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ZBTB16: A new biomarker for primitive neuroectodermal tumor element / Ewing sarcoma

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ABSTRACT

Primitive neuroectodermal tumor (PNET) traditionally encompasses two different classes of tumors with similar morphology - PNET of the peripheral nervous system (pPNET) and PNET of the central nervous system (cPNET). The latter also includes germ cell tumor-derived PNET (gPNET). There are currently no specific markers for gPNET. This study seeks to investigate the expression of ZBTB16 in PNET and other small round blue cell tumors as well as its potential diagnostic utility. Immunohistochemical expression of the ZBTB16 was studied in a total of 27 PNETs (12 pPNETs, 8 cPNETs, 3 primary testicular gPNETs, and 4 metastatic gPNETs) and 38 small round blue cell tumors. Positive expression for ZBTB16 was seen diffusely in 9/12 (75%), moderately in 2/12 (17%) and focally in 1/12 (8%) of pPNETs, diffusely in 3/7 (43%) and moderately in 4/7 (57%) of gPNETs, and diffusely in 2/8 (25%), moderately in 2/8 (25%) and focally in 4/8 (50%) of cPNETs. Whereas, all of the 38 non-PNET small round blue cell tumors were nonreactive. The results suggest that ZBTB16 is a highly sensitive and specific biomarker for both pPNET and gPNET/cPNET. ZBTB16 effectively differentiates PNETs from other small round blue cell tumor mimics, including the two most common germ cell tumor-derived somatic malignancies - rhabdomyosarcoma and nephroblastoma. Of note, compared to the expression of ZBTB16 in pPNET/Ewing sarcoma and gPNET, the expression of ZBTB16 in cPNET was more variable, which appears consistent with the heterogeneity of cPNET. The close proximity of ZBTB16 and FLI-1 genes on chromosome 11q may explain the overexpression of ZBTB16 in PNET, especially in pPNET with t(1122) translocation.

1. Introduction

Primitive neuroectodermal tumors (PNET) are a group of malignant small round blue cell tumors that have historically encompassed two different classes of tumors with similar morphologies [1]: PNET of the peripheral nervous system (pPNET) and PNET of the central nervous system (cPNET). The pPNETs are thought to originate in peripheral nerves or soft tissues, including the more differentiated end of a spectrum of neoplasms - skeletal and extraskeletal Ewing's sarcoma.

cPNETs are a molecularly heterogeneous group of tumors [2–4], comprising a variable specific embryonal tumor entities (e.g medulloblastoma, embryonal tumor with multilayered rosettes C19MC-altered, embryonal tumor with multilayered rosettes NOS, medulloepitheliomas, etc.). Although locations, pathophysiology, molecular biology, and natural history may differ, PNETs share similar morphologic appearances and are composed of small round blue neoplastic cells. Recently, PNET has also been described as one of the most common somatic non-germ cell malignancies in germ cell tumors [5–7]. Germ cell tumor-derived PNETs (gPNET) are thought to be analogous to cPNETs in both morphological and molecular features [5–7]. Characteristic t(1122) translocation is seen in 85% of pPNET and they often express CD99 and Fli-1 [8,9]; whereas, cPNET and gPNET lack such translocation and have weak to no expression of CD99, and are negative for Fli-1 [5]. At present, there are no specific markers for gPNET. Owing to the lack of specific markers and frequent occurrence of diverse and heterogeneous elements in mixed germ cell tumors, it is a challenge to diagnose de novo and metastatic gPNET. As a germ cell tumor derived somatic malignancy, the presence of gPNET predicts aggressive behavior and has a poor prognosis, especially when it is identified in a metastasis [5,6]. Unlike typical germ cell tumors, gPNET is resistant to cisplatin-based therapy and PNET specific chemotherapy or surgical resection is required for improved outcome [5,6,10]. Therefore, it is critical to correctly identify the PNET component as well as to differentiate it from other small round blue cell tumor mimics, so that patients can be appropriately managed.

