CLINICAL STUDY

Survivin: a novel neuroendocrine marker for pheochromocytoma

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Abstract

Objective: To study survivin expression in human adrenal medulla and in benign and malignant pheochromocytoma tissue as a tool to predict tumor metastatic potential and prognosis.

Design: Blinded study to assess the role of the anti-survivin antibody in chromaffin cells.

Methods: We performed immunohistochemistry with a purified rabbit-polyclonal anti-survivin antibody on 39 formalin-fixed and paraffin-embedded pheochromocytoma/paraganglioma specimens, and on 10 normal adrenal medulla samples from patients unaffected by a chromaffin cell tumor. Fourteen samples were from 14 patients with benign pheochromocytoma (<8 year follow-up, mean 5.2 years), 18 specimens were from 12 patients with malignant pheochromocytoma (<13 year follow-up, mean 6.3 years), 5 samples were from 2 patients with malignant paraganglioma (<6 year follow-up, mean 4 years), and 2 specimens from 2 patients with benign paraganglioma (<7 year follow-up, mean 5.5 years). Malignancy was defined by metastases in non-chromaffin tissues. Staining intensity with the anti-survivin antibody was scored from 0 (none) to 3+ (heavy). Tissues from human kidney, breast, and melanoma served as controls.

Results: All pheochromocytoma/paraganglioma specimens stained either 2+ or 3+. By analysis of variance (ANOVA), there was no statistically significant difference between the staining intensity of benign and malignant samples. All normal adrenal medulla specimens stained positively with anti-survivin but to a lesser degree than the chromaffin cell tumors ($P < 0.01$).

Conclusions: Based on these findings, we conclude that (i) survivin may represent a novel neuroendocrine marker for chromaffin cell tumors, and (ii) survivin does not appear to reliably distinguish benign from malignant pheochromocytomas/paragangliomas and thus does not identify patients at risk of recurrent disease.

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Introduction

Survivin is an inhibitor of apoptosis (IAP) and is overexpressed (mRNA and protein) in malignancies originating in lung, colon, esophagus, breast, prostate, in hematopoietic malignancies including large cell non-Hodgkin lymphoma, and in melanoma (1–8).

Current evidence suggests that normal tissues surrounding solid tumors do not express survivin, in contrast to other anti-apoptotic molecules such as bcl-2 (9, 10). Overexpression of survivin in cancer may provide an anti-apoptotic advantage for tumor progression. The gene for survivin is located on chromosome 17q25 and consists of 4 exons (1). In adult human tissues, survivin expression is absent except from placenta and thymus (11).

Pheochromocytomas are neuroendocrine tumors which arise in intra- and extra-adrenal chromaffin tissue. In up to 40% of cases, these tumors are malignant as defined by the presence of metastases in non-chromaffin tissue, most commonly in bone, lung, and liver (12–14). At present, there are no markers that can reliably distinguish benign from malignant pheochromocytoma at the time of initial surgery (15). We sought to investigate the presence of survivin in normal and tumorous chromaffin cells.

Materials and methods**Materials**

After approval by the Institutional Review Board, we obtained 39 formalin-fixed and paraffin-embedded

pheochromocytoma and paraganglioma specimens from the Departments of Pathology at the University of Münster, Germany, and the University of Michigan, Ann Arbor, USA. The diagnosis of pheochromocytoma or paraganglioma was established by clinical data and histopathological examination including positive immunohistochemistry with antibodies directed to chromogranin and synaptophysin. All specimens were cut at 5 microns, placed on positively-charged slides and air-dried. Fourteen pheochromocytoma samples were from 14 patients diagnosed with benign pheochromocytoma (absence of metastases at presentation, follow-up period from 2 to 7 years), 18 specimens were from 12 patients diagnosed with malignant pheochromocytoma (metastases in non-chromaffin tissue at presentation or recurrence during follow-up, follow-up period from 0 to 13 years), 5 samples were from 2 patients

with malignant paraganglioma (follow-up period from 3 to 5 years), and 2 specimens were from 2 patients with benign paraganglioma (follow-up period from 5 to 6 years). In some of the patients with malignant pheochromocytoma or paraganglioma, samples from primary tumor and metastatic lesions were available (see Table 1). One of the 30 patients had a neurofibromatosis type 1-associated pheochromocytoma with metastases to the vertebral bone, and another one had a multiple endocrine neoplasia type 2-associated pheochromocytoma without evidence of metastases at 5 year follow-up. The remaining 28 patients had sporadic chromaffin cell tumors.

