ORIGINAL ARTICLE

Immunohistochemical expression patterns of S100, synaptophysin, chromogranin A and neuron specific enolase in predicting malignant behaviour in paragangliomas

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Summary

Purpose: The purpose of this study was to evaluate the role of immunohistochemical markers in the prediction of malignancy in paragangliomas.

Methods: Our institute's patient records between 1990-2012 were retrieved in order to identify patients who were treated for paragangliomas. Size and location of the tumour, existence of concurrent metastatic disease, patient demographics and survival were recorded. Haematoxylin-eosin stained slides were reviewed and all tumours were stained specifically for neuron specific enolase (NSE), chromogranin, synaptophysin and S100 protein positivity. Positivity and expression patterns of the above markers were evaluated and compared between malignant and benign tumours. Malignant behaviour was defined when patient had concurrent or subsequent lymph node involvement, local recurrence and/or metastases.

Results: A total of 22 patients with a diagnosis of paraganglioma were treated in our institutes. Female to male ratio was 1.75: 1. The mean age was 43.5 and 51.6 years for women and men, respectively. In 5 patients the tumors had malignant clinical behavior. Their mean size was 3.65 cm for benign and 4.56 cm for malignant neoplasms. NSE expression was diffuse in 47.1% and 0% for benign and malignant tumors, respectively (p=0.10). S100 expression in the periphery of the tumour was typical in 88.2% and 0% for benign and malignant tumors, respectively (p<0.001).

Conclusion: Immunohistochemical profile from the combination of NSE, synaptophysin chromogranin and S100 staining patterns can serve as a cheap and valuable tool for correctly distinguishing between malignant and benign paragangliomas with high diagnostic accuracy.

Key words: chromogranin, immunohistochemistry, malignant paragangliomas, neuron-specific enolase, S100, synaptophysin

Introduction

Paragangliomas represent rare tumours arising from sympathetic or parasympathetic neuronal tissue and are chromaffin cell tumours. They develop from neural crest cells and comprise of 10-20% of all chromaffin cell tumours [1].

Paragangliomas arising from ganglia of the parasympathetic system are almost always located in the neck and base of the skull, mostly deriving from the carotid body. On the contrary, paragangliomas originating from ganglia of the sympathetic nervous system can be located along the distribution of the sympathetic nervous system from the head and neck, along the aorta through the mediastinum and in the retroperitoneal space, up to

the prostate gland and the urinary bladder. Most of them are located in the abdomen (75%), mostly at the site of the origin of the inferior mesenteric artery where the Zuckerkandl organ is located.

The majority of sympathetic system paragangliomas secrete catecholamines, which can be a life-threatening condition, and are associated with hormone secretion syndromes. On the other hand, parasympathetic paragangliomas do not often secrete catecholamines.

Paragangliomas may have malignant behaviour, with a prevalence of 2-26% [2]. The most common metastatic sites are lymph nodes, bone and lung. Although benign paragangliomas have a very good 5-year overall survival of more than 90%, paragangliomas with malignant behaviour have 5-year survival of around 50% [3,4]. It is therefore imperative that these tumours should be recognized early so that more aggressive surgical and medical treatment can be adopted.

Although many attempts have been made to identify clinical, biochemical and pathological markers suggestive of malignancy, none of them seem to be quite sensitive in predicting malignancy, leading to undertreatment of these patients. Immunohistochemical markers have shown insufficient concordance. Therefore, attempts are addressed towards designing multi-parameter scoring systems for risk stratification.

The purpose of this study was to retrospectively evaluate the expression and diagnostic accuracy of immunohistochemical markers and their combination for the prediction of malignancy of paragangliomas.

Methods

Patient records between 1990-2012 were retrieved in order to identify those treated in our departments with a diagnosis of paraganglioma.

Patient gender, age, size and location of the tumor, the presence of lymph node involvement or metastatic disease and the final pathology report were recorded.

