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NKX3–1 Is a Useful Immunohistochemical Marker of *EWSR1-NFATC2* Sarcoma and Mesenchymal Chondrosarcoma

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Abstract

NK3 homeobox 1 (NKX3–1) is widely accepted as a highly sensitive and specific marker for prostatic adenocarcinoma. Prompted by published transcriptome data showing upregulation of *NKX3–1* mRNA expression in *EWSR1-NFATC2* sarcoma, we explored the utility of NKX3–1 immunohistochemistry in sarcoma diagnosis. We applied NKX3–1 immunohistochemistry to 11 *EWSR1-NFATC2* sarcomas and 168 mimics using whole tissue sections. All *EWSR1-NFATC2* sarcomas consisted of uniform small round or ovoid cells, all except one showing at least focally the typical growth pattern of nests, cords, or trabeculae within a fibrous/myxoid background. A variable eosinophilic infiltrate was common. NKX3–1 was expressed in 9 out of 11 (82%) *EWSR1-NFATC2* sarcomas, often diffuse and of a moderate or strong intensity. All 12 mesenchymal chondrosarcomas tested were also positive for NKX3–1, with over half showing

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Disclosure/Conflict of Interest

None declared.

diffuse staining and moderate or strong intensity. The positive staining was seen only in the primitive small round cell component, whereas the cartilaginous component was mostly negative. Although 1 of 30 osteosarcomas showed focal NKX3–1 positivity, all the remaining 155 cases tested, including 20 Ewing sarcomas, 20 myoepithelial tumors, 11 ossifying fibromyxoid tumors, and 1 *FUS-NFATC2* sarcoma were negative for NKX3–1. Our study provides the first evidence that *EWSR1-NFATC2* sarcoma and Ewing sarcoma could be distinguished immunohistochemically, adding to the accumulating data that these tumors are phenotypically distinct. We suggest that NKX3–1 may have a diagnostic utility in the evaluation of sarcoma and we also call attention to potential pitfalls in the use of this well-known marker of prostatic adenocarcinoma.

Keywords

Sarcoma; *EWSR1-NFATC2*; Immunohistochemistry; mesenchymal chondrosarcoma; NKX3–1

Introduction

Ewing sarcoma is a prototypical small round cell sarcoma. Immunohistochemically, Ewing sarcomas are consistently positive for CD99, with most examples also being positive for NKX2–2 and PAX7.[1, 2] It is genetically defined by specific gene fusions involving either *EWSR1* or *FUS* partnering one of the *ETS* transcription family genes (e.g. *FLI1* or *ERG*). Small round cell sarcomas that resemble Ewing sarcoma to some degree, yet lacking a canonical *EWSR1*/*FUS-ETS* fusion, have been colloquially referred to as “Ewing-like sarcoma”.

Recent advancements in molecular genetic studies have disclosed that such “Ewing-like sarcoma” represents a heterogeneous group including multiple separate tumor entities. The best-established subsets are sarcoma with *CIC* rearrangements and sarcoma with *BCOR* gene abnormalities, each of which is associated with distinct clinicopathological and molecular features.[3] Less well studied is a group characterized by the gene fusion between *EWSR1* and non-*ETS* genes, with the reported partners including *NFATC2*, *PATZ1*, *SP3*, and *SMARCA5*.[3–5] *EWSR1-NFATC2* sarcoma is the best characterized among them. Since its discovery by Szuhai et al,[6] single reports and small series (up to 6 cases) have been documented, now accumulating more than 30 cases.[1–3, 6–17] The literature suggests that *EWSR1-NFATC2* sarcoma occurs more commonly in bone than in soft tissue of adult patients with a peak incidence of third to fourth decades and a male predilection.[9] Most *EWSR1-NFATC2* sarcomas share with Ewing sarcomas uniform small round cell cytology and frequent co-expression of CD99, NKX2–2, and PAX7,[1, 2, 13] but unlike Ewing sarcoma they often form nests, cords or trabeculae in a fibrous or myxoid stroma, mimicking myoepithelial tumors.[7–10, 12, 13, 15, 17] Some data suggest that *EWSR1-NFATC2* sarcoma follows a more indolent clinical course than Ewing sarcoma,[7–9, 13] despite poor responsiveness to Ewing-type chemotherapy regimens.[9, 13] Overall, despite controversy,[18, 19] distinctive histology, mRNA expression and methylation analysis suggest that *EWSR1-NFATC2* sarcoma represents a separate entity from Ewing sarcoma.[14, 17, 20]