As controls, adrenal medulla specimens from 10 patients unaffected by pheochromocytoma, and normal human tissue (breast and kidney to serve as negative tissue control) and human tumor (melanoma

Table 1 Immunohistochemical results of anti-survivin in pheochromocytoma/paraganglioma.

Patient no.	Age (years) Gender	Follow-up (years)	Tumor*	Staining intensity	% of immunopositive neoplastic cells
1	51/M	7	Benign	2+	30
2	58/F	5	Benign	2+	30
3	80/F	3	Benign	2+	30
4	31/M	4	Benign	2+	40
5	43/F	5	Benign	2+	40
6	51/M	5	Benign	3+	40
7	58/F	5	Benign	3+	90
8	41/M	5	Benign	3+	30
9	76/M	5	Benign	2+	20
10	30/F	5	Benign (MEN 2)	3+	20
11	22/M	5	Benign	3+	50
12	65/M	4	Benign	2+	30
13	47/M	3	Benign	2+	20
14	36/F	2	Benign	3+	80
15	31/F	1	Malignant	3+	80
			Malignant	3+	30
			Malignant	3+	30
16	43/F	13	Malignant	3+	20
			Malignant	3+	40
17	72/M	8	Malignant	3+	20
			Malignant	3+	20
18	25/M	7	Malignant	3+	60
19	51/M	5	Malignant	2+	30
20	32/F	5	Malignant	3+	20
21	45/F	5	Malignant	3+	40
22	69/F	4	Malignant	3+	50
			Malignant	3+	30
23	43/M	2	Malignant (NF1)	3+	20
24	68/M	9	Malignant	3+	20
25	58/M	7	Malignant	2+	40
26	56/F	9	Malignant	2+	20
			Met (Heart)	1+	50
27	17/F	3	Para, malignant	2+	40
			Met (Vertebra)	3+	20
28	59/F	5	Para, malignant	2+	90
			Recurrence	2+	20
			Recurrence	3+	25
29	46/F	6	Para, benign	3+	40
30	67/F	5	Para, benign	2+	25

* Intraadrenal pheochromocytoma except when indicated otherwise: Para, paraganglioma; Met, metastasis; recurrence, tumor/metastasis at the same location.

Table 2 Immunohistochemical results of anti-survivin in pheochromocytoma tumors arranged according to their incidence of immunopositivity.

Tissue	Staining intensity											
	0			1+			2+			3+		
	Incidence	No.	% Positivity	Incidence	No.	% Positivity	Incidence	No.	% Positivity	Incidence	No.	% Positivity
Pheochromocytoma	0/39	0	0	1/39	1	50	15/39	4	20	23/39	8	20
								1	25		1	25
								5	30		4	30
								4	40		4	40
								1	90		2	50
											1	60
											2	80
											1	90

0, no staining; 1+, weak staining; 2+, moderate staining; 3+, strong staining.

to serve as positive control) were obtained from surgical and autopsy specimens that were fixed in formalin and embedded in paraffin. These specimens were cut at 5 microns, placed on positively-charged slides, air-dried and stored in a closed container at room temperature until needed. The negative reagent control consisted of the substitution of each test article with species- and isotype-matched irrelevant normal rabbit IgG antibody at the same concentration to control for non-specific interactions. The negative tissue control refers to the tissue section to which the rabbit isotype control antibody was applied.

Immunohistochemistry and reagents

Two antibodies to survivin were used: one (RB anti-survivin, rabbit polyclonal IgG, concentration 2.5 mg/ml) was obtained from Dr Altieri's laboratory (Yale University, USA) and previously characterized with regard to specificity, and the other (Novus RB anti-survivin, concentration 1 mg/ml) was from Novus Biologicals, Inc. (Littleton, CO, USA). The antibodies were diluted to working concentrations (as dictated by titration analyses) with Primary Antibody Diluent (Research Genetics, Huntsville, AL, USA) to the same working concentration as the test article. All samples were tested with the antibody from Yale University. In addition, the 3 samples from patient 15

were tested with the Novus antibody delivering identical results in staining.

