Diagnostic Utility of PHOX2B in Primary and Treated Neuroblastoma and in Neuroblastoma Metastatic to the Bone Marrow

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Context.—Neuroblastoma (NB) is the most common extracranial tumor of childhood. Although most cases have a distinctive histology, a subset of primitive cases require immunohistochemical studies to distinguish them from other small round blue cell tumors of childhood. Immunohistochemistry is also used to detect small amounts of tumor metastatic to the bone marrow and in posttreatment samples with obscuring fibrosis, calcification, or inflammation. The transcription factor PHOX2B is essential for the differentiation and survival of sympathetic neurons and chromaffin cells, and therefore is highly specific for the peripheral autonomic nervous system.

Objective.—To determine the diagnostic utility of PHOX2B immunohistochemistry as a marker of primary, treated, and metastatic NB.

Design.—Neuroblastoma tissue microarrays were stained with PHOX2B, CD57, and synaptophysin. Arrays containing rhabdomyosar coma, Ewing sarcoma, and Wilms tumor were stained with PHOX2B, and negative bone marrow samples were stained with PHOX2B and CD57.

Results.—PHOX2B and CD57 were similar to synapto-physin in their ability to detect NB. PHOX2B and CD57 similarly showed robust staining in posttreatment NB and NB metastatic to the bone marrow. In contrast to the cytoplasmic staining pattern seen with synaptophysin and CD57, clear and strong nuclear PHOX2B permitted identification of individual tumor cells. PHOX2B staining was absent in all cases of rhabdomyosarcoma, Ewing sarcoma, and Wilms tumor, and in the negative bone marrow.

Conclusions.—PHOX2B and CD57 are useful markers of NB. PHOX2B is specific for NB in its differential diagnosis with other small round cell tumors, and its nuclear staining may be helpful for accurate bone marrow tumor quantification.


Neuroblastoma (NB) is the most common extracranial tumor of childhood. It has a diverse clinical behavior ranging from metastatic disease with dismal patient outcome to tumor maturation or spontaneous regression. Primary tumor morphology and age at diagnosis have an impact on clinical outcome, with more differentiated tumors and younger age at diagnosis considered favorable. Although most cases of NB show distinct and recognizable histology, a subset of primitive neuroblastomas with small round blue cell morphology require a panel of immunohis-
associated with tumors of neural crest origin, including NB, and with Hirschsprung disease.

Bielle et al studied a variety of neoplasms and found PHOX2B to be expressed specifically in tumors of autonomic nervous system origin, including 6 of 6 undifferentiated NBs. No other small round blue cell tumors showed PHOX2B reactivity, highlighting the utility of this marker for distinguishing undifferentiated NB from its mimics.

CD57 (Leu-7/HNK-1) is a carbohydrate epitope expressed on adhesion molecules of migrating neural crest progenitor cells,9,10 and NB has been shown to express this antigen by flow cytometry.11 CD57 immunohistochemical studies have demonstrated conflicting results, including restriction of CD57 staining to ganglioneuromas (GNs)5 and expression of this antigen by neuroblastomas, as well as neuroectodermal tumors, Ewing sarcomas, and Wilms tumors.2 The clinical diagnostic utility of CD57 has yet to be thoroughly evaluated.

In this study we optimized commercially available PHOX2B, CD57, and synaptophysin antibodies for immunoperoxidase staining of formalin-fixed, paraffin-embedded material and used tissue microarrays (TMAs) to examine their utility in the diagnosis of NB at various stages of differentiation. We also investigated the ability of PHOX2B and CD57 to identify NB metastatic to the BM and posttreatment NB. PHOX2B expression was also examined in other small round blue cell tumors of childhood.

MATERIALS AND METHODS

Paraffin-embedded TMAs, including 30 poorly differentiated NBs, 18 differentiating NBs, 6 ganglioneuroblastomas, 28 GNs, and 34 posttreatment NBs, were constructed. Neuroblastoma cases from 1992 to 2011 were obtained from the surgical pathology database per institutional review board protocol. An additional TMA was constructed from cases collected between 1995 and 2007; these included 4 undifferentiated NBs, 15 rhabdomyosarcomas, 15 Wilms tumors, and 11 Ewing sarcomas. Original hematoxylin-eosin stain slides and, when available, immunohistochemical stains, were reviewed and the diagnosis was confirmed using standard diagnostic criteria.13 A Beecher Instruments Manual Tissue Arrayer (Sun Prairie, Wisconsin) was used to prepare tissue cores from selected regions of archival tissue blocks. To create the TMAs, three to four 1-mm cores were prepared for each tumor case and re-embedded into a gridded paraffin block.