Follow-up data were collected either from the oncology service department of our institutes or after direct communication with the patients, when data were not available.

Metastatic behavior was determined based on the evaluation of lymph node involvement, distant organ involvement and recurrence of disease.

The study protocol was approved by the hospitals' Ethics Committees.

Immunohistochemistry protocol

Tissue samples were fixed in 10% buffered formalin and were embedded in paraffin. Sections of 5 μm were stained with hematoxylin-eosin. Immunohistochemistry was performed on deparaffinized 3-5 μm sections on a Ventana Automatic System (Benchmark XT) with the use of iVIEW DAB Detection kit - an indirect biotin-streptavidin system using DAB as chromogen. Protocols and primary antibodies used for specific staining of NSE, chromogranin A, S100 protein and synaptophysin are described below in more detail.

For NSE we used a mouse monoclonal antibody (clone: BBS/NC/VI-HI4- Dako, Denmark), with a dilution of 1:400, followed by 8-min of epitope retrieval and 16-min incubation of the antibodies.

For chromogranin A staining we used a mouse monoclonal antibody (clone: LK2H10- Biocare Medical, Concord, CA, USA), with a dilution of 1:50, followed by 30-min of epitope retrieval and 20-min of incubation of the antibody.

For synaptophysin, a rabbit monoclonal antibody (clone: SP11- Thermo Scientific, Fremont CA, USA) with dilution of 1:80 was used, followed by 30-min of epitope retrieval and 28-min incubation of the primary antibody.

Finally, for S-100 protein staining we used a mouse monoclonal antibody (clone 4C4.9- Cell Marque Rocklin, CA, USA) with a dilution of 1:100, followed by 30-min of epitope retrieval and 8-min of primary antibody incubation.

Staining pattern interpretation protocol

All cases were reviewed by a single expert pathologist (A.P). Immunohistochemical staining patterns were characterized according to Table 1. Each individual stain pattern was scored and the total score was calculated and evaluated for being predictive of malignancy.

Statistics

Results were expressed as mean ± SD. Comparison between groups was performed using the x^2 test for qualitative data and the unpaired t-test for quantitative data.

Sensitivity (true positive/true positive + false negative), and specificity (true negative/true negative + false positive) were calculated. Statistical analyses were per-

Table 1. Scoring of immunohistochemical staining patterns

Immunohistochemical stain	Staining pattern	Score
Neuron specific enolase	Sporadic cells	1
	Foci of cells	2
	Diffuse stain	3
Synaptophysin	Sporadic cells	1
	Foci of cells	2
	Diffuse stain	3
S100	No positivity	1
	Sporadic cells	2
	Peripheral staining of tumors	3
Chromogranin A	Sporadic cells	1
	Foci of cells	2
	Diffuse stain	3

formed using a commercially available statistical software package (SPSS 17.0 for Windows; SPSS Inc., Chicago, IL, USA). A p value less than 0.05 was considered statistically significant.

Results

Twenty-two patients were found with tumors classified as paragangliomas. Patient records, imaging studies, pathology workup and follow up were identified in 17 patients with benign disease and in 5 patients with paragangliomas with malignant behavior either at first presentation or during patient follow up. Fourteen patients were female and 8 male, with a mean age of 45.2±14.2 and 50.6±19.4 years, respectively. Mean size of the neoplasms was 3.6±1.5 cm for benign and 4.5±2.7 cm for malignant tumors (p=0.34). Data are summarized in Table 2.

