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ZBTB16 is a sensitive and specific marker in detection of metastatic and extragonadal yolk sac tumor

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ABSTRACT

AIMS: Accurate histologic diagnosis and classification of germ cell tumors (GCTs) is key to informing successful therapeutic and surveillance strategy. Modern therapeutic approach for yolk sac tumor (YST) is highly curative. Because YST takes on a large morphologic spectrum, it can be confused for other GCT subtypes as well as somatic carcinomas, particularly when YST presents in an extragonadal or a metastatic setting. Currently available immunohistochemical markers are limited by suboptimal sensitivity and specificity. We recently reported that ZBTB16 is a sensitive and specific marker for testicular YST. ZBTB16 is absent in other GCTs and in most common somatic carcinomas, including these of gastrointestinal, pancreatobillary, respiratory, genitourinary and gynecologic tracts. The purpose of this study is to investigate the diagnostic utility of ZBTB16 in the settings of metastatic and extragonadal YST.

METHODS AND RESULTS: We studied 32 archived metastatic and 4 extragonadal primary YSTs as well as 51 somatic malignancies for their immunohistochemical expression of ZBTB16. For comparison, AFP and Glypican-3 were also studied in parallel. Our results demonstrated an overall sensitivity of 91.6% for ZBTB16 in detecting metastatic and extragonadal YSTs. The non-YST elements (teratoma and embryonal carcinoma) in 15 YST-containing metastatic mixed GCTs were nonreactive. With exception of occasional myoepithelial cells of salivary gland carcinoma, all the 51 somatic malignancies were negative for ZBTB16.

CONCLUSION: ZBTB16 is a sensitive and specific marker for YST and is diagnostically superior to AFP and Glypican-3 in metastatic and extragonadal settings.

Key words: ZBTB16, yolk sac tumor, metastatic, extragonadal

INTRODUCTION

Accurate histologic diagnosis and classification of germ cell tumors (GCTs) is key to informing successful treatment and surveillance strategy. Modern therapeutic approach for yolk sac tumor (YST) is highly curative with a survival rate of greater than 90% (1). Because it takes on a large morphologic spectrum, YST has been the most commonly underdiagnosed element among the testicular GCTs (2-4). It can be confused for other GCT subtypes as well as primary somatic neoplasms, particularly when YST presents as an extragonadal primary or as a metastasis to other organs. This could lead to improper selection of therapeutic as well as biochemical surveillance strategy. Several ancillary markers have been used to facilitate the diagnosis of GCT, including the early marker, like placental-like alkaline phosphatase (PLAP), and the more recent ones – OCT4, SOX2 and SALL4. Although these markers are sensitive for GCTs, they lack specificity for subtyping GCTs (5, 6). There are currently limited YST immunohistochemical markers. α-fetoprotein (AFP) is a marker moderately specific for YST, but with a relatively low sensitivity, particularly for YST with glandular pattern (2-4). In addition, protein expression of AFP in YST may be lost in metastatic or post-treatment settings (7, 8). Furthermore, AFP is also expressed in some primary somatic neoplasms, such as hepatocellular carcinoma, hepatoid adenocarcinoma of the stomach, and clear cell carcinomas of mullerian origin (9-11). Another recently identified YST marker is GPC-3 (12, 13); however, its expression has been reported in other GCT subtypes, such as choriocarcinoma and, less frequently, teratoma and embryonal carcinoma (12, 13), as well as some primary somatic neoplasms (12, 14-17). Because of these limitations, a diagnostic

immunohistochemical panel, including markers of pluripotentiality (SALL4 and LIN28) and endodermal identity (AFP, GPC-3 and villin), is recommended to overcome the diagnostic challenges of multiple patterns of differentiation in YSTs (18).

