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Aberrant Nuclear Immunoreactivity for TFE3 in Neoplasms With *TFE3* Gene Fusions

A Sensitive and Specific Immunohistochemical Assay

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We report the aberrantly strong nuclear immunoreactivity for the C-terminal portion of TFE3 protein in tumors characterized by chromosome translocations involving the *TFE3* gene at Xp11.2. This group of tumors includes alveolar soft part sarcoma and a specific subset of renal carcinomas that tend to affect young patients. They contain fusion genes that encode chimeric proteins consisting of the N-terminal portion of different translocation partners fused to the C-terminal portion of TFE3. We postulated that expression of these fusion proteins may be dysregulated in these specific tumors and detectable by immunohistochemistry. We performed immunohistochemistry using a polyclonal antibody to the C-terminal portion of TFE3 in 40 formalin-fixed, paraffin-embedded tumors characterized by *TFE3* gene fusions, including 19 alveolar soft part sarcoma (of which nine were molecularly confirmed) and 21 renal carcinomas with cytogenetically confirmed characteristic Xp11.2 translocations and/or fusion transcripts involving *TFE3* (11 *PRCC-TFE3*, 7 *ASPL-TFE3,* 3 *PSF-TFE3*). We also screened 1476 other tumors of 64 histologic types from 16 sites for TFE3 immunoreactivity using tissue microarrays and evaluated a broad range of normal tissues. Thirty-nine of 40 neoplasms characterized by TFE3 gene fusions (19 of 19 alveolar soft part sarcoma, 20 of 21 renal carcinomas) demonstrated moderate or strong nuclear TFE3 immunoreactivity. In contrast, only 6 of 1476 other neoplasms labeled for TFE3 (sensitivity 97.5%, specificity 99.6%). Nuclear immunoreactivity in normal tissues was extremely rare. We then applied this assay to a set of 11 pediatric renal carcinomas for which only paraffin-embedded tissue was available, to assess if morphologic features could predict TFE3 immunoreactivity. Of the eight cases in which we suspected that a *TFE3* gene rearrangement might be present based on morphology, seven scored positive for nuclear TFE3 labeling. Of the three tumors whose morphology did not suggest the presence of a *TFE3* gene fusion, none showed nuclear TFE3 labeling. In summary, we find that nuclear immunoreactivity for TFE3 protein by routine immunohistochemistry is a highly sensitive and specific assay for neoplasms bearing *TFE3* gene fusions. Furthermore, the finding in our set of test cases (i.e., that morphologic features can be used to predict TFE3 immunoreactivity) further supports the notion that renal carcinomas with *TFE3* gene fusions have a distinctive morphology that corresponds to their genetic distinctiveness. Carcinomas associated with *TFE3* gene fusions may account for a significant proportion of pediatric renal carcinomas, and this immunohistochemistry assay may help to clarify their true prevalence.

Key Words: Chromosome translocation—Immunohistochemistry—Alveolar soft part sarcoma—Carcinoma.

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A subset of human cancers is characterized cytogenetically by recurrent chromosomal translocations.[7,38] Such chromosomal translocations are often the only karyotypic abnormality identified in these neoplasms, suggesting that they likely are necessary and possibly sufficient for tumorigenesis.[37] These chromosomal translocations result in gene fusions that encode novel chimeric proteins, many of which function as chimeric transcription factors that are overexpressed and more active compared with their normal counterparts.[7,26,38] Recurrent chromosomal translocations that involve transcription factors are most common in hematopoietic neoplasia[17] and sarcomas,[7,26] but some have recently been described in specific carcinomas.[18,24]

Chimeric fusion proteins represent novel targets for potential tumor-specific diagnostic immunohistochemistry (IHC) assays. There are several potential approaches to detecting translocation fusion proteins, as recently reviewed in detail by Falini and Mason.[17] One approach is to develop antibodies to the fusion protein breakpoint, which would in theory serve as absolutely specific markers for the translocation because the amino acid sequence

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at the fusion point should not be present in any other tissue, normal or neoplastic. However, the difficulty in raising specific antibodies to these amino acid sequences, coupled with the known variation in fusion protein structure within given tumor types, has limited this approach.[17] A second approach is to use robust antibodies to a portion of one of the proteins that is retained in the fusion because translocations often result in overexpression of the fusion protein relative to its normal counterparts and/or aberrant expression in novel tissue types. This second approach has been more successful; examples include ALK1 immunoreactivity as a marker for a subset of anaplastic large cell lymphoma[13,17] and inflammatory myofibroblastic tumor[12,14,28] and immunoreactivity for the C-terminus of WT1 as a marker of the desmoplastic small round cell tumor.[6,10,19]

Recently, the gene fusion resulting from the specific chromosome translocation of alveolar soft part sarcoma (ASPS) has been identified.[27] The specific der(17)t(X;17)(p11.2;q25) results in a fusion between the *TFE3* transcription factor gene on chromosome Xp11.2 and the novel *ASPL* gene on 17q25. Although the function of ASPL remains unknown, the *TFE3* gene is also implicated in translocations involving Xp11.2 in a subset of renal cell carcinomas that preferentially arise in children and young adults. The most common translocation in these renal carcinomas is a t(X;1)(p11.2;q21) in which *TFE3* is fused to the *PRCC* gene,[1,40,43] although variant translocations have also been described.[11] Our group has recently shown that the renal carcinomas bearing a t(X;17)(p11.2;q25) translocation bear the identical *ASPL-TFE3* gene fusion as ASPS, although these renal carcinomas are clinically and pathologically distinct from ASPS and from renal carcinomas with *PRCC-TFE3*.[1,2] A common feature of all of the translocations involving Xp11.2 is that a portion of the *TFE3* gene is placed under the control of a novel promoter, which differs in each specific translocation-associated tumor because the *TFE3* portion is at the 3′ end of each fusion gene. Based on experience with other tumor-specific chromosome translocations, we hypothesized that the TFE3 fusion protein may be overexpressed in these neoplasms and that IHC detection of overexpression of TFE3 may prove to be a useful diagnostic marker.

We report the utility of an IHC assay for TFE3 protein in archival formalin-fixed, paraffin-embedded tissue sections. The assay demonstrates high specificity and sensitivity when applied to a series of tumors characterized by Xp11.2 translocations and resulting *TFE3* gene fusions, and a large series of other tumors and tissues. Additionally, we applied the assay to evaluate a set of pediatric renal carcinomas in our files that lacked cytogenetic or molecular data to begin to assess the prevalence of *TFE3* gene fusions in this clinicopathologic setting.

MATERIALS AND METHODS

Positive Control Cases

As a positive control group for TFE3 IHC, we selected a series of tumors that are characterized genetically by chromosome translocations involving Xp11.2 that result in gene fusions with *TFE3,* namely, ASPS and three types of renal carcinomas.