Paraffin-embedded blocks for 15 decalcified BM core biopsies and particle preparations involved by metastatic NB were obtained from the surgical pathology database spanning the years 1997 to 2012. The diagnosis was confirmed by review of original hematoxylin-eosin slides and immunohistochemical stains, when performed. Included were cases with large tumor volume and cases with very small foci of tumor initially requiring immunohistochemistry (IHC) for diagnosis. Nine negative BM samples obtained with very small foci of tumor initially requiring immunohistochemistry were used for visualization of the PHOX2B antibody. Synaptophysin and CD57 required the Bond Polymer Refine detection system for visualization. The TMAs and involved BM biopsies were stained with PHOX2B, CD57, and synaptophysin. The negative sarcoma margins were stained with PHOX2B and CD57.

PHOX2B staining for cellular samples was scored on a 4-point scale as follows: 0, no staining of any cells within the tumor; 1+, weak, focal nuclear staining (<5% of cells) or very faint staining diffusely; 2+, strong nuclear staining in 5% to 50% of cells; and 3+, strong nuclear staining in more than 50% of cells. For diagnostic purposes, scores 2+ and 3+ were considered “positive,” whereas scores 0 and 1+ were considered “negative.” For GN samples or posttreatment samples with a low relative volume of neuronal cells, a 2-point scale was used as follows: 0, no staining of any cells within the tumor; 1+, any nuclear staining. Samples composed entirely of Schwannian stroma were excluded.

Synaptophysin and CD57 staining were scored on a 4-point scale as follows: 0, no labeling of any cells within the tumor; 1+, weak, focal cytoplasmic labeling of cells (<5%) or granular cytoplasmic blush in many cells; 2+, strong cytoplasmic staining in 5% to 50% of cells; and 3+, strong cytoplasmic staining in more than 50% of cells. For diagnostic purposes, scores 2+ and 3+ were considered “positive,” whereas scores 0 and 1+ were considered “negative.”

RESULTS

PHOX2B showed strong staining across all stages of NB differentiation and in lesions metastatic to the BM, with essentially the same expression pattern as CD57 and synaptophysin (Figure 1). PHOX2B was positive in neuroblasts and ganglion cells and showed reactivity in 75% of undifferentiated NBs, 100% of poorly differentiated NBs, and 96% of differentiating NB/ganglioneuroblastomas. This marker provided a crisp nuclear signal that allowed confident identification of tumor cells. No PHOX2B reactivity was identified in negative BM margins or in cases of rhabdomyosarcoma, Ewing sarcoma, or Wilms tumor (Table).

Ganglioneuromas, although usually diagnosed without the aid of IHC, were also tested for PHOX2B expression. Ganglioneuromas showed reactivity, in agreement with a previous report,6 but it was slightly decreased in comparison with other stages of NB differentiation, with only 74% of tumors labeling with PHOX2B. Low numbers of ganglion cells per GN core, variable PHOX2B nuclear reactivity, or loss of transcription factor expression in fully mature cells may have been responsible for this reduced staining rate.

Synaptophysin showed granular, cytoplasmic staining in all tumors, and CD57 showed strong cytoplasmic staining in 100% of poorly differentiated NBs, 96% of differentiating NBs/ganglioneuroblastomas, and 93% of GNs (Table).

PHOX2B, similar to synaptophysin and CD57, showed robust staining in decalcified BM biopsies. PHOX2B stained 88% of involved BMs, compared with 100% staining by synaptophysin and CD57. In contrast to the cytoplasmic staining pattern seen with synaptophysin and CD57, PHOX2B staining provided a discrete nuclear signal that permitted better discrimination of tumor cells from background hematopoietic precursors and stromal cells (Figure 2). In BM samples, PHOX2B was more specific for NB than CD57 was; although strong CD57 cytoplasmic staining was seen in 22% (2 of 9) of negative BM margins, no PHOX2B staining was seen in negative control tissue. CD57 also showed strong membranous staining in scattered small cells, likely natural killer cytotoxic T lymphocytes.
In posttreatment NB samples, synaptophysin, CD57, and PHOX2B performed similarly. Synaptophysin reactivity was seen in 100% of cases, CD57 staining was seen in 91% of cases, and posttreatment neuroblasts and ganglion cells showed PHOX2B reactivity in 94% of cases. The staining intensity was as robust for these markers as it was in untreated cases.