Zinc finger and BTB (Broad/complex/Tramtrack/Bric a Brac) domain containing 16 (ZBTB16) was first identified in a patient with acute promyelocytic leukemia. It is a zinc finger transcription factor belonging to the POZ (POxvirus and Zinc finger) -Krüppel family that binds to specific DNA sequences with its carboxy-terminal zinc fingers and suppresses transcription by recruiting co-repressors with its aminoterminal POZ domain (19, 20). ZBTB16 affects diverse signaling pathways, including cell cycle, differentiation, and programmed cell death pathways in hematopoietic cells as well as solid tumors (20-23). It is involved in major developmental and biological processes, such as spermatogenesis and stem cell maintenance, hind limb formation, hematopoiesis, immune regulation, and oncogenesis (19-21). In normal testes, ZBTB16 is expressed specifically in undifferentiated spermatogonial cells (19, 24). Studies have implied that ZBTB16 serves to promote spermatogonial stem cell self-renewal (19, 24) and plays a key role in the maintenance of normal spermatogenesis. Infertility is observed in ZBTB16-null mice due to the impairment of spermatogonial stem cell self-renewal and subsequent exhaustion of spermatogonia (24, 25). Although the function of ZBTB16 in spermatogenesis is relatively well studied, expression of ZBTB16 in GCTs has just been explored. We recently reported that, in testis, ZBTB16 is specifically expressed in YST, carcinoid and spermatocytic tumor but not in other GCT subtypes. Somatically, ZBTB16 is mainly expressed in prostate adenocarcinoma (23) and some carcinoid tumors (26). It is absent in most of the other common somatic tumors, including those of gastrointestinal, pancreatobillary, respiratory, genitourinary and gynecologic tracts (27).

The aim of this study was to investigate the diagnostic utility of immunohistochemical expression of ZBTB16 in the detection of metastatic and extragonadal YSTs. The diagnostic value of ZBTB16 will also be compared against AFP and GCP-3.

MATERIAL AND METHODS

Tissue samples

This study was approved by the relevant institutional review boards. A total of 32 metastatic YST-containing GCTs and 4 extragonadal primary YSTs accessioned between 2010 - 2016 were retrieved from the archives of two academic institutions. Among the 32 metastatic GCTs, 17 were pure YSTs, and 15 were mixed GCTs containing 5%-95% of YST element. The non-YST components in the metastatic mixed GCTs included teratoma (13 cases) and embryonal carcinoma (6 cases). Of the metastatic YSTs, 24 were obtained prior to chemotherapy, 8 were recurrent YSTs after chemotherapy. Most of the YSTs exhibited multiple morphologies (e.g. cystic, glandular, papillary, and solid). The sites of the metastases included retroperitoneum (22 cases), mediastinum/neck (5 cases), lower abdomen and pelvis (5 cases), and lung (1 case). The primary non-testicular extragonadal YSTs included 1 mediastinal, 1 retroperitoneal, 1 liver and 1 urinary bladder primaries. All the extragonadal YSTs were pure YSTs. The diagnosis of YST was established by clinical history including serum markers, histomorphology and/ or the results of immunohistochemical markers - AFP, GPC-3, SALL4, OCT4, PLAP, CD117, D2-40, and /or CD30.

Besides the somatic tumors that we have previously studied (23, 27, 28), fifty-one primary somatic malignancies, many of which exhibit morphologic overlap with YST, were evaluated for the immunohistochemical expression of ZBTB16. They included 8 breast carcinomas (4 ductal and 4 lobular carcinomas), 8 melanomas, 5 lymphomas (1 diffuse large

cell lymphoma, 1 Hodgkin's lymphoma, 1 T cell lymphoma, 2 CD30-positive malignant lymphomas), 3 pancreatobiliary carcinomas, 6 combined small cell carcinomas and urothelial carcinomas of urinary bladder, 7 head neck carcinomas (1 poorly differentiated non-keratinizing squamous cell carcinoma , 6 salivary gland carcinomas), 3 mesotheliomas, 2 synovial sarcomas, 2 renal cell carcinomas, 2 poorly differentiated carcinomas of mullerian origin, 2 colorectal carcinomas, 2 merkel cell carcinomas, and 1 adrenocortical carcinoma.