The ASPS-positive control cases were 19 well-characterized tumors from which paraffin blocks were available. These included nine previously reported cases of ASPS in which we had demonstrated an *ASPL-TFE3* gene fusion by reverse transcriptase polymerase chain reaction (RT-PCR).[25,27]

The renal carcinoma-positive control group consisted of 21 molecularly or cytogenetically confirmed *TFE3*-related renal tumors, i.e., tumors that contained a characteristic chromosome translocation with a breakpoint at Xp11.2 by cytogenetic analysis and/or a gene fusion involving the *TFE3* gene by RT-PCR. Based on availability of paraffin blocks, we selected seven renal carcinomas associated with the t(X;17)(p11.2.;q25) translocation, including six tumors in which we had demonstrated the *ASPL-TFE3* gene fusion by RT-PCR. Five of these cases were previously reported in the initial characterization of this entity.[2] We also selected 11 previously reported renal carcinomas containing the t(X;1)(p11.2;q21), consisting of 10 tumors that had positive cytogenetics and one tumor in which we had demonstrated the characteristic *PRCC-TFE3* gene fusion by RT-PCR.[1] Finally, we also studied three renal carcinomas that demonstrated a t(X;1)(p11.2;p34) translocation on cytogenetic analysis, known to generate a *PSF-TFE3* gene fusion.[11] Frozen material was available in one of these three cases, and the presence of a *PSF-TFE3* fusion transcript was also confirmed by RT-PCR (M. Y. Lui, M. Ladanyi, unpublished data). Thus, the 21 cases in the renal carcinoma-positive control group consisted of 7 *ASPL-TFE3* carcinomas, 11 *PRCC-TFE3* carcinomas, and 3 *PSF-TFE3* carcinomas.

Screening Cases

To determine the prevalence of nuclear immunoreactivity for TFE3 in a wide variety of neoplasms, we used two strategies. First, to study significant numbers of relatively common tumors, we analyzed a series of well-characterized organ-specific tissue microarrays (TMAs) created at Memorial Sloan-Kettering Cancer Center and the Johns Hopkins Hospital. High-density TMA blocks made at Memorial Sloan-Kettering Cancer Center contained 70–270 cores, ranging from 3 to 6 cores per tumor, with a core diameter of 0.4–1.0 mm. Low-density TMA blocks made at Memorial Sloan-Kettering Cancer Center contained 27–35 cores (single core per tumor),

each measuring 3 mm in diameter. Each TMA block made at The Johns Hopkins Hospital contained 99 spots of tumor with a diameter of 2 mm. When positive or weak positive staining was noted in specific TMA spots, the corresponding "donor blocks" were retrieved when available and whole sections were immunostained for TFE3. Second, to analyze certain uncommon tumors for which TMAs were not available, we obtained unstained sections containing tumor and normal tissue. The total numbers of different tumor types evaluated are listed in Table 1.

Test Cases: Archival Pediatric Renal Carcinoma Cases

This group consisted of a series of pediatric renal carcinomas from the consultation files of the authors (P.A., V.E.R.) for which molecular analysis could not be performed because of lack of frozen tissue. Based upon morphologic findings, IHC for cytokeratin, epithelial membrane antigen, vimentin, and renal cell carcinoma marker,[5] and our prior morphologic characterization of these tumor types,[1,2] we subdivided these tumors into those in which we suspected a *TFE3* gene fusion was present and those in which we did not suspect the presence of a *TFE3* gene fusion. These tumors were then evaluated for TFE3 nuclear immunoreactivity.

TFE3 Antibody

We used the P-16 polyclonal antibody to TFE3 (catalog no. sc-5958; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The portion of TFE3 used to generate this polyclonal antibody is the manufacturer's proprietary information. However, by testing this antibody on western blots of protein extracts from cells transfected with expression plasmids encoding full-length ASPL-TFE3 (types 1 and 2), we found that this antibody binds to the C-terminal portion of TFE3 protein downstream of the region encoded by exon 4 (Fig. 1). Because the *TFE3* exon involved in the type 1 *ASPL-TFE3* fusion is presently the most distal known fusion point in any *TFE3* fusion gene, it follows that the binding site of this TFE3 polyclonal antibody should be retained in all known TFE3 fusion proteins.

IHC Method

Four-μm sections were mounted onto positively charged slides. Slides were deparaffinized in xylene for 30 minutes, rehydrated using graded ethanol concentrations, and steamed for 30 minutes at 98–99°C in EDTA buffer in a vegetable steamer. Following quenching with hydrogen peroxidase and biotin blocking using avidin, sections were incubated overnight with a 1:600 dilution of the polyclonal antibody to TFE3 in phosphate-buffered saline. Detection of antibody binding was achieved using a biotinylated secondary antibody and horseradish peroxidase-conjugated streptavidin (Dako, Carpinteria, CA, USA) and 3′,3′-diaminobenzidine as chromogen.

Scoring of TFE3 Nuclear Immunoreactivity

TFE3 nuclear immunoreactivity was scored from 0 to 3+, and examples of 1+ to 3+ staining are shown in Figure 2. Tumors scored as positive for TFE3 demonstrated nuclear immunoreactivity that was readily apparent at low-power magnification (4× objective). These cases were subdivided into moderately (2+) and strongly (3+) positive based upon the intensity of labeling (Fig. 2). Cytoplasmic immunoreactivity was ignored because native TFE3 and its fusion proteins are known to localize to the nucleus[44] (M. Y. Lui, M. Ladanyi, unpublished data). Cases showing weak/equivocal nuclear immunoreactivity (1+) demonstrated nuclear immunoreactivity that was subtle at low power and typically required higher power magnification to be appreciated. Such cases were considered negative for statistical analysis and were combined with cases showing no (0+) nuclear labeling. Tumor cells that showed cytoplasmic reactivity, possibly resulting from endogenous biotin, were considered negative unless the immunostaining of the nuclei was clearly more intense than that of the cytoplasm.

RESULTS

Normal Tissues

Normal tissues evaluated included lung, thyroid, lymph node, breast, colon, liver, gallbladder, pancreas, uterus, ovary, bone, kidney, bladder, adrenal, prostate, and skin. None of these tissues consistently demonstrated detectable TFE3 nuclear protein by IHC, but rare sporadic immunoreactivity was observed. Normal pancreatic acini in one of 18 cases of chronic pancreatitis demonstrated weak (1+) immunoreactivity on two of two microarray spots of this case. TFE3 IHC on the donor block from this pancreas specimen yielded similar weak and focal immunoreactivity. In a minority of cases, sinus histiocytes of lymph nodes and nuclei within the glomerular mesangium also showed weak (1+) reactivity, but this was not a consistent finding.