### Expression of PHOX2B, CD57, and Synaptophysin in Neuroblastoma (NB) and PHOX2B in Selected Small Round Blue Cell Tumors of Childhood

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<th>PHOX2B</th>
<th>CD57</th>
<th>Synaptophysin</th>
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<tr>
<td>Undifferentiated NB</td>
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<td>Wilms tumor</td>
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* — indicates stain was not performed.

### COMMENT

The PHOX2B gene encodes a paired homeodomain transcription factor with expression limited to the autonomic nervous system. PHOX2B is essential for the differentiation and survival of neurons, is present during growth and development, and persists into adulthood. Neuroblasts are derived from sympathoadrenal lineage neural crest cells and therefore require and constitutively express PHOX2B. PHOX2B IHC, as a marker of neural crest derivation, has been shown to be sensitive and specific for undifferentiated NB in the differential diagnosis with other small round blue cell tumors of childhood. In this study we confirm PHOX2B to be a sensitive marker of NB in all stages of differentiation. We find the neural crest progenitor cell surface protein CD57 to be a sensitive marker of NB as well.

A striking finding in this study is the specificity of PHOX2B for NB in the small round blue cell tumor differential of childhood. Cases of Wilms tumor, rhabdomyosarcoma, and Ewing sarcoma were uniformly and unequivocally negative for this marker. Considered together with the pediatric tumor panel performed by Bielle et al, the accumulating evidence shows that this marker is specific for autonomic nervous system tumors and will be clinically valuable in this differential diagnosis.

The identification of NB metastatic to the BM is critical for accurate staging and disease surveillance. Using IHC for quantification of NB in the BM at diagnosis and during induction chemotherapy provides prognostic information that can identify patients with very high-risk disease who may then be considered for experimental therapy. Evaluating BM biopsies for metastatic NB with synaptophysin IHC can be difficult because of nonspecific background staining and heterogeneous expression within tumor cells. Synaptophysin may occasionally stain subsets of erythroid precursors in a granular cytoplasmic and nuclear fashion mimicking clusters of tumor, and can also show reactivity within plasma cells, osteoclasts, and osteoblasts. Nuclear PHOX2B staining permits discrimination of tumor cells from BM hematopoietic precursors or fibroblasts. While synaptophysin staining may lead to tumor quantity overestimation, the strong nuclear signal provided by PHOX2B may be better suited for quantifying the degree of marrow involvement by NB. In BMs with very small amounts of tumor, however, stains including synaptophysin and CD57

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Figure 1. A case of poorly differentiated neuroblastoma stained with (A) synaptophysin, (B) PHOX2B, and (C) CD57 (original magnification ×600).
Neuroblastoma is a common tumor of childhood for which multiple immunohistochemical stains can be employed to aid in diagnosis. PHOX2B is sensitive and specific for NB and shows diagnostic utility for detecting this disease at all stages of differentiation. CD57 shows a similar staining profile to PHOX2B and synaptophysin. This crisp nuclear staining provided by PHOX2B allows confident discrimination of tumor cells from nonspecific background staining. This is especially helpful in posttreatment samples, where treatment effect may make detection of residual tumor difficult by hematoxylin-eosin alone, and in the small foci of tumor metastatic to the BM. PHOX2B, in combination with a cytoplasmic stain such as synaptophysin, can help detect NB and will likely help accurately quantify the tumor burden in primary, metastatic, and posttreatment lesions.

We wish to thank Melissa Downing, Anthony Frazier, and the Vanderbilt University Medical Center Translational Pathology Shared Resource for outstanding technical expertise in preparing the neuroblastoma TMA and in immunohistochemical staining. This work was supported in part by NIH K12 grant CA 0902513.

### References