Diagnosing *EWSR1-NFATC2* sarcoma is primarily based on molecular identification of the fusion gene. One useful proxy is the amplification of the *EWSR1* 5' sequence, which is consistently present when tested using *EWSR1* break-apart fluorescence in situ hybridization (FISH) assays.[6, 10, 13] However, no immunohistochemical markers have been developed that facilitate differential diagnosis. A recent large transcriptome study by Watson et al identified *NKX3–1* as one of the most differentially upregulated genes in *EWSR1-NFATC2* sarcoma compared to other selected types of sarcomas.[14] NK3 homeobox 1 (NKX3–1, also known as NKX3.1) is a transcription factor that is essential for prostate development, being expressed in normal prostatic epithelium as well as Sertoli cells and mucous cells in salivary and bronchial glands.[2122–26] NKX3–1 is widely accepted as a highly sensitive and specific marker for prostatic adenocarcinoma,[26–28] and outside the prostate, only a subset of breast carcinomas, tracheal mucinous adenocarcinoma, and rare tumors in the testis and ovary have reportedly expressed this marker.[21, 24, 26, 29] The expression of NKX3–1 in sarcomas has not been extensively studied, except for a tissue microarray analysis by Gelmann et al, in which none of the selected types of soft tissue sarcomas was positive.[24] The goal of this study is 1) to clarify the NKX3–1 expression status in *EWSR1-NFATC2* sarcoma; and 2) to explore the utility of NKX3–1 immunohistochemistry in the differential diagnosis of small round cell sarcomas.

Materials and Methods

The study was approved by the institutional review board. We retrieved 11 *EWSR1-NFATC2* sarcoma samples from departmental and consultation archives. *EWSR1-NFATC2* fusion was previously confirmed by RNA sequencing and/or FISH assay for each case. In addition, we retrieved samples for "control" tumors, which were felt to mimic *EWSR1-NFATC2* sarcoma. After the observation of consistent NKX3–1 expression in mesenchymal chondrosarcoma (see Results), conventional osteosarcoma and chondrosarcoma cases were also included. We also tested a single sarcoma sample with *FUS-NFATC2* fusion, a potentially related fusion gene to *EWSR1-NFATC2*. Adamantinoma was further included, because one DNA methylation study suggested a similarity between these tumors.[30] The final comparison cohort comprised 1 *FUS-NFATC2* sarcoma, 20 Ewing sarcomas (18 tumors with *EWSR1-FLI1* fusion and 2 tumors with *EWSR1-ERG* fusion), 20 myoepithelial tumors (including 4 tumors with *EWSR1* fusion with non-*NFATC2* partners and 12 tumors tested negative for *EWSR1* rearrangement; the remaining 4 tumors molecularly uncharacterized expressed S100 protein), 11 ossifying fibromyxoid tumors (OFMT, including 5 tumors with *PHF1* rearrangement), 10 *CIC*-rearranged sarcomas, 10 *BCOR-CCNB3* sarcomas, 10 poorly-differentiated synovial sarcomas (all tumors harbored *SS18* rearrangement), 8 extraskeletal myxoid chondrosarcomas (all tumors harbored *NR4A3* rearrangement), 8 sclerosing epithelioid fibrosarcomas (all expressed MUC4; 4 tumors with *EWSR1* rearrangement and 3 tumors with *FUS* rearrangement; 1 tumor failed to hybridize), 5 alveolar rhabdomyosarcomas, 5 spindle cell/sclerosing rhabdomyosarcomas, 5 desmoplastic small round cell tumors (all showed *WT1* rearrangement and/or WT1 C-term immunoreactivity), 5 small cell carcinomas of the lung, 12 mesenchymal chondrosarcomas (all tumors harbored *NCOA2* rearrangement), 30 osteosarcomas including 5 small-cell variant, 5 conventional chondrosarcomas, and 3 adamantinomas.