Zinc finger and BTB (Broad/complex/Tramtrack/Bric a Brac) domain containing 16 (ZBTB16) was first identified in a patient with acute promyelocytic leukaemia. It is a suppressive zinc finger transcription

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factor belonging to the POZ (POxvirus and zinc finger) - Krüppel family [11,12]. ZBTB16 affects diverse signaling pathways, including cell cycle, differentiation and programmed cell death pathways in hematopoietic cells, as well as solid tumors [11,13]. It is involved in major developmental and biological processes, such as spermatogenesis and stem cell maintenance, hind limb formation, hematopoiesis, immune regulation and oncogenesis [11,12]. In normal testes, ZBTB16 is expressed specifically in undifferentiated spermatogonial cells [11,13,14]. Studies have implied that ZBTB16 serves to promote spermatogonial stem cell self-renewal [11,13,14] and plays a key role in the maintenance of normal spermatogenesis. We recently reported that, in testis, ZBTB16 is expressed specifically in yolk sac tumor and spermatocytic tumor but not in other germ cell tumor subtypes [15,16]. Somatically, ZBTB16 is expressed mainly in prostatic adenocarcinoma, and it is absent in most of the other common somatic tumors [17]. The aim of this study is to investigate the expression of ZBTB16 in PNETs and other small round blue cell tumors and to evaluate its diagnostic utility.

2. Materials and methods

2.1. Tissue samples

This study was approved by the relevant institutional review board. A total of 27 PNETs and 38 small round blue cell tumors of various types accessioned between January 2014 and June 2019 were retrieved from our archives. Among the 27 PNETs, 3 were associated with primary testicular teratoma-containing mixed germ cell tumors; 4 were found in metastatic sites (2 in the retroperitoneum, 1 in the abdomen and 1 in the mediastinum). All patients with the diagnosis of metastatic gPNET had a previous history of teratoma-containing testicular germ cell tumor. Eight of the 27 cases were from adults with a diagnosis of glioblastoma multiforme with PNET/neuronal component. Twelve of the 27 cases were soft tissue / bone PNETs / Ewing sarcomas. Of the 38 small round blue cell tumors, 11 were small cell neuroendocrine carcinomas, 5 rhabdomyosarcomas, 4 poorly differentiated squamous cell carcinomas, 6 synovial sarcomas, 3 Merkel cell carcinomas, 2 melanomas, 1 testicular granulosa cell tumor, 1 testicular malignant sex cord stromal cell tumor, 3 malignant peripheral nerve sheath tumors, and 2 nephroblastomas. Both of the nephroblastomas arose from retroperitoneal metastatic teratoma-containing mixed germ cell tumor. The diagnosis of PNET was established by tumor location, histomorphology, t(1122) translocation status, and / or the results of immunohistochemical studies (CD99, FLi-1, Synaptophysin, chromogranin, CD57, GFAP, S100, AE1/AE3, CD45, MyoD1, Melan A, etc.). The diagnosis of specific subtypes of the small round cell tumors was made based on histomorphology and / or the results of molecular tests and / or immunohistochemistry. Although “cPNET” is no longer recommended for clinical use, for simplicity, we here kept the general term ‘cPNET” in reference to the group of central nerve system embryonal tumors.

2.2. ZBTB16 immunohistochemistry

Following deparaffinization and rehydration, charged slides with 5-μm thick sections of tissue were treated with 3% hydrogen peroxide (H_2O_2) to eliminate endogenous peroxidase activity, then processed for antigen retrieval with 10 mm citrate buffer pH 6.0 using a pressure cooker (Pascal; Dako Cytomation, Glostrup, Denmark) for 1 min at 125 °C, followed by slow cooling. The rest of the procedure was conducted in a Dako automated instrument. All sections were rinsed with phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM potassium chloride, 4.2 mM sodium phosphate and 1.5 mM potassium phosphate) and reacted with mouse anti-ZBTB16 antibody (D-9, sc-28319; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1.5 h at 1:500 dilution in PBS containing 1% bovine serum albumin (BSA) and 5% normal goat serum at room temperature. The sections were then incubated for 20 min with EnVision + System horseradish peroxidase-labelled polymer conjugated with biotinylated anti-mouse secondary antibody and 3,3′-diaminobenzidine substrate. The slides were counterstained with hematoxylin, dehydrated and cover-slipped. ZBTB16 immunohistochemistry was validated by using normal testicular tissue containing ZBTB16 positive spermatogonial cells and or ZBTB16-expressing yolk sac tumor as we have previous reported [16] and was performed along with clinical samples on an automated DAKO platform. Only nuclear staining for ZBTB16 was considered positive.