Pre-treatment of paraffin-embedded specimens was performed to break the formalin-crosslinking of surface proteins and to enhance specific antibody-antigen interactions. Using a reagent concentration of 20 µg/ml, four alternate pre-treatment methods were compared with untreated material using the positive tissue control, melanoma. The pre-treatments were: pepsin, trypsin, proteinase K, and heat-induced epitope retrieval (HIER) with sodium citrate neutral buffer.

For the anti-survivin antibody, sodium citrate HIER gave the best combination of morphological preservation and staining intensity.

The purpose of a titration analysis was to select the highest titer of anti-survivin antibody in order to detect antigen in tissues that may express low levels, but that minimizes nonspecific binding. In addition, the test article titer was selected to ensure the highest combination of staining intensity and percentage of positively staining cells. An initial titration analysis for the anti-survivin antibody from Yale University was performed and it was determined that a titer of 20 µg/ml gave the best results. A subsequent equivalency titration analysis was performed for the anti-survivin antibody purchased from Novus Biologicals, and it was determined that a titer of 10 µg/ml gave

Table 3 Immunohistochemical results of anti-survivin in normal adrenal medulla.

Tissue	Staining intensity											
	0			1+			2+			3+		
	Incidence	No.	% Positivity	Incidence	No.	% Positivity	Incidence	No.	% Positivity	Incidence	No.	% Positivity
Adrenal medulla	0/10	0	0	2/10	2	80	6/10	2	10	2/10	1	20
								2	20		1	40
								1	30			
								1	80			

0, no staining; 1+, weak staining; 2+, moderate staining; 3+, strong staining.

Figure 1 Immunohistochemistry with anti-survivin on a specimen from a benign pheochromocytoma (patient no. 8): 3+ staining, 30% of neoplastic cells.

comparable results regarding highest combination of staining intensity and percentage of positively staining cells without significant background.

Immunohistochemical studies were performed using an indirect peroxidase-conjugated immunohistochemical detection technique, the DAKO Envision+ System (DAKO Corporation, Carpinteria, CA, USA), according to the manufacturer's instructions. Slide-mounted paraffin-embedded tissue sections were deparaffinized and rehydrated, followed by rinses in water and phosphate-buffered saline (PBS; Amresco, Solon, OH, USA). The slides were then pre-treated with citrate buffer (10 mmol/l, pH 6.2) for 40 min at 95 °C, then allowed to cool for 15 min at room temperature. Tissue sections were then washed in PBS. Endogenous peroxidase activity was quenched with a 5-min hydrogen peroxide solution provided with the Envision+ kit, followed by PBS washes. The slides were incubated with the anti-survivin antibody or the isotype-matched control antibody for 30 min at room temperature, followed by PBS washes. The slides were then incubated with an anti-rabbit antibody conjugated to a peroxidase-labeled dextran polymer provided in the Envision+ kit for 30 min at room temperature, and washed in PBS. The peroxidase reaction was visualized by incubating for 5 ± 1 min with 3,3'-diaminobenzidine tetrahydrochloride solution. The slides were thoroughly washed with tap water, counterstained with modified Harris hematoxylin (American Master Tech. Scientific Inc., Lodi, CA, USA), dipped in 0.25% acid alcohol, blued in 0.2% ammonia, dehydrated through graded alcohols, cleared in xylene, and coverslipped.

Interpretation of stained slides was performed by microscopic examination. In general, a morphological review of the tissue on the slide determined whether an adequate amount of tissue was present, and

Figure 2 Immunohistochemistry with anti-survivin on a specimen from a 56-year-old woman with a right adrenal pheochromocytoma, 8 cm in size on presentation (patient no. 26). (A) 2+, 20% of cells. (B) Heart metastasis: 1+, 50% of tumor cells.

whether the designated tissue was appropriately represented. Samples failing to meet the above standards were rejected from the analysis and excluded from this study. All slides were reviewed by 3 different pathologists blinded to the clinical data (benign or malignant pheochromocytoma) regarding the respective tumor. Two pathologists were evaluating and scoring the slides simultaneously using a double-headed microscope. Later, a third pathologist looked at both the slides and respective scores and agreed with the evaluation.