A representative paraffin section from each case was immunostained with an anti-NKX3–1 polyclonal antibody (dilution 1:500; Athena Enzyme Systems, Baltimore, MD, USA). This is the same antibody as was well characterized and used in previous studies.[21, 22, 26, 31] Antigen retrieval was performed in citrate buffer (pH 6.0) using an autoclave (121°C, 10 min). The slides were incubated with the primary antibody for 1h and subsequently labeled using the EnVision system (Dako, Glostrup, Denmark). Nuclear staining was considered as positive. The intensity of staining was graded as weak, moderate, or strong. When staining intensity was heterogeneous, the greatest intensity was recorded. The extent of staining was scored as negative (0% or <5% of cells positive), focal (5–50% of cells positive), and diffuse (>50% of cells positive). A metastatic prostatic adenocarcinoma was used as a positive control. In addition, during the process of validating antibody specificity we confirmed the previously reported observation that NKX3–1 expression in human adult tissue is restricted to prostatic luminal cells, testicular Sertoli cells, and respiratory and salivary mucous cells.[21, 24, 26]

Results

Summary of EWSR1-NFATC2 sarcomas and FUS-NFATC2 sarcoma

Clinicopathological summary of *EWSR1-NFATC2* sarcomas is provided in Table 1. Eleven tumors occurred in 7 men and 4 women with a median age of 39 years old (range, 27–78 years), arising from bone (N = 6) or soft tissues (N = 5). All *EWSR1-NFATC2* sarcomas histologically consisted of uniform small round or oval cell proliferation, 10 of which showed a known characteristic growth pattern, at least focally, showing nests, cords, or trabeculae within a fibrotic, hyalinized, or myxoid background (Figures 1A-C, 2A, 2C, 2E). Uncommon findings included a pseudoacinar architecture (Figure 1C), diffuse growth pattern (Figure 1D), and necrosis. Many cases showed cytoplasmic clearing of tumor cells and variable degrees of eosinophilic infiltrate were common (Figure 1E). One soft tissue case demonstrated a previously undescribed peripheral cuff of mature bone, and this finding, along with reticular architecture of round cells, led to the original diagnosis of OFMT (Figure 1F). Four of 4 cases previously tested co-expressed CD99, NKX2–2, and PAX7.

The single *FUS-NFATC2* sarcoma occurred in the femoral diaphysis of a 49-year-old man with soft tissue extension, and histologically showed dense proliferation of uniform oval cells without a cord or nested pattern (Figure 3A). The tumor morphology changed in the post-chemotherapy resection specimen to intersecting fascicular proliferation of anaplastic spindle cells (Figure 3B). No myxoid or myxohyaline matrix suggestive of chondroid differentiation was observed, unlike some reported cases,[14] and the tumor was negative for CD99 and NKX2–2. The patient died of disease a year and a half after the diagnosis with lung and brain metastasis.

NKX3–1 immunohistochemistry

The results are summarized in Table 2. NKX3–1 was expressed in 9 out of 11 (82%) *EWSR1-NFATC2* sarcomas (Table 1, Figure 2). The staining was observed in 20–90% of tumor cells, categorized as diffuse in 7 tumors and focal in 2 tumors. Its intensity was strong in 6 tumors, moderate in 2 tumors, and weak in 1 tumor. One *FUS-NFATC2* sarcoma was

negative for NKX3–1 (Figure 3C). Among other tumors, 12 out of 12 (100%) mesenchymal chondrosarcomas were positive for NKX3–1 (Figure 4). Only the primitive small round cell component showed NKX3–1 positivity, whereas the cartilaginous component stained negative or only faintly in a few cells. The staining was observed in 20–90% of primitive tumor cells, categorized as diffuse in 7 tumors and focal in 5 tumors, and its intensity was strong in 4 tumors, moderate in 7 tumors, and weak in 1 tumor. One of 30 osteosarcomas (an osteoblastic osteosarcoma with a non-small-cell pattern) showed focal (~10%) NKX3–1-positive pleomorphic spindle cells (Figure 5). All the remaining 155 tumors, including Ewing sarcoma, myoepithelial tumor, and OFMT were negative for NKX3–1 (Figure 6). One each of osteosarcoma, desmoplastic small round cell tumor and myoepithelioma showed < 5% of positive cells in a weak or moderate intensity, which were categorized as negative. There was no evidence of NKX3–1 expression in normal or reactive connective tissues.