Immunohistochemistry

For 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 minute at 125°C, followed by slow cooling. The rest of the procedure was done in a DAKO automated instrument. All sections were rinsed with phosphate-buffered saline (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) for 1.5 hours at 1:500 dilution in phosphate-buffered saline containing 1% bovine serum albumin (BSA) and 5% normal goat serum at room temperature. The sections were then incubated for 20 minutes with EnVision+ System horseradish peroxidase-labeled polymer conjugated with biotinylated anti-mouse secondary antibody and 3,3'-diaminobenzidine substrate. Laboratory validated AFP and GPC3 immunohistochemistry was performed in an automated DAKO platform in the same way as clinical samples. All slides were counterstained with hematoxylin, dehydrated, and cover slipped.

Analysis of immunohistochemical staining

Tumor cells were analyzed for ZBTB16 immunoreactivity in a semiquantitative way. Each histologic component of the mixed GCT, when present, was independently evaluated. Histologic patterns/subtypes of YST were assessed individually for their immunoreactivity with ZBTB16, AFP and GPC-3, respectively. Only nuclear staining for ZBTB16 and cytoplasmic staining for AFP and GPC-3 were considered positive. Based on the extent of the immunoreactivity, the staining was graded as: virtually no (<1% cells) staining (negative), 1-25% of cells staining (focal), 25%-50% of cells staining (moderate extent) and >50% of cell staining (extensive/diffuse). For ZBTB16, normal testicular tissue sections containing ZBTB16-positive spermatogonial cells and ZBTB16-negative stromal cells were used as positive and negative testing controls, respectively.

RESULTS

I. Expression of ZBTB16 in metastatic YST

Twenty-nine of the 32 metastatic YSTs showed immunoreaction with ZBTB16: 13/32 (41%) had diffuse, 9/32 (28%) had moderate, and 7/32 (22%) had focal nuclear expression. Three (9%) cases had no immunoreactivity with ZBTB16 (1 reticular, 1 solid, and 1 polyvesicular). ZBTB16 reactivity/grade appeared not associated with specific histologic growth patterns. In 15 cases of the mixed GCTs, all non-YST GCT components (teratoma and/or embryonal carcinoma) were nonreactive with ZBTB16 (0/15, 0%). The overall sensitivity of ZBTB16 in detecting metastatic YST was 29/32 (91%). Seven of 8 (88%) postchemotherapy metastatic YSTs were immunoreactive for ZBTB16 with 50% (4/8) of the cases exhibiting diffuse cell staining, 25% (2/8) with moderate extent of cell staining, and 13% (1/8) with focal cell staining. Only one (13%) postchemotherapy metastatic YST did not stain. Twenty-two of 24 (92%) metastatic YSTs without prior treatment were positive for

ZBTB16 with 38% (9/24) of the cases showing diffuse, 29% (7/24) moderate, and 15% (6/24) focal staining. Only two cases (8%) were nonreactive. ZBTB16 showed no statistically significant difference in sensitivity for detecting metastatic YSTs between the non-treated and the chemotherapy treated YSTs ($p<0.005$). The results of ZBTB16 expression in extragonadal primary and metastatic YSTs are summarized in Table 1. Immunohistochemical expression of ZBTB16 in a representative case of metastatic YSTs is illustrated in figure 1. The overall sensitivity of ZBTB16 in detecting primary extragonadal YST was 100% (4/4). The overall sensitivity of ZBTB16 in detecting both metastatic YST and extragonadal YST was 91.6% (33/36).