The scoring system included an analysis of staining intensity with exclusively cytoplasmic staining being typical for the anti-survivin antibody. The staining intensity of tumors and controls was judged relative to the intensity of a control slide containing an adjacent section stained with an irrelevant, negative control antibody that is species- and isotype-matched to the test article. One component of this semiquantitative

Figure 3 Immunohistochemistry with anti-survivin on a specimen from a 17-year-old girl with right retroperitoneal paraganglioma, 15 cm in size (patient no. 27). (A) Primary tumor: 2+, 40% of tumor cells. (B) Vertebral metastasis: 3+, 20% of tumor cells.

scheme is a value (ranging from 0 to 3+) that indicates the amount of epitope present on individual cells as assessed by the intensity of a colorimetric reaction product. Staining of the section labeled with the negative reagent control was considered 'background.' A 0 indicates no staining relative to background, 1+ indicates minimal staining or a blush of stain, 2+ indicates moderately heavy staining, and 3+ indicates intensely heavy staining. Staining intensity was reported at the highest level of intensity observed. Another component of the scoring scheme is the percentage of cells staining at the indicated intensity. With respect to individual tumor specimens, a percentage less than 10 was usually considered insignificant, whereas a percentage of 80 or more was considered 'universal' staining. In considering individual normal human tissue specimens, the same generalizations applied; however, the heterogeneity of cell types typically found in the

Figure 4 Immunohistochemistry with anti-survivin on a specimen from a 59-year-old woman with a left para-aortal paraganglioma, 6 cm in size on presentation (patient no. 28). (A) Primary tumor: 2+ staining intensity for survivin, 90% of tumor cells. (B) Recurrent tumor (3.5 cm): 2+, 20% of tumor cells. (C) Interaortocaval recurrent tumor (5 cm): 3+, 25% of tumor cells.

samples had to be taken into account and the specific cellular element(s) stained had to be identified.

Results

Among the 39 pheochromocytoma specimens, a range of staining intensities from weak (1+) to strong (3+) was observed (Tables 1 and 2). Similarly, of the 10 normal adrenal gland/medulla specimens, staining intensity ranged from 1+ to 3+ (Table 3). However, a higher proportion of tumors (53%, 16/30) displayed intense staining (3+) with the anti-survivin antibody compared with normal adrenal medulla (20%, 2/10). Using the unpaired *t*-test, the mean staining intensity of normal adrenal medulla samples was 2.0 ± 0.21 S.D./0.666 S.E., whereas the mean staining intensity of tumors was 2.53 ± 0.05 S.D., 0.009 S.E. ($P < 0.0114$).

Using a one-way analysis of variance (ANOVA) at 0.05 confidence level (probability level = 0.197 when corrected for ties), there was no statistically significant difference in mean staining intensity of benign and malignant pheochromocytoma specimens.

The mean staining intensity in benign pheochromocytoma specimens was 2.43+ with a mean percentage of neoplastic cells staining of 39%, as opposed to 2.75+ and 37% in malignant pheochromocytoma samples. With inclusion of the benign and malignant paraganglioma specimens, these values changed to 2.44+ (38%) in benign chromaffin cell tumors and 2.64+ (41%) in malignant chromaffin cell tumors. Within the 4 paraganglioma sample group, staining intensity values for survivin were also similar in benign and malignant tumors. Representative images are shown in Figs. 1–4.

The initial negative tissue control (used for pre-treatment analysis) was normal human kidney. Using the anti-survivin antibody provided from Yale University, moderate (2+) to strong (3+) specific staining of renal tubule epithelia was observed and, therefore, the negative tissue control was changed to normal human breast which only occasionally revealed focal staining of ductal epithelial cells. On the positive tissue control (melanoma), the anti-survivin antibody performed as expected from the published literature and showed a moderate (2+) to strong (3+) cytoplasmic staining intensity.

Discussion

At present, there are no markers that can reliably distinguish between benign and malignant pheochromocytomas (15). Such a distinction, however, would be very important in assisting the management of patients presenting with a pheochromocytoma. We here investigated the role of survivin, a recently characterized mammalian IAP that has been shown to be

overexpressed in many malignancies, in the diagnosis of pheochromocytoma.

Since survivin expression is present in neural-crest derived cells in fetuses, it is not surprising that in our study survivin expression was found in normal adrenal medulla, although much less intense than in chromaffin cell tumors. Thus, survivin may represent a novel neuroendocrine marker for pheochromocytoma. Compared with current neuroendocrine markers such as chromogranin, synaptophysin, and neuron-specific enolase, however, the utility of survivin in the diagnosis of pheochromocytoma and paraganglioma has yet to be confirmed in a larger series and its utility in the diagnosis of other neuroendocrine tumors has yet to be proven.