Discussion

EWSR1-NFATC2 sarcoma shares with classic Ewing sarcoma the small round cell cytology and co-expression of CD99, NKX2–2, and PAX7.[1, 2] This has led to some controversy as to whether *EWSR1-NFATC2* sarcoma should be considered a subtype of Ewing sarcoma.[18, 19] However, accumulating evidence from case reports and small series suggest that *EWSR1-NFATC2* sarcomas demonstrate a distinct histological architecture from Ewing sarcoma, comprising cords, nests, or trabeculae in a fibromyxoid background,[7–10, 12, 13, 17] and these patterns were indeed observed, at least focally, in 10 out of 11 cases that we examined. A variable degree of eosinophilic infiltrate was also characteristic of this tumor, as described previously.[10, 17] Recent transcriptome and methylation analyses have further suggested their distinctiveness from Ewing sarcoma.[14, 17, 20, 30] The present study now provided the first evidence that these tumors can also be distinguished immunohistochemically. Over 80% of *EWSR1-NFATC2* sarcomas expressed NKX3–1 mostly with a moderate or strong intensity, whereas none of the Ewing sarcomas, with either *EWSR1-FLI1* or *EWSR1-ERG* fusions, expressed NKX3–1. The result is in agreement with a study showing significant *NKX3–1* upregulation in *EWSR1-NFATC2* sarcoma.[14]

NKX3–1 was not expressed in most other tumor types. It is particularly important that all myoepithelial tumors we tested, one of the closest histological mimics of *EWSR1-NFATC2* sarcoma, showed negative reactivity. Along with S100 protein, which is often expressed in myoepithelioma but not expressed in *EWSR1-NFATC2* sarcoma, NKX3–1 staining can thus help this separation. One of the *EWSR1-NFATC2* sarcomas arising in soft tissue was originally interpreted as OFMT, because of the peripheral cuff of mature bone. NKX3–1 was negative in all OFMTs tested, suggesting application to this distinction as well. As a corollary, NKX3–1 staining could be used as a rapid screening method to identify *EWSR1-NFATC2* sarcomas among the archival tumor samples that originally received a diagnosis of Ewing sarcoma, myoepithelioma, or OFMT. A single *FUS-NFATC2* sarcoma showed no nested or corded pattern and was negative for CD99, NKX2–2, and NKX3–1. Only a small number of these tumors have been well characterized,[7, 9, 14] and they showed transcriptome profiles distinct from *EWSR1-NFATC2* sarcomas in one study.[14] Two *EWSR1-NFATC2* sarcomas were negative for NKX3–1. Although the reason for this is unclear, these 2

samples were unstained FFPE sections that were stored for some periods and such pre-analytical conditions may have contributed to some extent.

We unexpectedly observed that all mesenchymal chondrosarcoma samples tested showed NKX3–1 expression. The expression was restricted to the primitive component, whereas the cartilaginous element was mostly negative. In mice, *Nkx3.1* is transiently expressed in the sclerotome and plays a coordinated role with *Nkx3.2* in the development of ventral vertebra by regulating spatial organization and differentiation of chondrogenic cells.[32, 33] Contrasting NKX3–1 expression between two components of mesenchymal chondrosarcoma may reflect its early-stage specific role in chondrogenesis, because mesenchymal chondrosarcoma has been hypothesized to recapitulate chondrogenic developmental processes.[34] Mesenchymal chondrosarcoma occasionally shows arborizing perivascular collagenization that compartmentalizes small round tumor cells, which may impart some similarity to *EWSR1-NFATC2* sarcoma, but at least focal chondroid differentiation, hemangiopericytomatous vascularity, and coarse granular chromatin pattern should enable distinction in most instances. Frequent expression of CD99 and NKX2–2 in mesenchymal chondrosarcoma could be a potential pitfall,[35, 36] but it consistently lacks PAX7 expression unlike *EWSR1-NFATC2* sarcoma.[2] Our data also suggest that NKX3–1 staining may be useful to distinguish mesenchymal chondrosarcoma from poorly-differentiated synovial sarcoma and osteosarcoma.