II. Expression of ZBTB16 in extragonadal YST:

The 4 extragonadal primary YSTs presented either as solid pattern, partially glandular or a mixture of both, mimicking moderately to poorly differentiated somatic malignancies. All the 4 extragonadal primary YSTs were strongly and diffusely reactive with ZBTB16 (4/4, 100%). Figure 2 illustrates a liver primary YST (hematoxylin-eosin) and immunohistochemical expression of ZBTB16 in tumor cells. The results of ZBTB16 immunohistochemical expression in the metastatic YST-containing GCTs and primary extragonadal YSTs are summarized in table 1.

III. Comparison of the immunoreactivity of ZBTB16 with AFP and GPC3

Of the thirty-two cases of metastatic YSTs in our case series, twenty-nine were available for immunohistochemical study with AFP and GPC3. The results are summarized in table 2. The detecting sensitivity of ZBTB16, AFP and GPC-3 for those 29 cases was 89.7%, 58.6% and 86.2%, respectively. Although there was no association in the immunoreactivity of AFP and GPC-3 with specific YST histologic patterns, AFP displayed a

trend for nonreactivity in solid. Both AFP and GPC3 were nonreactive in the sarcomatoid YST case, with which, interestingly, ZBTB16 showed focal reactivity. Nevertheless, those pattern-related immunoreactivity differences warrant further validation with a larger number of cases.

IV. Expression of ZBTB16 in somatic malignancies

We have previously demonstrated that ZBTB16 nuclear expression was absent in most of the somatic carcinomas, except reactivity in prostate cancers and weak and focal positivity in approximately one third of hepatocellular carcinomas (23, 28). With the exception of occasional ZBTB16 positive myoepthelial cells in the salivary gland carcinomas (adenoid cystic and acinar cell carcinoma), all the 51 somatic malignancies in this study were negative for ZBTB16. These results indicate that ZBTB16 is highly specific for YST. The results of ZBTB16 expression in the somatic malignancies are summarized in table 3.

DISCUSSION

It is estimated that 3% to 5% of newly diagnosed cancers present without established primary sites (29). YST notoriously displays diverse histologic patterns, which can mimic many different types of tumors – GCTs and somatic malignancies alike. Proliferative or hyperplastic endodermal glandular epithelium of teratoma and somatic adenocarcinoma can be mistaken for glandular YST (2-4, 30). Seminoma and poorly differentiated somatic malignancies can be simulated by solid YST (31), particularly in small biopsies or suboptimal specimens. In later recurrence of GCTs, YST commonly presents as a glandular and/or solid tumor, which, without prior clinical information, may lead to erroneous diagnosis of a somatic-type malignancy. All those make YST one of the most challenging malignancies to diagnose, particularly in the setting of extragonadal location and unknown clinical history.

Distinction of YST from other GCTs (especially metastatic teratoma and seminoma) as well as from somatic malignancies is of critical importance because of different implications for treatment as well as prognosis and tumor biomarker surveillance. For example, metastatic teratoma is usually treated by surgery alone, while the presence of YST requires additional adjuvant chemotherapy (32-34). Furthermore, presence of YST in metastases increases the chances of recurrence despite additional therapy and portends an overall worse prognosis. Sensitive and specific markers for YST are therefore extremely valuable in helping reach correct diagnosis and informing optimal management strategy.

AFP is a useful traditional marker for YST, nevertheless, it is limited by low sensitivity with an immunoreactive positive rate as low as 60% (11). In addition, its staining is frequently focal and patchy (9, 35), contributing to false negative immunoreactivity in small biopsy specimens (9, 35). AFP has also been reported to stain embryonal carcinoma and teratoma (36, 37) as well as somatic tumors, such as ovarian serous adenocarcinoma (38), clear cell adenocarcinoma (9), pancreatic adenocarcinomas (7), hepatocellular carcinoma (8), and gastric carcinoma (39)

GPC-3 is another GCT marker that was recently identified to be valuable for diagnosis of YST. It has been reported that GPC-3 is more sensitive than AFP (12, 14), but might not be as specific as AFP for YST. Expression of GPC-3 has been reported in embryonal carcinoma, choriocarcinoma, and teratoma as well as somatic tumors, including hepatocellular, gastric, and lung carcinomas (12, 14-17).