Positive Control Cases: Tumors With Known Xp11.2 Translocations or *TFE3* Gene Fusions

Among the 40 positive control tumors, 39 demonstrated moderate or strong nuclear immunoreactivity for TFE3 protein (Table 1). As described in *Materials and Methods*, we scored only 2+ (moderate) or 3+ (strong)

TABLE 1. *Nuclear immunoreactivity for TFE3 in screening cases and positive control cases (preceded by +)*

Organ System	Tumor type	Total cases	TFE3 IHC			
			0+	1+	2+	3+
Soft tissue	+ Alveolar soft part sarcoma	10	0	0	1	18
	Rhabdomyosarcoma	48	48	0	0	0
	Leiomyosarcoma	4	4	0	0	0
	Fibromatosis	24	24	0	0	0
	Low-grade myxofibrosarcoma	6	6	0	0	0
	Low-grade fibrosarcoma	8	8	0	0	0
	High-grade fibrosarcoma	5	5	0	0	0
	Malignant fibrous histiocytoma (MFH)	17	17	0	0	0
	Pleomorphic liposarcoma	13	13	0	0	0
	High-grade leiomyosarcoma	4	4	0	0	0
	High-grade myxoid and pleomorphic sarcoma, NOS	1	1	0	0	0
	High-grade myxofibrosarcoma	2	1	0	1	0
	High-grade malignant giant cell tumor	3	3	0	0	0
	High-grade sarcoma, NOS	3	3	0	0	0
	Glomus tumor	2	2	0	0	0
	Hemangiopericytoma	4	4	0	0	0
	Solitary fibrous tumor	4	4	0	0	0
	Schwannoma	2	2	0	0	0
	Neurofibroma	2	2	0	0	0
	Malignant peripheral nerve sheath tumor	3	2	1	0	0
	Paraganglioma	5	5	0	0	0
	Epithelioid sarcoma	4	4	0	0	0
	Granular cell tumor	8	5	1	0	2
	Synovial sarcoma	2	2	0	0	0
	Dermatofibrosarcoma protuberans	2	2	0	0	0
Renal	+ *PRCC-TFE3* renal carcinomas	11	0	1	8	2
	+ *ASPL-TFE3* renal carcinomas	7	0	0	5	2
	+ *PSF-TFE3* renal carcinomas	3	0	0	0	3
	Conventional (clear cell) renal carcinoma	21	21	0	0	0
	Papillary renal cell carcinoma	22	22	0	0	0
	Chromophobe renal carcinoma	20	20	0	0	0
	Unclassified renal cell carcinoma	21	21	0	0	0
	Oncocytoma	20	20	0	0	0
	Wilms tumor	33	33	0	0	0
	Clear cell sarcoma of the kidney	10	10	0	0	0
	t(6;11) (p21;q12) renal tumors[3]	2	2	0	0	0
	Angiomyolipoma	4	4	0	0	0
	"Henle loop tumors"[34,41]	3	3	0	0	0
Bone	Chordoma	4	2	2	0	0
	Chondrosarcoma	3	3	0	0	0
	Osteosarcoma	2	2	0	0	0
Adrenal	Adenoma	12	12	0	0	0
	Adrenal cortical carcinoma	60	57	1	1	1
Prostate	Adenocarcinoma	36	36	0	0	0
Bladder	Urothelial carcinoma	70	69	1	0	0
Uterus	Endometrial carcinomas	150	150	0	0	0
	Uterine leiomyomas	2	2	0	0	0
	Uterine leiomyosarcoma	4	4	0	0	0
Ovary	Carcinomas	99	99	0	0	0
Breast	Carcinomas	135	135	0	0	0
Lung	Adenocarcinomas	18	18	0	0	0
Thyroid	Papillary carcinomas	41	41	0	0	0
Skin	Malignant melanoma	50	50	0	0	0
Biliary tract	Distal bile duct carcinomas	15	14	0	1	0
	Intrahepatic cholangiocarcinomas	10	10	0	0	0
	Gallbladder carcinomas	15	15	0	0	0
	Metastatic adenocarcinoma to liver	79	79	0	0	0
Intestinal tract	Colorectal adenocarcinomas	115	115	0	0	0
	Gastrointestinal stromal tumors	4	4	0	0	0
Pancreas	Adenocarcinomas	72	72	0	0	0
	Acinic cell carcinoma	14	14	0	0	0
	Solid cystic papillary tumor	8	8	0	0	0
	Pancreatoblastoma	4	4	0	0	0
	Undifferentiated pancreatic carcinoma with osteoclastic giant cells	3	3	0	0	0
	Intraductal papillary mucinous neoplasms	3	3	0	0	0
	Mucinous cystic neoplasms	4	4	0	0	0
Lymphoid	Non-Hodgkin's lymphoma	93	93	0	0	0
	Hodgkin's lymphoma	19	19	0	0	0

NOS, not otherwise specified.

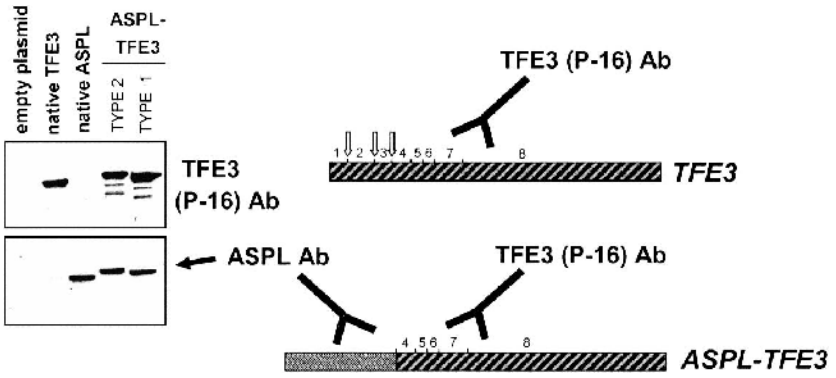

FIG. 1. Confirmation and mapping of TFE3 (P-16) polyclonal antibody binding using expression plasmids encoding ASPL and ASPL-TFE3. Protein was extracted from HeLa cells (human cervical carcinoma) transfected with expression plasmids encoding native TFE3, native ASPL, ASPL-TFE3 type 1, or ASPL-TFE3 type 2. The two types of ASPL-TFE3 fusions differ by the presence of an additional exon of TFE3 in type 2, resulting in a slightly larger protein in that type. Western blots of these transfectants were stained with the TFE3 (P-16) polyclonal antibody used for IHC, as well as with a custom polyclonal antibody to ASPL (M. Y. Lui, M. Ladanyi, unpublished data), the latter used as a control for the western blot results. As illustrated in the accompanying diagram of native TFE3 and the ASPL-TFE3 type 1 fusion products, the results indicate that the TFE3 (P-16) polyclonal antibody used for IHC binds to the portion of TFE3 retained in ASPL-TFE3 fusion proteins. This portion of TFE3 encoded by exons 4 to 8 is also known to be included in PRCC-TFE3 and PSF-TFE3 fusion proteins. The portions of the TFE3 protein encoded by specific exons are shown and the positions of possible fusion points in the known TFE3 fusion proteins are shown by arrows.