2.3. Analysis of immunohistochemical staining

Tumor cells were analyzed for ZBTB16 immunoreactivity in a semiquantitative manner. Based on the extent of the immunoreactivity, the staining was graded as: virtually none (< 1% cells) staining (negative / 0), 1–25% of cells staining (focal / 1+), 25–50% of cells staining (moderate extent / 2+) and > 50% of cell staining (extensive / diffuse / 3+).

3. Results

3.1. Expression of ZBTB16 in PNETs

All the 27 PNETs were immunopositive for ZBTB16. Among the 12 primary soft tissue PNETs/Ewing sarcomas, 9 were diffusely reactive with ZBTB16, irrespective of the presence (Fig. 1A-B) or absence (Fig. 1C-D) of translocation t(11:22); 2 were moderately and 1 focally/scattered immunoreactive with ZBTB16. Of the 3 primary testicular PNETs, 1 was diffusely positive and the remaining 2 had moderate / patchy positivity for ZBTB16 (Fig. 1E-F). Of the 4 gPNETs at metastatic sites, 2 were diffusely positive and 2 had moderate / patchy positivity for ZBTB16. In the 3 primary testicular PNET cases, all had concurrent ZBTB16-negative teratoma, 2 also had concurrent ZBTB16-negative embryonal carcinoma and seminoma, and 2 had concurrent ZBTB16-positive yolk sac tumor. Of the 8 brain cPNETs, 2 was diffusely positive (Fig. 1G-H), 2 moderately positive and 4 focally/scattered positive for ZBTB16. The associated glial cells were also sparsely immunoreactive with ZBTB16. Of note, 1 of the testicular primary PNETs from outside institution was originally mis-interpreted as seminoma.

The immunohistochemical, molecular, and clinical profiles are shown in Table 1 and the expression of ZBTB16 in pPNET, cPNET and gPNET are summarized in Table 2.

3.2. Expression of ZBTB16 in other small round blue cell tumors

Other small round blue cell tumors were all nonreactive for ZBTB16, including small cell neuroendocrine carcinoma, rhabdomyosarcoma, poorly differentiated squamous cell carcinoma, synovial sarcoma, Merkel cell carcinoma, melanoma, testicular granulosa cell tumor, malignant sex cord stromal cell tumor, malignant peripheral nerve sheath tumor and nephroblastoma. The nephroblastoma was mainly composed of blastema with scattered immature tubular structures and mesenchymal elements. All these components were nonreactive with ZBTB16. Of note, the nephroblastoma element in 1 of the 2 cases was initially diagnosed as PNET. The results of immunohistochemical expression of ZBTB16 in other small round blue cell tumors are summarized in Table 3, and representatively illustrated in Fig. 2.

4. Discussion

Albeit sharing similar morphologic appearance, the family of small round blue cell tumors encompasses a diverse group of malignancies [18] with differing biological behavior and treatment. Accurate diagnosis of each type of these tumors is therefore essential for an optimal outcome. Thanks to decades of work, with a few exceptions, most of the small round blue cell tumors can be accurately classified based on their

