the development of an aggressive tumor phenotype. They found that Cox-2 expression was positive in normal adrenal cortex and negative in adrenal medulla. Cox-2 expression in all malignant pheochromocytomas was moderate or strong, while in 75% of the benign pheochromocytomas and in 79% of the borderline pheochromocytomas was negative or weak. In our research, Cox-2 staining was seen in 23.68% of the benign versus 83.33% of the malignant cases ($P < 0.05$), and none of the normal controls appeared positive for Cox-2. The positive predictive value of Cox-2 alone was 0.45 according to our research. The result was similarly to that of the previous studies.

Although HPA-1 and Cox-2 were previously reported to be prognostic indicator of malignancy in pheochromocytomas, it was not sufficient on their own to be useful clinically at present. In our study, using both HPA-1 and Cox-2 combined as the prognostic indicator, the positive predictive value of malignant pheochromocytoma was magnificently increased to 0.72, compared to 0.44 by HPA-1 or 0.45 by Cox-2 alone.

The value of angiogenesis in tumor growth, invasion, and metastasis has been confirmed by a wealth of studies since Folkman, and microvessel density (MVD) is regarded as golden standard in evaluating tumor angiogenesis [21]. MVD also can be used as an important marker to indicate the biological behavior of the malignant tumors, such as invasion and metastasis. Recently, tumor-derived vascular endothelial cells are usually marked by the antibodies CD34, CD31, VIII factor, etc. Our results demonstrated that expression of MVD was highest in malignant pheochromocytomas, the next in benign, and the last in normal adrenal medulla tissues.

Presumably, the mechanism HPA-1 promote tumor metastasis is multimodal. Tumor production of the enzyme facilitates degradation of the heparan sulfate moieties in the basement membrane and ECM, allowing migration of malignant cells through these structures and distant dissemination to other organs [9, 10]. However, tumor spread also depends on angiogenesis as the malignant cells implant in distant sites. Quiros et al. [5] suggested that HPA-1 could promote angiogenesis and its overexpression was associated with malignancy in pheochromoctomas. However, they did not evaluate the correlation between HPA-1 expression and tumor angiogenesis in adrenal pheochromocytomas. In this study, we demonstrated that expressions of HPA-1 in benign and malignant pheochromocytomas were directly correlated with MVD expression, which confirmed HPA-1's important role in angiogenesis. Elkin et al. [22] have considered HPA-1 as a mediator in angiogenesis. Investigations on breast cancer [23], esophageal cancer [24], and gastric cancer [25] revealed that HPA promotes the expression of Cox-2 mRNA. In this study, we found HPA correlated with Cox-2 expression directly, which indicated that HPA-1 may promote angiogenesis by upregulating Cox-2 expression. It has been reported that low metastatic murine T-lymphoma and melanoma cells transfected with the heparanase cDNA acquired a highly metastatic phenotype in vivo, reflected by a massive liver and lung colonization, and this procedure can be inhibited by using HPA inhibitors [9]. The results provide a theoretical basis in the targeted therapy for malignant pheochromocytomas.

In conclusion, HPA-1 and Cox-2 expressions are higher in malignant pheochromocytomas than in benign pheochromocytomas or normal tissue. HPA-1 and Cox-2 may contribute to the invasive characteristics of malignant pheochromocytomas. Using HPA-1 and Cox-2 combined can improve the positive predictive value of malignant pheochromocytoma. The combination of HPA-1 and Cox-2 is better than their own to be used as a biomarker to distinguish malignant from benign pheochromocytoma.

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