immunoreactivity as positive for aberrant TFE3 expression. Weak to equivocal (1+) nuclear staining was considered negative for aberrant TFE3 expression. Thus, all 19 ASPS labeled positively for TFE3; 18 of 19 ASPS demonstrated strong (3+) labeling, and one demonstrated moderate (2+) labeling. All seven t(X;17)(p11.2;q25) (*ASPL-TFE3*) renal carcinomas were scored as positive, two demonstrated strong (3+) immunoreactivity, and five

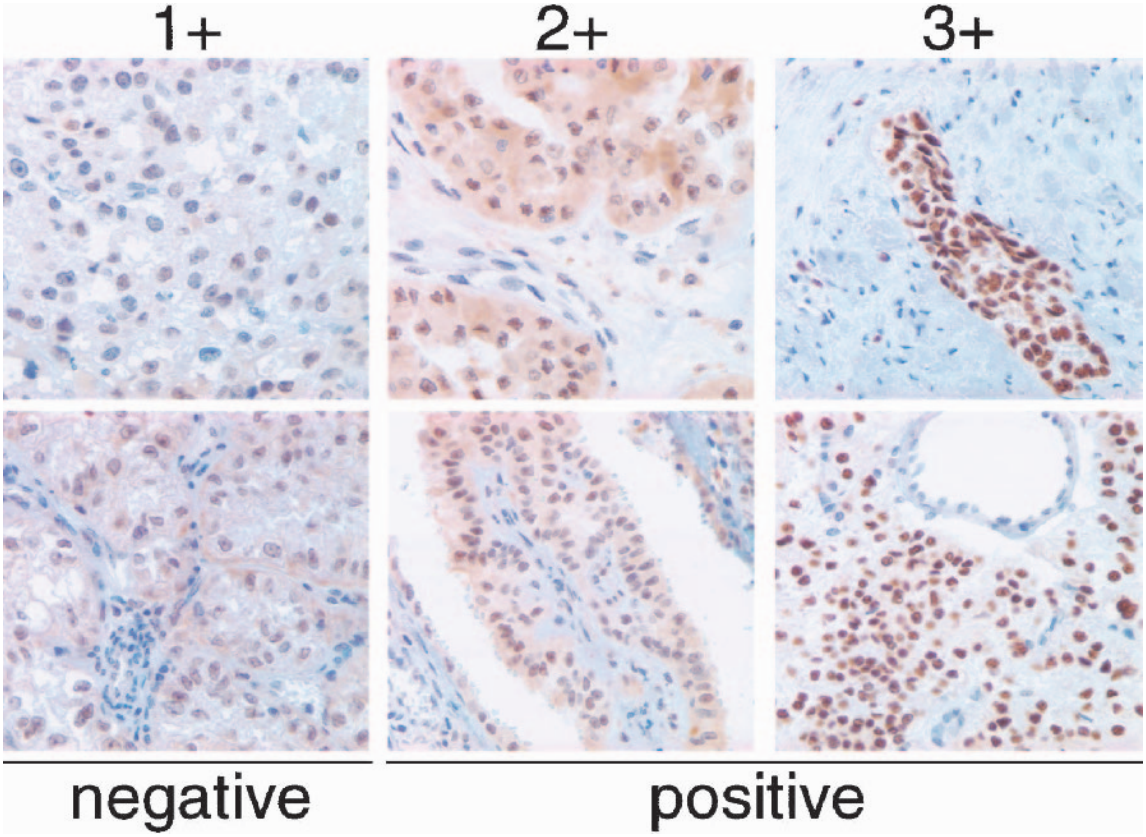

FIG. 2. Scoring of 1+, 2+, and 3+ TFE3 nuclear immunoreactivity. Two examples of each score are shown. Tumors scored as positive for TFE3 demonstrated nuclear immunoreactivity that was readily apparent at low power magnification (4× objective) and could be subdivided into moderate (2+) and strong (3+) positivity, as illustrated in these higher power views. Cases scored as 1+ showed nuclear immunoreactivity that was equivocal at low power. On high power, it was apparent that the hematoxylin staining of these nuclei was obscured by pale brown immunostain. Nonetheless, such cases were considered negative for statistical analysis and were combined with cases showing no (0+) nuclear labeling.

demonstrated moderate (2+) labeling. All three t(X;1)(p11.2;p34) (*PSF-TFE3*) renal carcinomas demonstrated strong (3+) nuclear labeling. As reported previously,[1] 10 of 11 t(X;1)(p11.2;q21) (*PRCC-TFE3*) renal carcinomas labeled positively for TFE3; two were strongly positive (3+), whereas eight were moderately positive (2+). The remaining t(X;1)(p11.2;q21) renal carcinoma demonstrated focal (1+) weak immunoreactivity for TFE3; this case was scored as a negative for statistical analysis (see *Materials and Methods*). Of note, this tumor was fixed in Bouin's acidic fixative. Examples of these tumors are shown in Figure 3. TFE3 antigenicity appears to be somewhat labile insofar as the TFE3 immunoreactivity was in some cases more intense at the periphery (but not at the edge) of the tissues, and more prominent at the subcapsular or leading edge of the tumor, than it was in the center.

Screening Cases

Among 1476 cases tested largely by TMA, all but six were negative for TFE3 (Table 1). Two tumor types yielded positive results in more than a single isolated case (Fig. 4). First, of 60 adrenal cortical carcinomas tested, one demonstrated strong (3+) nuclear labeling, one demonstrated moderate (2+) labeling, and a third demonstrated weak (1+) nuclear labeling. The donor block for the adrenal cortical carcinoma that demonstrated a 3+ result on the TMA demonstrated diffuse moderate to strong nuclear immunoreactivity that was readily distinguishable from cytoplasmic staining, consistent with the observation that both spots derived from this tumor on the TMA slide had labeled strongly. Second, of eight granular cell tumors studied, two demonstrated strong (3+) nuclear immunoreactivity and one demonstrated weak (1+) labeling. Both individual donor blocks for the granular cell tumors that labeled strongly on the array demonstrated only 1+ to 2+ nuclear immunoreactivity for TFE3. Two other single tumors labeled positively for TFE3. One of 15 distal common bile duct carcinomas demonstrated 2+ nuclear immunoreactivity in one of two spots on the array; the other spot from this tumor was negative. The donor block from this case demonstrated focal moderate labeling, consistent with the array result. Additionally, one of two cases of high-grade myxofibrosarcoma (myxoid MFH) demonstrated moderate (2+) nuclear immunoreactivity for TFE3 on the TMA slide; the donor block from this tumor was not available for repeat IHC.

Several additional tumors demonstrated weak (1+) nuclear immunoreactivity on the TMAs. These were 2 of 4 chordomas, 1 of 3 malignant peripheral nerve sheath tumors, and 1 of 70 high-grade invasive urothelial carcinomas. Donor blocks of these cases were not available for repeat analysis.