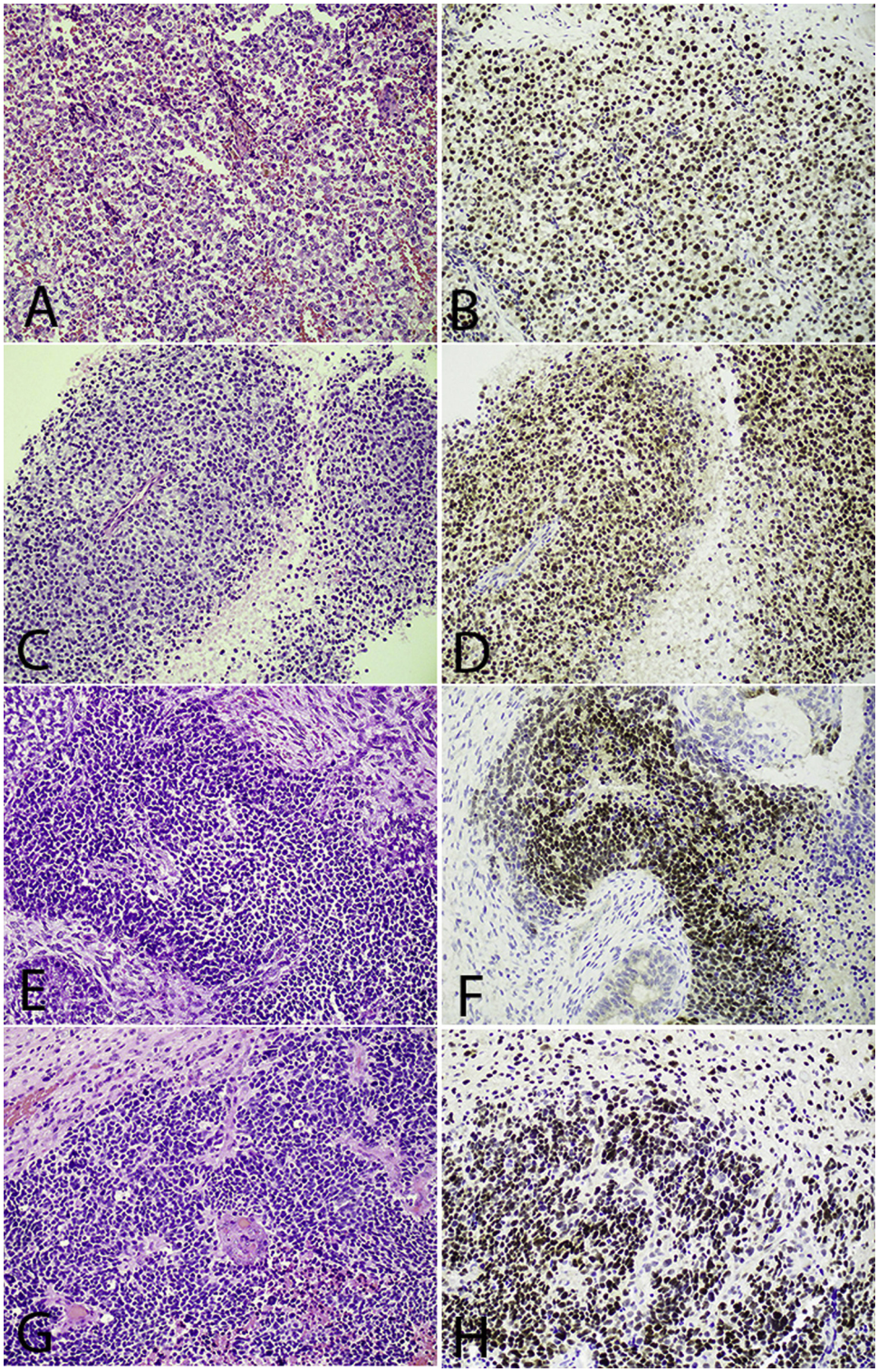

Fig. 1. Immunohistochemical expression of ZBTB16 in pPNET, cPNET and gPNET. pPNET with t(1122) (A: H&E and B: ZBTB16 immunostain) and without translocation (C: H&E and D: ZBTB16 immunostain), gPNET (E: H&E and F: ZBTB16 immunostain), cPNET (G: H&E and H: ZBTB16 immunostain).