NKX3–1 is an accepted marker of prostatic adenocarcinoma, and is widely recommended in evaluating a cancer of unknown primary origin.[26–28] Although mesenchymal chondrosarcomas are unlikely to be confused with metastatic carcinoma, some *EWSR1-NFATC2* sarcomas exhibit nested growth of cells in fibrotic stroma, which could potentially invite confusion with carcinoma (see Figure 2C). Although *EWSR1-NFATC2* sarcoma has never formed true glands, pseudoacinar structure has been uncommonly encountered (see Figure 1C). *EWSR1-NFATC2* sarcoma also commonly expresses cytokeratin, either focally or diffusely, but it is often dot-like,[9, 12, 13] in contrast to diffuse and solid cytoplasmic keratin expression in carcinoma. In rare difficult situations, other prostatic markers such as prostatic specific antigen (PSA) can be helpful, although PSA expression may be lost in poorly-differentiated carcinoma or at metastatic sites.[27, 37, 38] Notably, small cell carcinoma of the prostate may present as metastasis to the viscera or bone, and it may reportedly express NKX3–1 in up to 18% of cases,[31, 39] representing a potential pitfall. In addition, a small subset of breast carcinoma has been shown to express NKX3–1, with invasive lobular type having the highest reported rate (25–28%) of expression.[24, 26] Attention to clinical history should be adequate for discrimination, but careful histological observation (e.g., intracytoplasmic lumina and stromal properties) and other marker statuses (e.g., keratin expression pattern, NKX2–2, and PAX7) would help when difficulty might arise.

In summary, the present study demonstrated that NKX3–1 was frequently expressed in *EWSR1-NFATC2* sarcoma and mesenchymal chondrosarcoma, in contrast to most other histological mimics. Our study provides the first evidence that *EWSR1-FNATC2* sarcoma and Ewing sarcoma could be distinguished immunohistochemically, adding to the growing data that these tumors are phenotypically distinct. We suggest that NKX3–1 may have a

diagnostic utility in the evaluation of small round cell sarcoma and we also call attention to potential pitfalls in the use of this well-accepted marker of prostatic adenocarcinoma.

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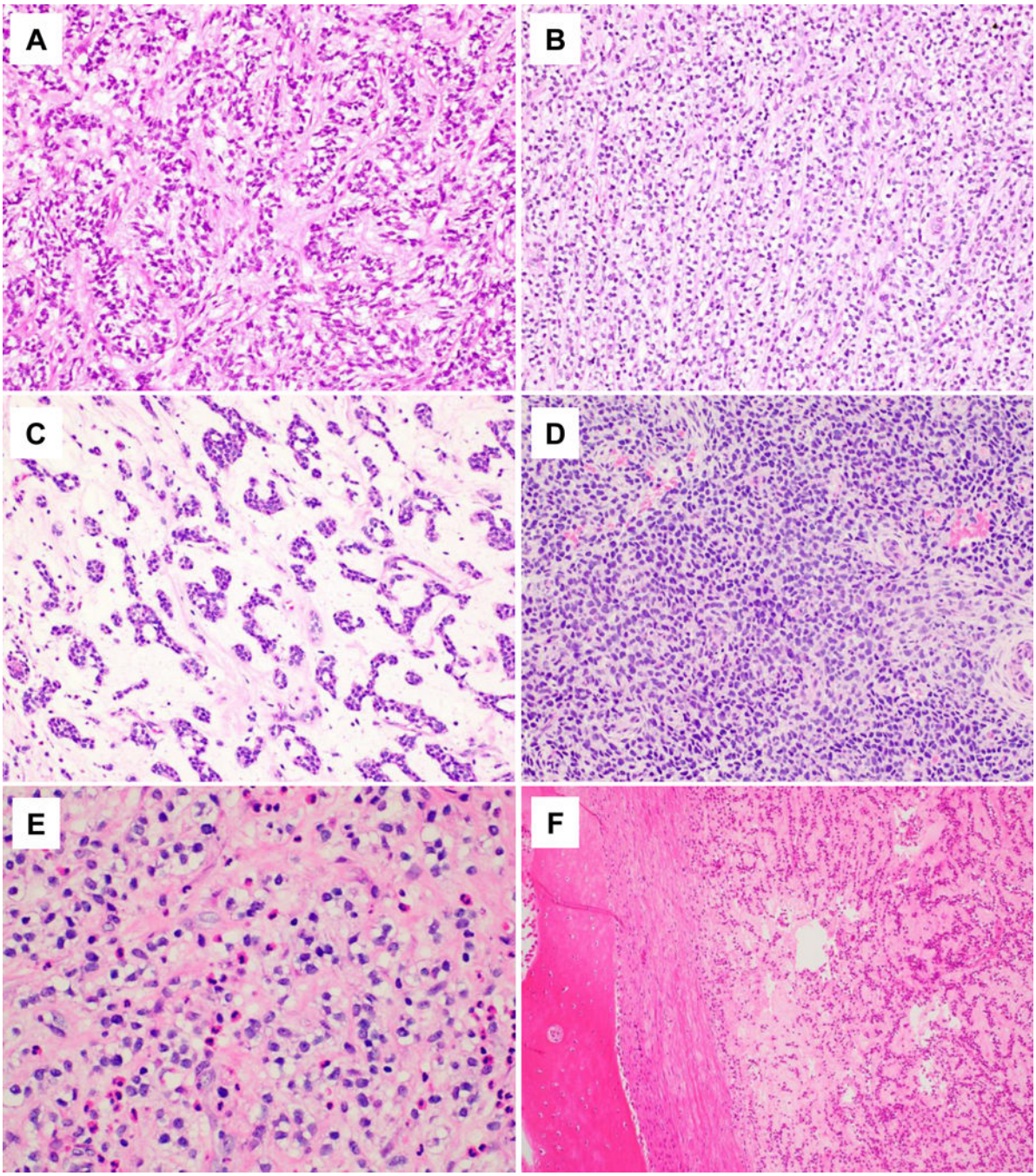