All of the remaining tumors were completely negative for nuclear TFE3 immunostaining. These included several specific cases chosen because their genetic or morphologic features suggested the possibility of a *TFE3* gene rearrangement. These included a Wilms' tumor with a t(X;7)(p11;p13) (kindly provided by Charles Timmons, MD, PhD, Dallas, TX, USA), selected for analysis because of the presence of the cytogenetic Xp11 breakpoint. On histologic review, this tumor proved to be a typical blastemal-predominant Wilms' tumor. Another negative lesion tested was a renal carcinoma with clear cell and papillary features arising in an adult with autosomal dominant polycystic kidney disease. We studied this case because the combination of clear cytoplasm and papillary architecture is a characteristic morphologic feature of Xp11.2-associated renal carcinomas.[1,2] The results of the TFE3 IHC are summarized in Table 1.

Test Cases: Relationship Between TFE3 Immunoreactivity and Morphology in Pediatric Renal Carcinomas

To test the strength of the relationship between morphologic subtypes of pediatric renal tumors and nuclear immunostaining for TFE3, we assembled a test set of 11 additional cases of pediatric renal cell carcinoma from our consultation files. Cytogenetic data and material suitable for molecular analysis were not available in any of these cases. On morphologic grounds, we predicted that eight contained TFE3 fusion proteins, including five suspected *ASPL-TFE3* renal carcinomas and three suspected *PRCC-TFE3* renal carcinomas. We then performed TFE3 IHC on these 11 cases. Of the eight tumors that we predicted to contain TFE3 fusion proteins, seven demonstrated definite TFE3 nuclear immunostaining. Examples of two positive cases thus identified are shown in Figure 5. These included all five neoplasms suspected of being *ASPL-TFE3* renal carcinomas on the basis of characteristic cytologic features (voluminous cytoplasm, vesicular chromatin with prominent nucleoli), architecture (nested, pseudopapillary, and papillary patterns), abundant psammoma bodies, and paucity of immunoreactivity for epithelial markers.[2] Of the three neoplasms suspected to be *PRCC-TFE3* renal carcinomas on the basis of hematoxylin and eosin morphology (more compact architecture, combinations of nested and papillary architectural patterns, clear and eosinophilic cytology, and relatively minimal immunoreactivity for epithelial markers[1]), two were scored as positive for TFE3. The other tumor demonstrated focal 1+ nuclear immunoreactivity (interpreted as negative). The clinical and pathologic features of these cases are summarized in Table 2. In the remaining three cases of pediatric renal carcinoma

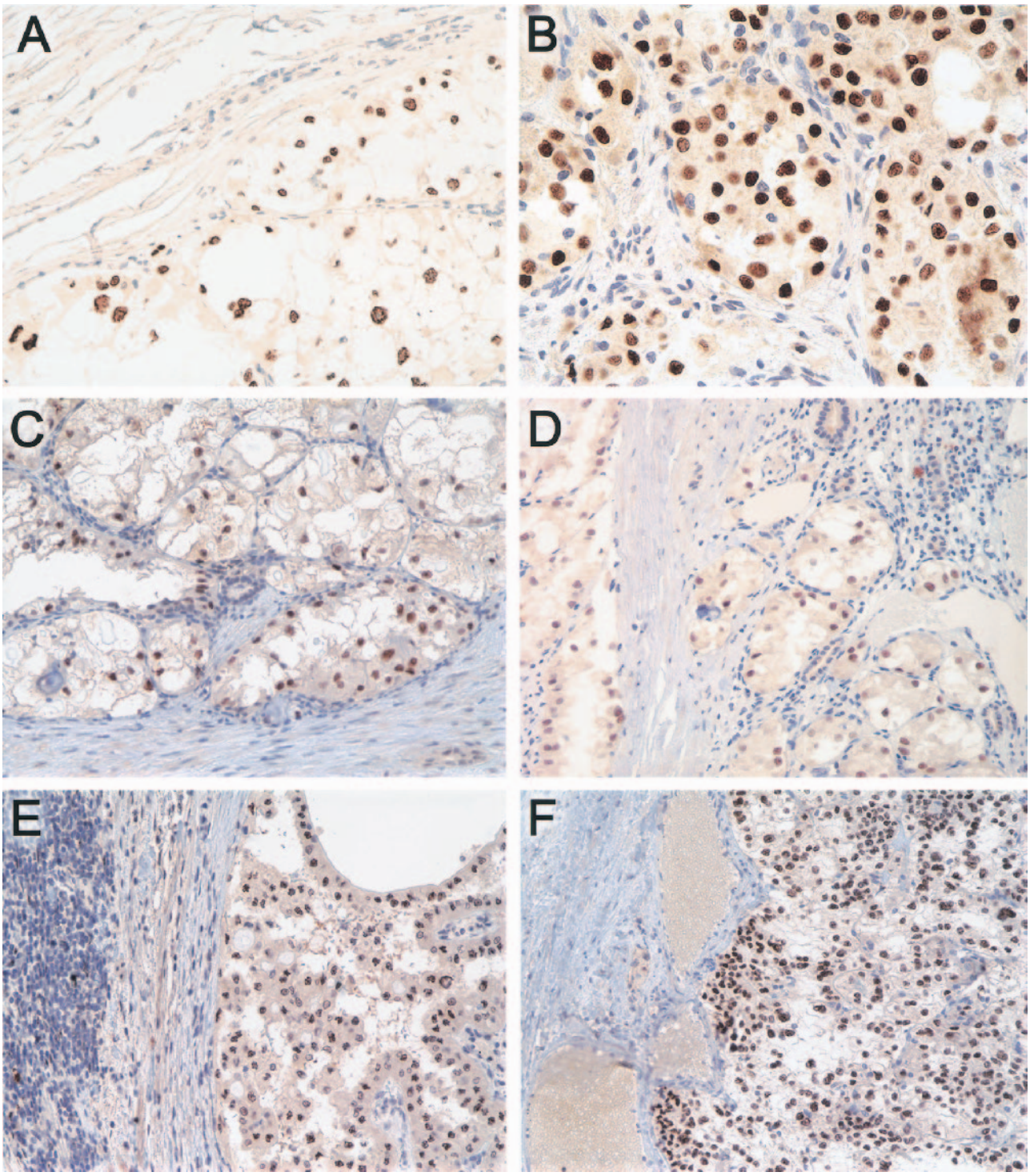

FIG. 3. *TFE3* nuclear immunoreactivity in tumors with confirmed Xp11.2 translocations and/or *TFE3* gene fusions. Note the strong nuclear immunoreactivity of tumor cells (3+) and absence of immunoreactivity of normal stroma and entrapped renal tubules. (**A** and **B**) Alveolar soft part sarcomas. (**C** and **D**) *ASPL-TFE3* renal carcinomas associated with the t(X;17)(p11.2;q25). (**E**) *PRCC-TFE3* renal carcinoma associated with the t(X;1)(p11.2;q21). (**F**) *PSF-TFE3* renal carcinoma associated with the t(X;1)(p11.2;p34).