NSE expression pattern was characteristic with foci of cells stained in both malignant and benign tumors. There was a trend towards more diffuse staining pattern in benign tumors, that, however, did not achieve statistical significance (p=0.10). The expression pattern of S100 was significantly different between groups with staining at the periphery of the tumors in the majority of benign tumors (p<0.001). This typical staining pattern was absent in all malignant tumours (Figures 1 and 2). Moreover, there was a significant difference in the expression of synaptophysin between benign and malignant tumors, with malignant neoplasms showing a more sporadic expression in contrast to benign tumors in which the stain was organized in cell foci or diffusely (p=0.003). No difference in the expression pattern of chromogranin A was identified between the two groups (Figure 3). Data are summarized in Tables 3 and 4.
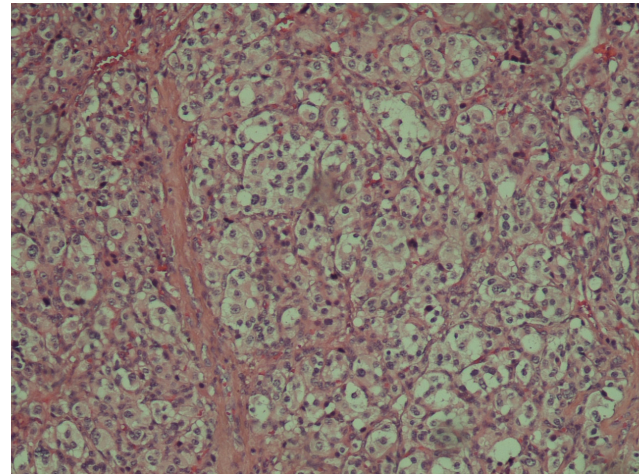

Figure 1. Histological section of a paraganglioma showing a positive S100 immunoreaction of the sustenctacular cells surrounding the neoplastic nests (immunostain S100 ×120). This is a case of a non malignant paraganglioma.
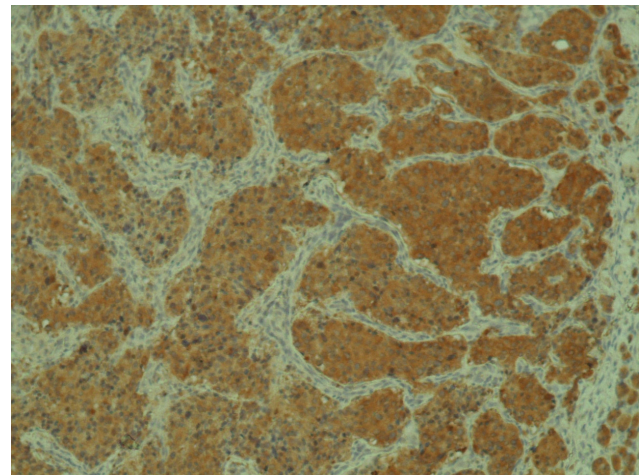

Figure 2. Histological section of a paraganglioma showing random positive S-100 immunoreaction of the sustenctacular cells (immunostain S100×120). This is a case of a metastatic paraganglioma.

Table 2. Demographic data and tumor characteristics (mean±SD)

Characteristics	Age (years)	Cervical location	Retroperitoneal location	Tumor size (cm)	Male/ Female
Benign	45.2±14.2	13	4	3.6±1.5	6/11
Malignant	50.6± 19.4	2	3	4.5±2.7	2/3
p	0.50	1.24	1.24	0.34	0.84

Table 3. Differences in immunohistochemical staining patterns of neuron-specific enolase and S-100

Tumour	Neuron specific enolase				S100	
	Sporadic cells	Foci of cells	Diffuse stain	Negative stain	Sporadic cells	Peripheral staining
Benign	1	8	8	0	2	15
Malignant	0	5	0	4	1	0
p			0.10			<0.001

Table 4. Differences in immunohistochemical staining patterns of synaptophysin and chromogranin