respective specific biomarkers. PNET is a small round blue cell tumor that includes pPNET as well as cPENT and gPNET, which differ in their clinical, molecular and immunohistochemical features [1,5]. pPNET and cPNET are well recognized entities. gPNET is a recently described tumor element and has been reported as a common form of non-germ cell malignancies in patients with testicular germ cell tumors. The finding gPNET in germ cell tumors, especially in metastases, has a poor prognosis [5,7,10]. Germ cell tumors with gPNET metastasize or relapse more frequently than those without gPNET [5,19]. In addition, gPNET is resistant to cisplatin-based treatment regimens and PNET specific chemotherapy regimens and surgery is required for effective treatment [5,20]. Recognition and correct identification of gPNET is crucial to appropriate patient management. Based on morphology alone, it is sometimes not easy to recognize gPNET and it can be mistaken for other somatic small round blue cell tumor mimics, especially in inexperienced hands.

Table 1
Clinical and immunohistochemical profiles and EWSR1 gene rearrangement in PNETs/Ewing sarcomas.

Cases	Immunohistochemistry		EWSR1 gene rearrangement (FISH)	ZBTB16 IHC	Location/ Association
pPNET/Ewing	CD99/FLI1	Other markers			Location
1	CD99 +, FLI1 +	AE1/AE3-CK7-Desmin-MyoD1- S100 +/-ERG-Melan A- CD45-	Positive	2+	Chest wall
2	CD99 +, FLI1 +	Chrom-Synap-	Positive	3+	Arm
3	CD99 +		Positive	1+	Arm
4	CD99 patchy +		Positive	3+	Lung
5	CD99 +		Positive	3+	Ulna
6	CD99 +, FLI1 +		Positive	3+	Thigh
7	CD99 +, FLI1 +		Positive	3+	Pelvis
8	CD99 +, FLI1 +		Positive	3+	Unknown
9	CD99 +		Positive	3+	Foot
10	CD99 +, FLI1 +		ND	2+	Epidural
11	CD99 +, FLI1 +		ND	3+	Arm
12	CD99 +, FLI1 +		Negative	3+	Leg
"cPNET"	Synaptophysin/CD99	Other markers			Association
1	Synap +	GFAP +/-P53 +/-Chrom-	ND	1+	GBM
2	Synap +		ND	1+	GBM
3	Synap +		ND	2+	GBM
4	Synap +		ND	3+	GBM
5	CD99 +, Synap +		ND	3+	GBM
6	Synap +		ND	2+	GBM
7	Synap +		ND	1+	GBM
8	CD99 +, Synap +		ND	1+	GBM
gPNET	CD99/FLI1	Other markers			Location
1	CD99 patchy +	Synap + CD57 + S100 +/-Chrom-	ND	2+	Testis
2	CD99 +, FLI1 +		ND	2+	Testis
3	CD99 focal +		ND	3+	Testis
4	CD99 focal +		ND	2+	Lung
5	CD99 focal +		ND	2+	Mediastinum
6	CD99-		ND	3+	Pelvis
7	CD99 focal +		ND	3+	Pelvis

IHC: Immunohistochemistry; ND: Not done; Synap: Synaptophysin; Chrom: Chromogranin; GBM: Glioblastoma; +: Positive; -: Negative.

Table 2
Summary of immunohistochemical expression of ZBTB16 in PNETs.

PNET (n = 27)	–	1+	2+	3+
Primary testis (n = 3)	0	0	2(67%)	1(33%)
Metastasis from testis (n = 4)	0	0	2(50%)	2(50%)
Primary brain (as part of GBM) (n = 8)	0	4(50%)	2(25%)	2(25%)
Primary soft tissue/bone (n = 12)	0	1 (8%)	2 (17%)	9(75%)

Table 3
Immunhoistochemical expression of ZBTB16 in other small round blue cell tumor mimics.