tested, we did not suspect an Xp11.2-associated renal carcinoma based upon morphology, and indeed these three cases did not label for TFE3. Two of these tumors did show unusual nucleolar reactivity in the absence of nuclear labeling, which we regarded as negative. We suspect that these latter tumors may be related to low-grade distal nephron carcinomas described previously.[34,41]

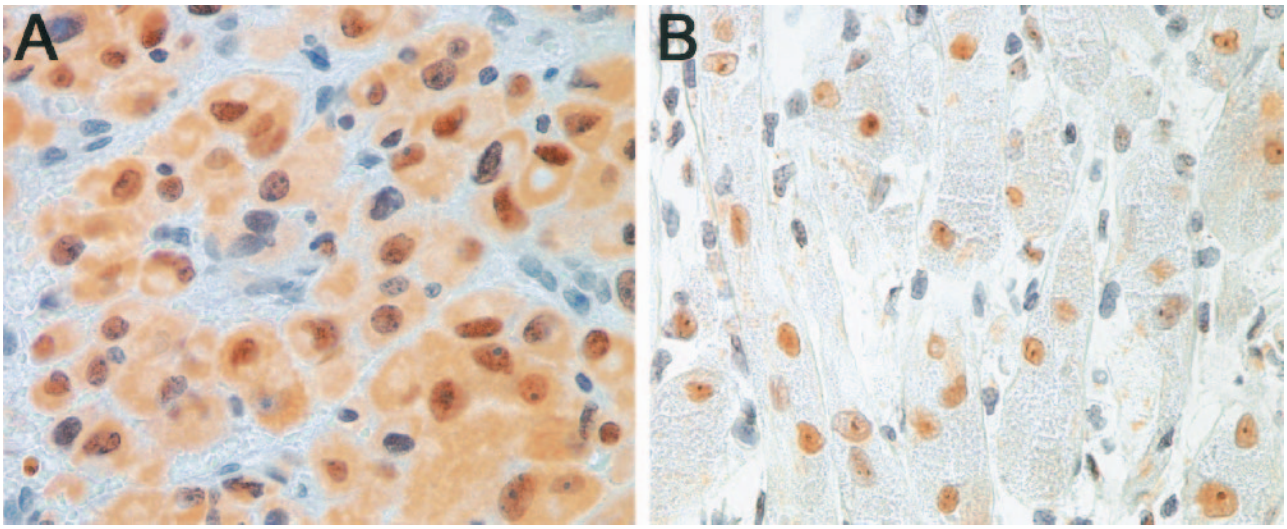

FIG. 4. Rare cases without known or suspected *TFE3* gene fusions showing TFE3 immunoreactivity. (**A**) Adrenal cortical carcinoma (3+ staining). Further molecular analysis of this tumor did not find evidence of a *TFE3* gene fusion. The vast majority of other adrenal cortical carcinomas were negative (Table 1). (**B**) Granular cell tumor (2+ staining). There was no frozen tissue available for further molecular analysis of this tumor. Most granular cell tumors were negative (Table 1).

DISCUSSION

We report the utility of an IHC assay for the C-terminal portion of TFE3 protein in distinguishing tumors characterized by *TFE3* gene rearrangements from genetically unrelated lesions. TFE3 is a transcription factor with a basic helix-loop-helix DNA binding domain and a leucine zipper dimerization domain. TFE3 contains a nuclear localization signal that maps to a portion of TFE3 retained within all known TFE3 fusion proteins.[42] Accordingly, the PRCC-TFE3 fusion protein has been shown to localize to the nucleus,[44] as has the ASPL-TFE3 fusion protein (M. Y. Lui, M. Ladanyi, unpublished data). TFE3 is ubiquitously expressed in humans and presumed to regulate many genes,[23,31,32] most of which remain to be identified. We find that native TFE3

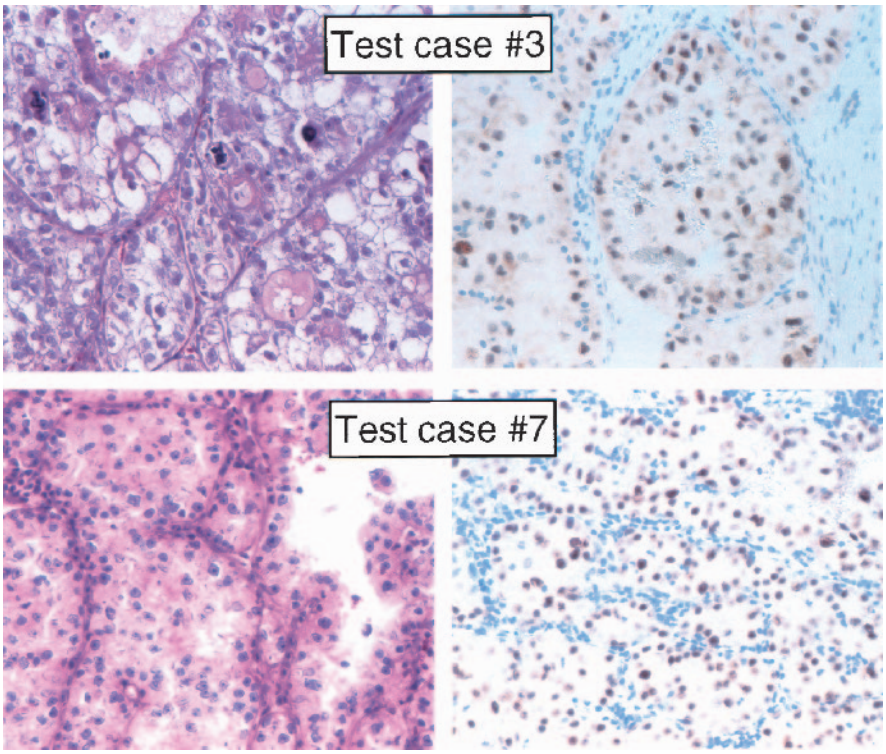

FIG. 5. TFE3 immunohistochemical analysis of test cases, as defined in *Methods*. (**A**) Test case no. 3 demonstrates classic features of a t(X;17) carcinoma; specifically, alveolar architecture, voluminous clear to eosinophilic cytoplasm, hyaline nodules, and psammomatous calcifications. (**B**) This tumor was strongly and diffusely (3+) immunoreactive for TFE3. Note the benign tubule to the right that does not label. (**C**) Test case no. 7 demonstrates typical features of a t(X;1) carcinoma; specifically, clear but less voluminous cytoplasm and nested to papillary architecture. (**D**) This tumor was also diffusely and strongly (3+) immunoreactive for TFE3.