In our previous study, we have showed that ZBTB16 is highly sensitive and specific for testicular YST (40). In this current study, we further demonstrated that ZBTB16 was also a sensitive marker for extragonadal and metastatic YSTs with an overall sensitivity of 91.6%, superior to AFP and GPC-3.

Although ZBTB16 is expressed in some somatic tumors, including low grade and a portion of high grade prostatic carcinoma (23), some carcinoid tumor (26) as well as about 30% of hepatocellular carcinomas (with focal and weak positivity) (28), ZBTB16 nuclear expression is rarely seen in other somatic malignancies, particularly the common insidious and specific-marker-lacking malignancies, such as pancreatobilliary and upper gastrointestinal carcinomas. Due to the availability of tissue specific markers for prostatic carcinoma, hepatocellular carcinoma and carcinoid tumor, distinction of YST from these ZBTB16-expressing somatic tumors should be less problematic. ZBTB16 is, therefore, an excellent adjunct marker with high sensitivity and specificity in distinguishing extra-testicular primary YST as well as metastatic YST from its mimics. The other advantage of ZBTB16 is its nuclear staining which makes it easy to interpret. Unlike the cytoplasmic staining of AFP and GPC-3, nuclear ZBTB16 immunostaining is less affected by hemorrhage, serum and secretions, or necrosis, thus the background staining is minimal. Moreover, when necessary, ZBTB16 in paneling with other GCT markers (e.g. SALL4, AFP or GPC-3) should further increase the sensitivity and confidence in the diagnosis of YST for challenging cases.

In summary, the results of this study demonstrate that ZBTB16 is a novel sensitive and specific nuclear marker in the detection of metastatic and extragonadal YSTs.

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Author contributions: GQX and MTI designed the project, the main conceptual ideas and proof outline. GQX drafted the manuscript. DSP and QY collected the samples and data and performed the study. DSP, MTI, CW and MA analyzed the results and revised the draft.

Conflicts of interest

The authors declare no competing financial interests.

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TABLES

Table 1: Immunohistochemical expression of ZBTB16 in primary extragonadal YSTs and metastatic YST-containing GCTs

Table 2. Immunohistochemical expression of ZBTB16 in somatic malignancies

Table 3. Growth patterns and immunoreactivity of metastatic YSTs with ZBTB16, AFP and GPC-3.

FIGURE LEGENDS

Figure 1: Expression of ZBTB16 in metastatic YST: A (Hematoxylin and Eosin) and B (immunohistochemistry) - Moderate extent of ZBTB16 positivity in metastatic retroperitoneal YST with reticular pattern. C (Hematoxylin and Eosin) and D (immunohistochemistry) - Diffuse ZBTB16 immunoreactivity in metastatic mediastinal YST with mostly solid growth pattern. E (Hematoxylin and Eosin) and F (immunohistochemistry) - Metastatic omental mixed germ cell tumor showing focal YST element being highlighted by ZBTB16 immunostaining.

Figure 2: Expression of ZBTB16 in primary liver YST from a 76 year-old male. A and B (Hematoxylin and Eosin, low and high magnification) - Poorly differentiated carcinoma with vague glandular formation. C (Immunohistochemistry) - Diffuse and strong immunoreactivity of tumor cells with ZBTB16.