	–	1+/2+/3+
Small cell neuroendocrine carcinoma (n = 11)	11	0
Rhabdomyosarcoma (n = 5)	5	0
Poorly differentiated squamous cell carcinoma (n = 4)	4	0
Synovial sarcoma (n = 6)	6	0
*Merkel cell carcinoma (n = 3)	3	0
Testicular granulosa cell tumor (n = 1)	1	0
*Melanoma (n = 2)	2	0
Malignant testicular sex cord stromal cell tumor (n = 1)	1	0
Malignant peripheral nerve sheath tumor (n = 3)	3	0
Nephroblastoma arising from teratoma (n = 2)	2	0

* These are additional cases to our previous report in ref. [15].

The majority of pPNET cases can be confirmed by t(1122) translocation and / or by a combination of morphology and immunoreactivity for both CD99 and Fli-1; however, small numbers of pPNET cases may not have this typical translocation or immunoprofile. In addition, the specificity of CD99 and Fli-1 for pPNET is relatively low. CD99 immunoreactivity is also seen in lymphoma and many other small round blue cell tumors [21–25]. Fli-1 nuclear positivity can also be seen in endothelial cells / vascular tumors, lymphoma [26], melanoma [27], Merkel cell carcinoma [28], and others. In contrast, cPNET and gPNET do not harbor EWS-FLI translocation and show only occasional immunoreactivity for CD99 [5]. Although CD57 and synaptophysin have been shown to be frequently positive in cPNET/gPNET [5], based on our experience, they are neither sensitive nor specific. At present, there are no reliable markers available for cPNET and gPNET as well as pPNET without the t(1122) translocation.

This study showed that ZBTB16 is a highly sensitive and specific novel biomarker for both pPNET and cPNET/gPNET among the small round blue cell tumors. Our findings suggest that ZBTB16 is more sensitive and specific than CD99 for pPNET and CD57 for cPNET/gPNET. ZBTB16 also effectively differentiated PNETs from the other small round blue cell tumor mimics, including the common testicular non-germ cell somatic malignancies - rhabdomyosarcoma and nephroblastoma. Of note, compared with expression of the ZBTB16 in pPNET/Ewing sarcoma, the expression of ZBTB16 in cPNET was more variable.

The mechanisms underlying the overexpression of the ZBTB16 gene and the role of ZBTB16 in PNET is yet to be determined. The pathognomonic t(1122) translocation in pPNET results from fusion of the EWS gene on 22q12 with the FLI1 gene on 11q24 [29]. Interestingly, ZBTB16 gene is located on 11q23.2 [30], in close vicinity to the FLI1 gene (on 11q24). Fusion of EWS and FLI1 could perturb the neighboring ZBTB16 gene due to proximity and result in upregulation of ZBTB16. cPNET is a molecularly heterogeneous group of tumors and a majority of tumors designated cPNET represent specific diagnostic entities [2–4,27,28]. The variable expression of ZBTB16 in the 8 cPNETs appears consistent with this heterogeneity.

In summary, ZBTB16 was expressed in both pPNET and cPNET/gPNET, but was absent in other small round blue cells tumor mimics. The high sensitivity and specificity of ZBTB16 for PNET suggests it is a valuable biomarker for the diagnosis of PNET, especially in cases of pPNET without EWS-FLI1 translocation and in the identification of

