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Urinary bladder paragangliomas: How Immunohistochemistry can assist to identify patients with *SDHB* germ line and somatic mutations

Alessio Giubellino¹, Karlana Lara¹, Victoria Martucci², T Huynh², Piyush Agarwal³, Karel Pacak², and Maria J Merino¹

¹Translational Surgical Pathology Section, Laboratory of Pathology, National Cancer Institute, National Institutes of Health. Bethesda, MD

²Eunice Kennedy Shriver, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD.

³Urologic Oncology Branch, National Cancer Institute, National Institutes of Health. Bethesda, MD

Abstract

Urinary bladder paraganglioma (paraganglioma) is a rare tumor of chromaffin cells of the sympathetic system of the urinary bladder wall. We studied 14 cases of this entity and investigated the usefulness of SDHB protein staining by immunohistochemistry (IHC) as a diagnostic tool to identify patients with bladder paragangliomas that could be associated with SDHB gene mutations, since these patients have a more aggressive disease. Eleven tumors from these patients were stained by IHC. Six out of 11 tumors were negative for SDHB staining by IHC with no cytoplasmic staining in tumor cells when compared with normal tissues. Five out of these 6 negative cases were confirmed to be positive for germline SDHB mutations. One case showed negative staining and no germline SDHB mutation, however, further investigation of the tumor revealed a somatic SDHB gene deletion. The remaining 5 cases showed strong cytoplasmic staining but they were negative for the presence of SDHB mutation. They were found to be either sporadic tumors or part of von Hippel-Lindau syndrome. Staining for SDH-A was positive in all cases. Our study confirms that there is very good correlation between the presence of an SDHB mutation, whether germline or sporadic, and negative SDHB IHC staining in urinary bladder paragangliomas, and represents the first study to demonstrate that somatic mutations can be recognized by IHC staining.

Background

Urinary bladder paraganglioma (paraganglioma) is an unusual tumor that originates from chromaffin cells of the sympathetic system of the urinary bladder wall [1]. It accounts for less than 0.05% of all bladder neoplasms, often occurs in young adults with a female prevalence, and can be sporadic or part of hereditary syndromes such as von Hippel-Lindau (VHL), multiple endocrine neoplasia (MEN), and succinate dehydrogenase (SDH)

syndromes [2]. Clinically, these patients present more frequently with hematuria, but they may present with catecholamine-related symptoms, such as hypertension, tachyarrhythmia, sweating, headache and micturition syncope. These tumors are histologically distinct from a high grade urothelial carcinoma and carcinoid tumors, with a classic zellballen pattern of growth embedded in a highly vascular fibrous network [3,4]. Several studies have explored the usefulness of different morphological, immunohistochemical, and other markers of malignancy [5-8], but there is still not unanimous consensus.

Bladder paragangliomas can occur as a component of hereditary tumor syndromes, which have been well defined in the last decade [2]. One of these familial paraganglioma syndromes is associated with distinct genetic mutations in one of the four subunits genes of the succinate dehydrogenase (SDH-A, B, C or D), an enzyme complex which is involved in the electron transport chain and in the Krebs cycle. These genes act as tumor suppressor genes and conform to the Knudson's two-hit model of tumorigenesis.

In particular, germline mutations in the *SDHB* gene have been associated with hereditary forms of bladder paraganglioma and their more aggressive and metastatic behavior. Somatic *SDHB* mutations have also been described [9]. Since *SDHB* mutations are associated with the highest rate of malignancy (greater than 50%) [10,11], recognition and accurate tumor characterization for *SDHB* gene mutation status are of utmost importance for the management, prognosis and proper follow-up of these patients and their families. This strongly suggests that ancillary studies are important in distinguishing these tumors from other hereditary paragangliomas.

Therefore, in the present study we investigated the usefulness of *SDHB* protein presence or absence as a diagnostic tool to identify bladder paragangliomas associated with *SDHB* gene mutations using staining by immunohistochemistry (IHC) in paraffin-embedded sections.

Materials and methods

Paraganglioma tumor tissue was obtained from patients accepted for protocol evaluation at the National Institutes of Health (NIH), in accordance with the principles and procedures outlined in the NIH IRB Guidelines, and this was approved by the Institutional Review Board of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD), NIH. All patients signed an IRB-approved consent that allowed for the collection of tissue samples.

Fourteen cases of urinary bladder paragangliomas were studied. Tumors were morphologically evaluated and stained for proliferative markers (MIB1), chromogranin and synaptophysin. Eleven cases were stained for SDHA and SDHB protein expression by IHC. One polyclonal antibody (HPA002868, Sigma-Aldrich) was used to recognize of the target human protein for SDHB (recognizing the C-terminal).