Table 1: Immunohistochemical expression of ZBTB16 in primary extragonadal YSTs and metastatic YST-containing GCTs

Diagnosis and %YST in GCT	number of cases	Composition of GCT	Location	ZBTB16 Positivity of YST element	ZBTB16 positivity of non-YST element
Primary extragonadal YST					
100% YST (pure YST)	4	YST (4/4)	Retroperitoneum (1/4) Mediastinum (1/4) Liver (1/4) Bladder (1/4)	3+ (4/4; 100%)	NA
Metastatic mixed GCT, no chemotherapy					
YST< 50%	6	T+ EC+ YST (3/6) T+YST (3/6)	Retroperitoneal LN (6/6)	3+ (1/6; 17%) 2+ (3/6; 50%) 1+ (2/6; 33%)	0 (0/6, 0%)
YST≥50%	18	YST (14/18) T+YST (3/18) EC+YST (1/18)	Retroperitoneal LN (10/18) Mediastinal LN (4/18) Abdomen (2/18) Lung (1/18) Pelvic LN (1/18)	3+ (8/18; 44%) 2+ (4/18; 22%) 1+ (4/18; 22%) 0 (2/18; 11%)	0 (0/4, 0%)
Metastatic mixed GCT, postchemotherapy					
YST< 50%	2	T+YST (2/2)	Retroperitoneal LN (1/2) Renal vein (1/2)	3+ (1/2; 50%) 1+ (1/2; 50%)	0 (0/2, 0%)
YST≥50%	6	YST (3/6) T+YST (1/6) T+EC+YST (1/6) EC+YST (1/6)	Retroperitoneal LN (4/6) Mediastinal LN (1/6) Pelvic LN (1/6)	3+ (3/6; 50%) 2+ (2/6; 33%) 0 (1/6; 17%)	0 (0/3, 0%)

GCT = germ cell tumor; YST = yolk sac tumors; EC = embryonal carcinoma; T = teratoma; LN = lymph node

Table 2: Growth patterns and immunoreactivity of metastatic YSTs with ZBTB16, AFP and GPC-3

Case No	Tumor growth patterns	ZBTB16	AFP	GPC-3
1	Myxomatous	2+	3+	2+
2	Reticular+macrocystic	2+	Neg	2+
3	Sarcomatoid	1+	Neg	Neg
4	Reticular	3+	3+	3+
5	Polyvesicular + glandular	3+	2+	2+
6	Endodermal sinus+reticular	3+	3+	2+
7	Reticular +glandular	2+	3+	2+
8	Reticular	3+	3+	2+
9	Reticular	1+	Neg	1+
10	Endodermal sinus +papillary	3+	3+	3+
11	Myxomatous	1+	1+	1+
12	Reticular	3+	Neg	1+
13	Reticular	3+	3+	3+
14	Solid+ endodermal sinus	2+	2+	2+
15	Polyvesicular+ macrocystic	2+	2+	2+
16	Reticular	Neg	Neg	1+
17	Reticular	1+	Neg	Neg
18	Papillary	3+	Neg	2+
19	Reticular+glandular	1+	Neg	2+
20	Reticular	3+	Neg	3+
21	Glandular	2+	1+	1+
22	Solid	Neg	Neg	1+
23	Polyvesicular+ reticular	3+	3+	1+
24	Polyvesicular	Neg	1+	Neg
25	Solid	2+	Neg	3+
26	Glandular+ reticular	2+	Neg	1+
27	Endodermal sinus	1+	2+	Neg
28	Myxomatous	1+	1+	1+
29	Endodermal sinus	3+	2+	2+

Neg. negative

Table 3: Immunohistochemical expression of ZBTB16 in additional somatic malignancies

Diagnosis	# of cases	ZBTB16 immunoreactivity
Breast carcinoma	8	Negative
Melanoma	8	Negative
Lymphoma	5	Negative
Pancreatobilliary carcinoma	3	Negative
Combined small cell carcinoma and urothelial carcinoma	6	Negative
Salivary gland carcinoma	7	Negative, occasional myoepithelial cell positive
Mesothelioma	3	Negative
Adrenocortical carcinoma	1	Negative
Synovial sarcoma	2	Negative
Renal cell carcinoma	2	Negative
Mullerian carcinoma	2	Negative
Colorectal carcinoma	2	Negative
Meckel cell carcinoma	2	Negative