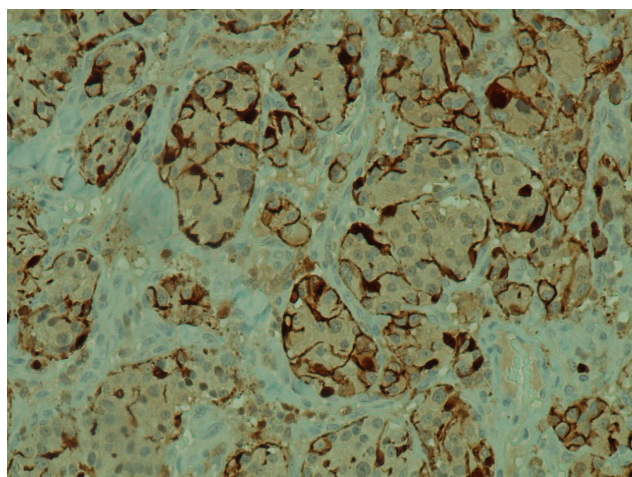| Tumour	Synaptophysin			Chromogranin		
	Sporadic cells	Foci of cells	Diffuse stain	Sporadic cells	Foci of cells	Diffuse stain
Benign	0	10	7	0	10	7
Malignant	3	1	1	0	4	1
p		0.03			0.61	

Figure 3. Histological section of a paraganglioma showing positive chromogranin immunoreactions of the cells (immunostain ×120).
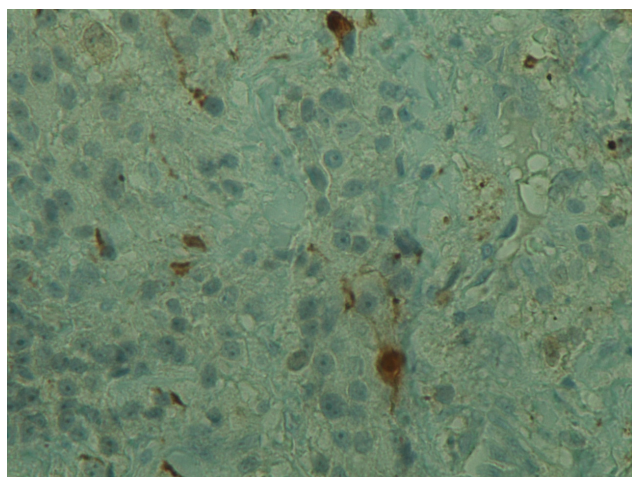

Figure 4. Histological section of a typical paraganglioma (Hematoxylin-Eosin ×120).

Table 5. Differences in total immunohistochemical staining pattern score between benign and malignant paragangliomas

Tumour	Total immunohistochemical pattern score	
	Score ≤8	Score ≥ 9
Benign	0	17
Malignant	5	0
p	<0.0001	

There was a significant difference in the total score of the immunohistochemical staining pattern between patients with benign and malignant disease (p<0.001). When using a cut-off value of 8 for the total immunohistochemical staining score, all patients with total scoring less than 8 had a tumor of malignant behavior, as opposed to patients with a score of 9 or higher whose tumor demonstrated a benign clinical course during follow-up. The absence of peripheral and typical S100 staining has a sensitivity of 100% and a specificity of 88.2% in predicting malignancy in our series. A total immunohistochemical pattern score less than 8 had a specificity and sensitivity of 100 in predicting malignancy, as shown in Table 5 and Figure 4.

Discussion

Paragangliomas are chromaffin cell tumors that develop from the neural crest cells. They are divided into tumors of the parasympathetic or sympathetic ganglia. The parasympathetic paragangliomas are found mainly in the neck and skull base and arise within the carotid body or the globus jugulotympanic. Parasympathetic paragangliomas are the most common type, while the rest are derived from the sympathetic ganglia and are located in the chest and retroperitoneal space and rarely to the head and neck region [5].

Both sympathetic and parasympathetic paragangliomas have similar histological characteristics, although there are data supporting that they are genetically different [6-8].

The majority of paragangliomas are benign. However, malignancy is thought to exist in up to 35% of sympathetic and, rarely, of parasympathetic (head and neck) paragangliomas. These figures can be even higher when specific genetic syndromes, such as succinate dehydrogenase gene mutations (SDHB, SDHC, SDHD), neurofibromatosis type 1 (NF-1), von Hippel-Lindau, the Carney triad, and others are taken into account [4,8].