Immunohistochemistry

Protein expression of SDH-A and SDH-B was evaluated by immunohistochemistry using a mouse monoclonal antibody against human SDH-A (Abcam) or a polyclonal antibody

against SDH-B protein (Santa Cruz Biotechnology). Paraffin-embedded sections (5 μ m) were deparaffinized in three Xylene baths and rehydrated in graded alcohols. Tissue sections were microwaved in Tris-EDTA buffer (10 mM Tris, 1mM EDTA) for 20 minutes and then allowed to cool down for other 20 minutes. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in phosphate buffered saline (PBS) for 15 minutes. Sections were then incubated with protein blocking buffer at room temperature for 30 minutes followed by incubation at 37 °C for one hour with the SDH-A or SDH-B antibody (1:100 final concentration). For negative controls, sections were incubated in parallel with TBST buffer instead of primary antibodies. After rinsing, the sections were incubated with a peroxidase-labeled polymer conjugated to goat anti-mouse and goat anti-rabbit immunoglobulins as secondary antibody for 45 minutes (EnVision™ Dual Link System-HRP Kit, Dako Cytomation, Carpinteria, CA). The staining was visualized with 3,3'-diaminobenzidine as chromogen and slides were counterstained with hematoxylin, dehydrated and finally mounted. IHC was interpreted as positive or negative when cytoplasmic staining was identified (see Results section). For MIB-1 immunohistochemistry, the specific antibody was used at 1:200 concentration, for 20 minute in citrate pH 6.0. Antigen retrieval was obtained in pressure cooker, followed by 32 minutes primary incubation on Ventana Benchmark XT with Ultra View DAB kit detection.

Sequencing analysis

Detection of large deletions was performed using a combination of MLPA based probes and Luminex® FlexMap technology. Seventeen sets of hybridization probes were designed to cover all 3 genes (SDHB, SDHC, SDHD) and five sets of hybridization probes were designed for housekeeping gene controls. The upstream probe of each set contained a universal 5' M13 sequence while the downstream probe carried a specific sequence designed to bind to an individual FlexMap bead, and a universal M13 -3' tail. Following ligation and amplification, PCR products were captured on Luminex® FlexMap beads carrying the complementary antitags to the 3' probe tags. The median fluorescent intensity (MFI) signal is detected. For each probe, significant deviations from a 1:1 ratio between signals from normal control samples and patient samples indicate possible deletions or insertions/duplications.

Figures 2 shows the peak ratios of averaged control signal to the signal generated in a run by control samples and the response ratios observed in the sample of a M865 patient with a complete deletion of the SDHB gene (A1). The horizontal green lines and the green shaded area on each graph indicate the expected normal range for the signal ratios (ratio between 0.8 and 1.2).

RESULTS

Immunohistochemical analysis

Six out of the 11 tumors stained were negative for SDHB staining by IHC showing no cytoplasmic staining in the tumor cells (Figure 1) when compared with the normal tissues. Of these 6 negative staining cases, 5 were confirmed positive for germline *SDHB* mutations, the remaining case was negative for *SDHB* germline mutation but further investigation

confirmed an *SDHB* somatic mutation as described below. Five of the 11 stained cases showing strong cytoplasmic staining were negative for the presence of *SDHB* mutations, but four were positive for *VHL* gene mutation (and one unknown). All the cases stained with *SDHA* by IHC were positive in our cohort (Figure 1). An illustrative micrograph from each staining profile is represented in Figure 1, including staining for *SDH-B* and *SDH-A* and a confirmatory immunohistochemistry stain for chromogranin.

Clinical characteristics

Table 1 summarized basic clinical characteristics of the patients evaluated in this study. A predominance of females is represented in our dataset, with a cohort of 11 women (78%) and 3 men (22%). This is consistent with the known female predominance reported in the literature. The mean age at the time of diagnosis was 31 years, with a range spanning from 6 to 64 years. This is also in line with the younger age of onset of these types of tumor as reported in the literature.

The patients in our cohort were evaluated for germline mutation analysis of the most common hereditary paraganglioma syndromes. Of these, 4 patients were found to have *VHL* mutations, 6 patients had *SDHB* mutations, (5 were available for staining). One patient was known negative for the germline mutation but it proved to have the somatic mutation. The remaining 4 patients had germline mutations in the *VHL* gene, and one was sporadic.

Histological characteristics

A summary of the demographic and genetic characteristics is included in Table 1. The average tumor size of the cases for which we had measurements information (11) cases was 2.59 cm, with a standard deviation of 2.09 cm. Two cases were excluded because only biopsies were available and not the full specimen, and for one case we were not able to have sufficient information. The mean tumor size was similar between the patients with *VHL* and *SDHB* germline mutations. We identified lymphovascular invasion in 2 cases, but none of the cases in our cohort had evidence of tumor necrosis or mitotic activity. MIB-1 stains for the Ki-67 proliferation index were performed in 11 cases. Evaluation of Ki67 was done following similar criteria for the other neuroendocrine tumors. We found that 8 cases had low proliferation index (up to 2%) and 3 cases ad an intermediate proliferation index (>2%). The cases with a higher proliferation index and larger size and vascular invasion were patients carrying an *SDHB* germline mutation.