TABLE 2. *TFE3 IHC results in test cases: pediatric renal carcinomas morphologically consistent with cases bearing Xp11.2 translocations/TFE3* gene fusions

Test case	Age (yr)/sex	Histologic features	Tumor diameter/AJCC stage	Routine immunophenotype	TFE3 IHC result
1	2/F	t(X;17)-like: voluminous cytoplasm, vascular invasion, abundant psammoma bodies	2 cm, pT1N1MX	Cam 5.2, EMA, Vimentin-; AE1/3 focal +	+++
2	9/F	t(X;17)-like: voluminous cytoplasm, abundant psammoma bodies	5.2 cm, pT1N0MX	Cam 5.2, EMA, Vimentin, S100, Desmin-; AE1/3 focal +	++
3	10/F	t(X;17)-like: voluminous cytoplasm, abundant psammoma bodies	2.9 cm, pT1N1MX	Cam 5.2-, CK7-; Vimentin ±; EMA focal +	+++
4	8/M	t(X;17)-like: voluminous cytoplasm, abundant psammoma bodies, vascular invasion	3 cm, pT2N1MX	Cam 5.2 and EMA-; RCC Marker+, AE1/3 and CK7 + in single cells, S100-, Vimentin patchy	+++
5	8/F	t(X;17)-like: voluminous cytoplasm, psammoma bodies, vascular invasion, but true papillary areas too	10 cm, pT2N0MX	Cam 5.2, AE1/3, EMA, Vimentin–	++
6	8/F	t(X;1)-like: compact, clear cells and papillary architecture; rare psammoma bodies	13 cm, pT2N1MX	Cam 5.2+ focal; RCC+, Vimentin patchy, AE1/3-, EMA-	++
7	10/F	t(X;1)-like: compact, eosinophilic and clear cells with nested architecture, no psammoma bodies	Unknown (biopsy only)	RCC Marker+, EMA+, AE1/3-	+++
8	6/F	t(X;1)-like: compact; clear cells with nested architecture, moderate psammoma bodies	4 cm, pT1NXM0	Cam5.2, EMA, CK7, EMA patch, +	+

protein is usually not detected by IHC, presumably because the levels are below the limits of assay detection in archival tissue. The half-life of transcription factors is often short and tightly regulated, and this may contribute to poor nuclear immunoreactivity.[30] The mechanism of TFE3 nuclear overexpression in tumors associated with Xp11.2 translocations remains to be determined. One possible mechanism is that the immunoreactivity seen represents overexpression of the TFE3 fusion protein relative to native TFE3, likely because the TFE3 fusion protein is now expressed from a different promoter. The consistency of this finding among tumors with different TFE3 fusion partners [ASPL in soft tissue ASPS and t(X;17) renal carcinomas, PRCC in t(X;1)(p11.2;q21) renal carcinomas, PSF in t(X;1)(p11.2;p34) renal carcinomas] suggests that fusion protein overexpression may be a common oncogenic mechanism in these neoplasms. Other possibilities are that the fusion protein degradation is impaired, that the region bound by our TFE3 polyclonal antibody somehow becomes more accessible in the context of these fusion proteins, or that native TFE3 synthesis is somehow upregulated in these neoplasms. The last possibility seems unlikely in that we were unable to detect TFE3 immunoreactivity in these tumors using an antibody to the N-terminal portion of TFE3 (B. Hutchinson, M. Ladanyi, unpublished data).

TABLE 3. *Sensitivity and specificity of TFE3 IHC assay*

TFE3 nuclear immunoreactivity	Tumors with known Xp11.2 translocations and/or *TFE3* fusion transcripts (Positive Control Cases)	Tumors not known or suspected to harbor *TFE3* gene fusions (screening cases)
Positive (2+, 3+)	39	6
Negative (0+, 1+)	1	1470

Sensitivity = true positive/true positive + false negative = 97.5%.

Specificity = true negative/true negative + false positive = 99.6%.

Strong nuclear immunoreactivity for TFE3 protein proved to be highly sensitive and highly specific for neoplasms with TFE3 fusion proteins (Table 3). For scoring purposes, we considered only 2+ (moderate) or 3+ (strong) immunoreactivity to be positive. Weak to equivocal (1+) nuclear labeling was considered negative for aberrant TFE3 expression. Using these criteria, the sensitivity was 97.5% (39 of 40 tumors labeled), with the one negative case carrying the caveat that it did show 1+ (weak/equivocal) immunoreactivity and was fixed in Bouin's. It is well known that this acidic fixative may render specific antigens (particularly nuclear ones like TFE3) less detectable by IHC, and we suspect that this may have been significant here.[9] The specificity was 99.6%, as only 6 tumors of 1476 tested showed unexpected significant TFE3 immunoreactivity. Alternatively, if we had scored 1+ immunoreactivity as positive, the sensitivity would rise to 100%, but the specificity would fall to 99.1%. We chose not to score 1+ immunoreactivity as positive given the equivocal nature of immunoreactivity in most cases and to maximize the specificity of what was scored as a positive result for cases with *TFE3* gene fusions. Despite the high specificity and sensitivity of the TFE3 IHC assay for tumors containing *TFE3* gene fusions, it evident from the

rare 2+ or 3+ cases of other tumor types (Fig. 4) that TFE3 nuclear immunoreactivity is not diagnostic in and of itself but is merely a useful confirmatory marker, to be interpreted in the context of compatible histologic findings.

Several features of TFE3 immunoreactivity merit mention. First, three of the ASPS blocks that yielded strong immunoreactivity were >20 years of age, indicating that the antigen on TFE3 does not typically decay within formalin-fixed paraffin tissue blocks in this time period. Second, one of the slides that yielded a strong positive (3+) score from a *PSF-TFE3* carcinoma block was an unstained section cut over a year before and stored at room temperature. This result indicates that the TFE3 antigen does not necessarily decay upon oxidation over this time period, a significant limitation of IHC for many antigens.[22] Third, we consistently noticed stronger immunoreactivity at the edges of intact sections of these tumors than in the center, suggesting that better antigen preservation from more complete fixation at the periphery of the tissue pieces may have enhanced labeling. This has recently been described for another nuclear protein, p27Kip1.[15] An alternative hypothesis is that TFE3 expression is more intense at the tumor edge, possibly due to differences in oxygenation.[8] Thus, although this assay is highly sensitive for tumors associated with *TFE3* gene fusions, a negative result may not entirely exclude the diagnosis. If a given tumor with morphology typical of a tumor associated with a *TFE3* gene fusion fails to label, one should consider the possibility of technical issues such as fixation in Bouin's or inadequate sampling. If frozen tissue is available, one should consider performing molecular testing for the presence of a *TFE3* gene fusion. Nonetheless, such cases should, in our opinion, be reevaluated morphologically to exclude alternative diagnoses.