To date there is no established clinical, biochemical, radiological or pathological features of malignancy for these tumors. According to cytomorphometric studies, predictors of malignancy in a paraganglioma are considered to be extra-adrenal

location, confluent tumor necrosis, vascular invasion, local invasion, coarse nodularity and absence of hyaline globules. The diagnosis of malignancy is made when regional lymph node metastasis or distant metastases are found either at diagnosis or during follow-up of these patients. Although there have been many attempts to correlate histological features with malignant potential of these tumors, all of them lack specificity and sensitivity. Multiparameter scoring systems have been developed for risk stratification, such as the Phaechromocytoma of the Adrenal Scaled Score (PASS) [9] and the Grading System for Adrenal Phaechromocytoma amd Paraganglioma (GAPP) [10]. Although the features integrated in these systems may have some validity, PASS was found to have very poor concordance between expert pathologists in a validation study [11], and GAPP has so far had no validation study results published. Malignant characterization remains of vital importance, as several management options have been made available, from 131I-MIBG treatment (and recently peptide receptor radionuclide treatment), to chemotherapy and external beam radiation therapy, even in the adjuvant setting after surgery [12]. In addition, prediction of malignancy in these tumors could lead to a more aggressive treatment which may potentially increase survival rates.

Several immunohistochemical markers have been widely incorporated in the pathology workup of neuroendocrine neoplasia, such as enzymes (NSE), proteins stored in the secretory granules (chromogranin A and HISL- 19 protein), resident proteins of the presynaptic vesicles, catecholamines and indolamines, neuropeptides, and molecules with unknown function (Myelin associated glycoprotein Leu- 7).

Chromogranins, in general, are considered to provide sufficient documentation of neuroendocrine differentiation. Chromogranin A, in particular, has been found to reflect both tumor burden and tumor secretion in neuroendocrine tumors [13].

S100 protein is used to identify sustentacular cells. In normal paraganglia, these cells exhibit various functions, such as serving as stem cells, and as glial-like supporting cells. In paragangliomas, however, their role is questioned, because they could be either neoplastic or benign migrants

from adjacent tissue or the circulation [14]. S100 staining in type II cells is localized at the periphery of the cytoplasm and of the nuclei. Abundance of type II cells may offer good prognosis of paragangliomas [15]. Interestingly, in the majority of published case series, a remarkable decrease in the immunoreactivity of S100 protein in malignant cases has been noted. S100 positive cells were noted in metastatic deposits, suggesting that the sustentacular cells had metastatic potential along with chromaffin cells and are an integral part of the tumour [9]. In our study, the expression pattern of S100 was significantly different between groups with a staining of the periphery of the tumors in the majority of benign tumors (p<0.001).

Locally aggressive tumor tissues express poorly chromogranin and S100 protein [16]. Synaptophysin expression is not limited by the stage of differentiation, as its monoclonal variant is very sensitive and specific for neuroendocrine tumors. In our patients' histochemical analysis, there was a significant difference in the expression of synaptophysin between benign and malignant tumors, with malignant neoplasms showing a more sporadic expression in contrast to benign tumors in which the stain was organized in cell foci or diffusely (p=0.003).

Pathologists consider that NSE is a sensitive marker for neuroendocrine tumors, although it has been reported to have a low specificity. Regarding neuroendocrine tumors' malignant behavior, previous studies have claimed that NSE may reflect cell necrosis, and raised NSE levels are associated with poorly differentiated tumors.

Conclusion

Distinguishing between benign and malignant paragangliomas remains a diagnostic challenge for every pathologist, due to the absence of established criteria. Combining immunohistochemical staining patterns of routine markers can be proved to be a cheap, readily available and reliable method of predicting malignancy in these tumours.

Conflict of interests

The authors declare no conflict of interests.

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