Survivin expression is correlated with a worse prognosis in neuroblastoma, bladder cancer, colorectal, esophageal, and gastric carcinomas (2, 3–7, 16, 17). By immunohistochemistry, the percentage of survivin-positive cases varies greatly among the different tumors studied. In gastric cancer, 30% of cells were positive, whereas 100% of cells were positive in malignant melanoma (6). In neuroblastoma, survivin expression was present in 47% of cases and correlated with disseminated disease and worse prognosis (4). In bladder cancer, survivin expression was present in 78% of cases and correlated with a high risk for disease recurrence (5).

In neuroblastoma, survivin overexpression may be due to gain of chromosome 17q including the survivin gene locus at 17q25. The cause for survivin's overexpression in other tumors needs to be elucidated.

According to the literature, by Northern hybridization, survivin mRNA transcripts are not detected in adult human tissues except for faint signals in placenta and thymus (1, 11). This expression pattern stands in contrast to other inhibitors of apoptosis molecules (3, 18). On the other hand, during human and mouse development, survivin is an abundant protein and prominently found in proximal tubules of the kidney, acinar tubules of the canalicular stage of the lung, endometrial glands, epidermis, thymic medulla, and the axial mesenchymal space surrounding the somites (1, 19). In fetuses, survivin is also found in neural-crest derived cells such as in neurons of the dorsal root ganglion, hypophysis, and choroid plexus (19). The latter findings, especially with regard to survivin's role in embryology, may explain why, in our study, survivin expression in pheochromocytoma, normal adrenal medulla, melanoma, and adult human kidney was positive. Other investigators recently found that survivin is expressed in normal colonic mucosa tissue (20).

Based on published studies that report survivin to be present in preneoplastic lesions such as colon polyps, Bowen's disease, and actinic keratosis, it seemed plausible to us that survivin (over)expression may appear early during malignant transformation

and may perhaps be different in pheochromocytomas that take a benign course and such chromaffin cell tumors that become metastatic/malignant. Chromaffin cell tumors such as pheochromocytoma and paraganglioma are neuroendocrine tumors and are generally slow growing (12, 14, 21). Survivin expression has not yet been reported in any neuroendocrine tumor.

In this immunohistochemical study, we attempted to determine whether survivin could be used as a marker to distinguish benign from malignant pheochromocytoma. Although all pheochromocytomas and paragangliomas in this study showed survivin expression, we did not find a statistically significant difference between benign and malignant chromaffin cell tumors. A limiting factor in this regard, however, are the short follow-up periods. In general, approximately 10% of all adrenal and 40% of all extraadrenal pheochromocytomas are reported to be malignant (13). The longer the follow-up, the more pheochromocytomas may eventually turn out to be malignant. For instance, in patients with multiple endocrine neoplasia type 2 (MEN 2), pheochromocytomas have been reported to occur in about 50% of patients with a wide range of malignancy rates (0–25%) depending upon the follow-up period, the longest one reported being 25 years (22–27). Therefore, pheochromocytomas classified as 'benign' in this study, may undergo reclassification as 'malignant' on longer follow-up.

On the other hand, if survivin is expected to be over-expressed in tumors with more malignant potential, all 'malignant' classified chromaffin tumors in our study should have had 3+ staining intensity. Although this was the case for the majority of malignant chromaffin cell tumors, there were 5 tumors (patients 19, 25, 26, 27, 28) which had only 2+ staining intensity. More importantly, patient 26 showed less staining intensity (1+) in the metastasis (heart) than in the primary tumor (adrenal gland, Fig. 2). This result questions the use of survivin expression as a marker to predict prognosis in pheochromocytomas. It may indicate that some metastases from pheochromocytomas have less inhibition of apoptosis, i.e. undergo cell death. Similar results are illustrated in Figs. 3 and 4.

In summary, the anti-survivin antibody recognizes epitopes present on chromaffin cell tumors such as pheochromocytomas and paragangliomas, and on normal adrenomedullary cells and epithelial cells in several normal tissue types including kidney. Staining of multiple normal tissue elements in the kidney is not consistent with published accounts of survivin expression, namely that it is almost exclusively expressed in tumors. Based on our findings, we conclude (i) that survivin appears not to distinguish reliably between benign and malignant pheochromocytoma/paraganglioma, and (ii) that survivin may represent a novel neuroendocrine marker for chromaffin cell tumors.

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