We suspect that detection of upregulated native TFE3 protein may have resulted in our few "false-positive" cases. This assertion is supported by the focality of immunoreactivity in some of these cases, by the absence of a consistent tumor type that labeled unexpectedly, and by the fact that still other cases showed equivocal weak labeling. These cases emphasize the need to optimize this assay on known positive and negative cases before applying it to clinical practice. Because TFE3 is ubiquitously expressed, an IHC technique that is too sensitive (i.e., due to excessive antigen retrieval, too high an antibody concentration, or excessive signal amplification) could lead to false-positive results. An analogy can be drawn to Her-2/neu labeling, where IHC assays are intended to detect overexpressed Her-2/neu and not native Her-2/neu produced by normal epithelia, but the oversensitivity of some IHC assays may blur this distinction and requires careful consideration of scoring issues.[21,39] Another possibility is that artifacts of fixation in these specific "false-positive" cases led to nonspecific staining reactions. We nonetheless performed additional PCR-based analyses to exclude the possibility of an unsuspected *TFE3* gene fusion in the most striking "false-positive" case: the adrenal cortical carcinoma that showed strong (3+) labeling. This was also the only one of the "false-positive" cases to have available frozen tissue for RNA extraction. RT-PCR analysis of this tumor RNA failed to demonstrate *PRCC-TFE3* or *ASPL-TFE3* fusion transcripts, and a PCR-based technique to identify the 5′ sequences fused to the 3′ portion of *TFE3* isolated only native *TFE3* sequences (M. Y. Lui, M. Ladanyi, unpublished data).

TFE3 immunoreactivity should prove to be useful in routine surgical pathology practice for confirming the diagnosis of ASPS. ASPS classically presents as a soft tissue mass in the extremities of a young adult and typically features sinusoidal capillaries that support dyscohesive, large, polygonal, epithelioid cells with vesicular chromatin and prominent nucleoli. Periodic acid–Schiff stain reveals needle-shaped cytoplasmic deposits that resist diastase digestion, which correspond to the characteristic membrane-bound, rhomboidal crystals that are seen on ultrastructural analysis.[16,29,33] In the usual setting, the diagnosis of ASPS is not difficult. However, the diagnosis is far more difficult in small samples, particularly because the characteristic crystals may not be found in limited material even if tissue is preserved for electron microscopy. When it presents in a visceral site, the diagnosis of ASPS is often not entertained at first, with the differential diagnosis focusing on more common histologic mimics that feature an alveolar pattern such as alveolar rhabdomyosarcoma, renal cell carcinoma, paraganglioma, granular cell tumor, and melanoma. Finally, ASPS may easily be dismissed as a histiocytosis or a granular cell tumor in the pediatric age group, where tumors often involve the head and neck region and have a more compact architecture.[16] All of these clinical settings might benefit from a specific, positive IHC marker of ASPS; TFE3 immunoreactivity fills this void nicely. We have shown that the tumors in the differential diagnosis of ASPS (with the exception of occasional granular cell tumors) are almost always negative for TFE3, which emphasizes the specificity of the assay. Finally, the assay detected both pediatric and adult tumors in this study, indicating that unusual morphologies of ASPS or unusual presentations of this neoplasm are unlikely to react differently.

TFE3 IHC also has several immediate applications to differential diagnoses in renal neoplasia. In the pediatric kidney, the distinction of clear cell sarcoma of the kidney (CCSK) from Xp11.2-related renal cell carcinomas may sometimes be problematic, particularly when the characteristic fine chromatin of CCSK nuclei is obscured by suboptimal fixation.[4] Cytokeratin labeling, which is con-

sistently negative in CCSK, may be negative in Xp11.2-associated renal carcinomas, further confounding matters.[1,2] As CCSKs are treated effectively with intensive chemotherapy regimens that include doxorubicin[4] and renal cell carcinomas may respond to interferons,[20,35] this distinction has major therapeutic implications. The CCSKs we tested in this study, as well as all other pediatric renal tumors, were completely negative for TFE3, establishing the utility of the assay in this setting. Another application is in the distinction of adult conventional (clear cell) renal carcinomas from the Xp11.2-related renal carcinomas, which they resemble morphologically. All adult-type RCCs were negative for TFE3 in this study. This distinction may prove to have therapeutic implications. Given their different underlying genetic alterations, Xp11.2-related carcinomas may not be responsive to the treatments currently given to patients with advanced-stage conventional renal cell carcinoma (including interferons), although this remains an open question at this time because too few confirmed Xp11.2 carcinomas have been studied. Only through the identification of larger numbers of Xp11.2-associated carcinomas can these tumors be studied in a meaningful way so that agents specifically active against them can be developed.

Finally, TFE3 IHC may finally allow the clarification of the prevalence of these tumors in adults and children. Rare t(X;1)-associated renal carcinomas have been reported in adults in their 60s.[1] Additionally, we have recently seen a genetically confirmed *ASPL-TFE3* carcinoma associated with the t(X;17) in a 68-year-old woman and tumors morphologically consistent with *ASPL-TFE3* renal carcinoma in a 38-year-old man and a 39-year-old woman, all three of which labeled for TFE3 protein, indicating that this neoplasm may also rarely occur in adults (P. Argani, M. Ladanyi, unpublished data). However, the fact that all of the unselected adult conventional-type (clear cell) renal cell carcinomas (which most closely resemble these tumors morphologically) and unclassified adult renal cell carcinomas in this study were negative for TFE3 protein suggests that the prevalence is quite low in adults. In contrast, we think that the proportion of Xp11.2 carcinomas is higher than previously thought among pediatric renal carcinomas, based upon the group of "test cases" in the present study and a review of a large number of other unselected consultation cases. The "test cases" group in the present study was not designed to be random and indeed was likely biased toward Xp11.2 carcinomas given our interest and ongoing work in this area. It is therefore unlikely that Xp11.2 carcinomas comprise the majority of pediatric renal carcinomas as the test group results might suggest. Nonetheless, other recent studies of pediatric renal cell carcinoma suggest that the prevalence is significant. For instance, Renshaw et al.[36] identified four "voluminous cell tumors" in a series of 24 pediatric renal carcinomas; the morphologic description of these tumors (voluminous cytoplasm, abundant psammoma bodies, minimal cytokeratin immunoreactivity) is identical to the appearance of the t(X;17) renal carcinomas. Indeed, both of the two tumors from this subgroup that we subsequently tested proved to have the *ASPL-TFE3* gene fusion.[2] Hence, we suspect that t(X;17) renal carcinomas comprised 17% of pediatric renal cell carcinomas in that series. Because t(X;1) renal carcinomas are thought to be more common that the t(X;17) carcinomas, it is possible that the overall percentage of Xp11.2-associated carcinomas in that series may exceed 30%. Although these data are suggestive, only a large study of an unselected series of pediatric renal carcinomas can definitively address this issue. The availability of TFE3 IHC, as demonstrated in the present study, should facilitate studies of this question. □

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