Sequencing analysis

Only one case showed negative staining and no germline *SDHB* mutation, however, further investigation of the tumor using complete genotyping of the *SDHB* gene using multiplex ligation-dependent probe amplification (MLPA) revealed a somatic *SDHB* deletion (Figure 2).

Conclusions

Urinary bladder paragangliomas are rare tumors, representing 1% of all paragangliomas and 0.05% of all bladder tumors. However, the urinary bladder is a common site of paragangliomas associated with hereditary syndromes. Paragangliomas associated with

SDHB syndrome have been known to have a more aggressive behavior, and potential for metastasis [12]. Hence, the importance to recognize patients that present with these tumors so proper treatment and genetic counseling is established. Several markers have been proposed and some authors have tentatively explored scoring systems to predict malignant behavior [7,8].

Up to 30% of all paragangliomas present as hereditary with specific germline mutations. Recently a single aberrant somatic mutation has been described in a bladder wall paraganglioma: a C299T mutation was found in the SDHB gene in tumor DNA and not in the normal tissue from the same patient, excluding an SDHB germline mutation [9]. This mutation results in the change of a nonpolar to an uncharged polar side chain in a highly conserved region in the SDHB protein, with potential consequence on the protein folding and overall structure. This discovery stressed the importance of studying the SDHB gene not only in inherited but also in sporadic paragangliomas. Building on these and other findings, here we identified a patient with a negative SDHB germline mutation and negative IHC SDHB staining for further analysis using complete genotyping of the SDHB gene using multiplex ligation-dependent probe amplification (MLPA). We found that the patient carried a somatic SDHB gene deletion. Staining by IHC for SDHB is an easy and practical way to identify patients with both, germline and somatic mutations.

Our findings are in agreement with a recent report [13] which has identified 3 out of 11 patients with loss of SDHB staining. One of the three patients also had a loss of SDHA mutation. We also stained our cohort for SDHA, but we did not find loss of SDHA staining in any of our patients. Our study shows the importance of sporadic mutations, usually more difficult to discover because no family history is present and no specific tests are performed on the patient. Moreover, we emphasize the importance to have SDHB stains in a pathology immunohistochemistry lab, considering the growing number of diseases where staining for SDHB can be a useful diagnostic or prognostic tool.

We evaluated for the proliferation index using the MIB-1 stain (ki67) and found that 8 cases had a low Ki67 rate (up to 2%) and 3 cases had an intermediate Ki67 rate (>2%). The 3 cases with an intermediate Ki67 rate were patients with confirmed SDHB germline mutations. We have previously reported the importance of MIB-1/Ki67 as a prognostic marker in other tumors [14]. However in this case, the small number of specimens is too limited for a conclusion, although there may be a correlation.

Our results indicate a direct correlation between the presence of an *SDHB* mutation, whether germline or somatic, and negative SDHB IHC staining in urinary bladder paragangliomas. These findings strongly support the use of SDHB IHC in the diagnostic algorithm (Figure 3) to recognize paraganglioma of urinary bladder associated with SDHB syndrome that may have potential for an aggressive behavior. Identification of these patients will also allow for family screening, genetic counseling and establishment of proper therapies. In most institutions staining for SDH-B is ordered by pathologists since clinicians may not be aware that immunohistochemistry for SDH-B is available. In conclusion, if questions arise, it may be recommended that the pathologist consults with the clinician who can discuss the

situation with the patient. Genetic testing should be recommended for cases that stain negative.

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Figure 1.

Representative micrograph of bladder paraganglioma. A. H&E stain of a bladder paraganglioma. Low power. B. High power H&E stain of a bladder paraganglioma. C. SDH-B positive cytoplasmic stain in a patient with VHL related paraganglioma. D. SDH-B negative paraganglioma. E. SDH-A positive stain in a bladder paraganglioma. F. Immunohistochemistry stain for chromogranin in a patient with bladder paraganglioma.

Figure 2. MLPA analysis. Only one case showed a negative staining and no germline SDHB mutation, however, further investigation of the tumor using complete genotyping of the SDHB gene using multiplex ligation-dependent probe amplification (MLPA) revealed a somatic SDHB gene deletion.

Figure 3.
Diagnostic algorithm for the identification of patient with SDH-B deficient paraganglioma.

Table 1

summarizes basic clinical characteristics of the patients evaluated in this study. A predominance of females is represented in our dataset, with a cohort of 11 women (78%) and 3 men (22%).

Total cohort	
Sex	
Female	11 (78%)
Male	3 (22%)
Age at diagnosis	
Mean	31 (SD: 19)
Range	6-64 years
Tumor characteristics	
Mean size	2.59 cm (SD: 2.09)
Lymphovascular invasion	2
Positive SDHA stain	11
Negative SDHA stain	0
Positive SDHB stain	5
Negative SDHB stain	6
Ki67 (MIB-1)	8 low (up to 2%); 3 intermediate (>2%)
Mutation analysis	
VHL germline	4
SDHB germline	6
SDHB somatic	1