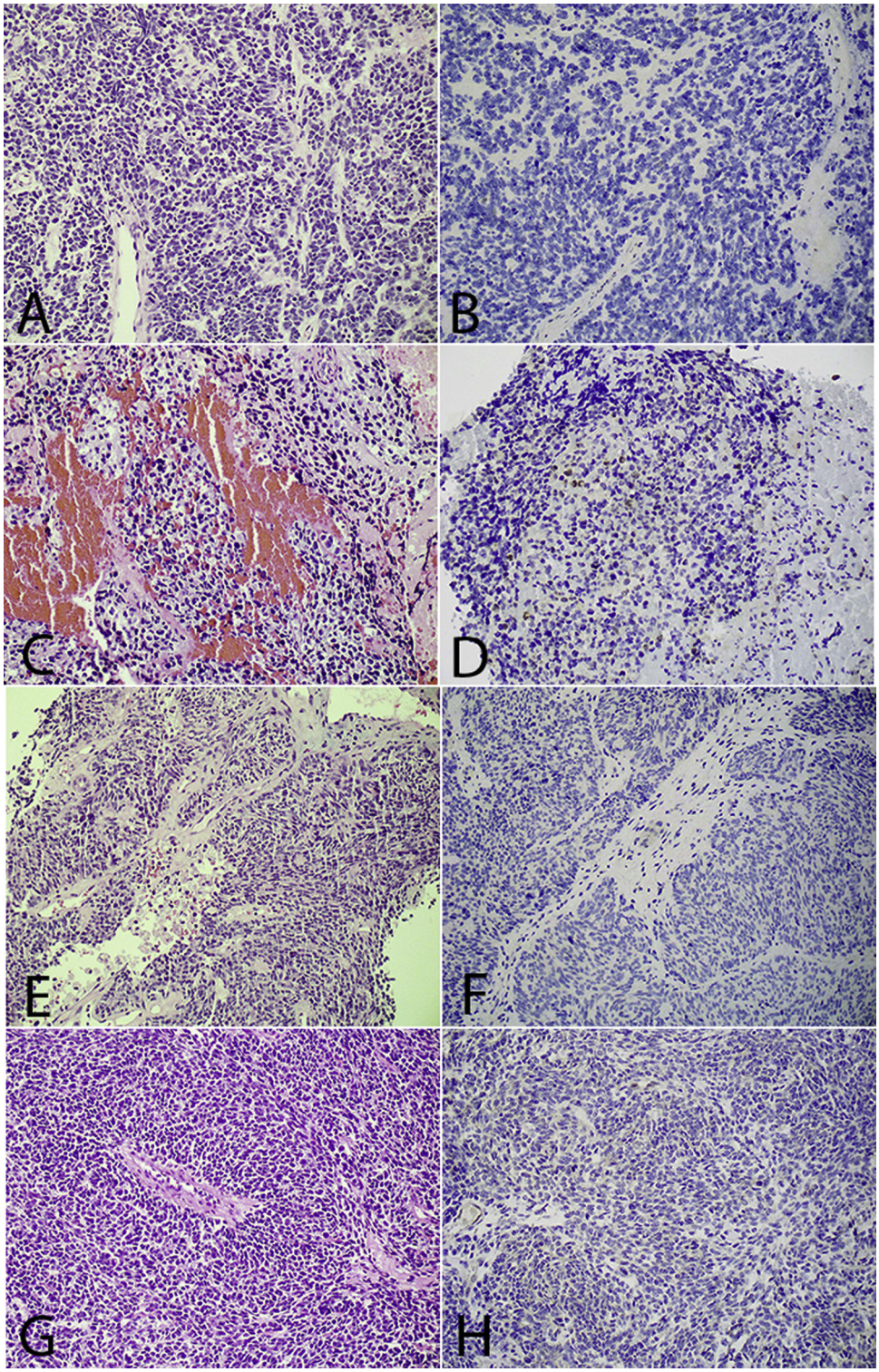

Fig. 2. Immunohistochemical expression of ZBTB16 in other representative small round blue cell tumors: Small cell neuroendocrine carcinoma (A: H&E and B: ZBTB16 immunostain), rhabdomyosarcoma (C: H&E and D: ZBTB16 immunostain), melanoma (E: H&E and F: ZBTB16 immunostain), nephroblastoma (G: H&E and H: ZBTB16 immunostain).

gPNET component in patients with a history of mixed germ cell tumors containing heterogeneous elements, particularly with respect to differentiating them from other small round blue cell tumors, including rhabdomyosarcoma and nephroblastoma. That said, this study was limited by a small number of cases and we have not tested it on some of the other rare recently described and genetically defined small round blue cell tumors, such as CIC-sarcomas, BCOR-sarcomas and EWSR-rearranged small round cell (non-Ewing sarcoma) tumors. Nevertheless, the results appear promising, and further broad studies with larger cohorts to validate the findings are warranted.

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