Figure 1.

Most *EWSR1-NFATC2* sarcomas showed a characteristic growth pattern, at least focally, showing nests, cords, and/or trabeculae within a fibromyxoid or sclerotic background (**A**, case 10**; B**, case 5). Uncommon findings included a pseudoacinar pattern (**C**, case 5) and diffuse proliferation (**D**, case 3). A variable degree of eosinophilic infiltrate in tumor tissue was common (**E**, case 5). One soft-tissue case demonstrated a peripheral cuff of mature bone closely simulating OFMTs (**F**, case 8).

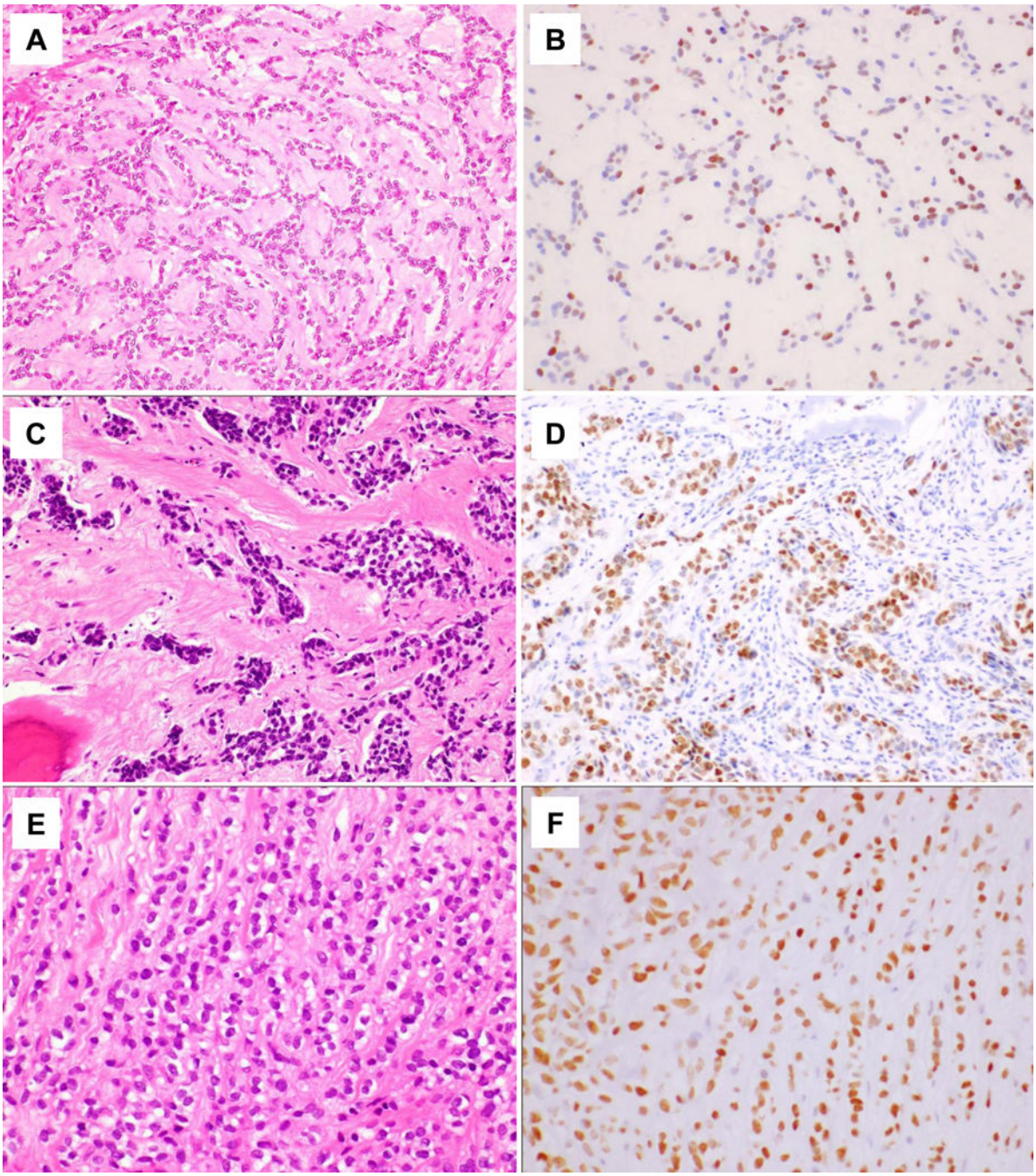

Figure 2.

NKX3–1 was expressed in 9 out of 11 *EWSR1-NFATC2* sarcomas. **B**, **D**, and **F** show positive NKX3–1 immunoreactivity for **A** (case 8), **C** (case 11), and **E** (case 9), respectively.

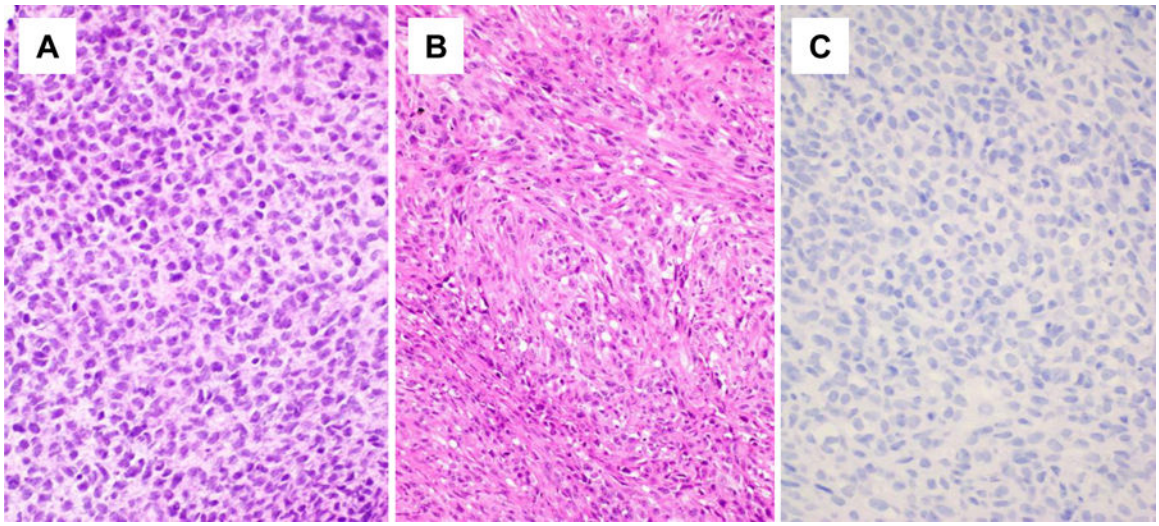

Figure 3.

One *FUS-NFATC2* sarcoma showed diffuse oval cell proliferation in biopsy (**A**) and fascicular proliferation of anaplastic spindle cells in post-chemotherapy resection (**B**). The tumor was negative for NKX3–1 (**C**).

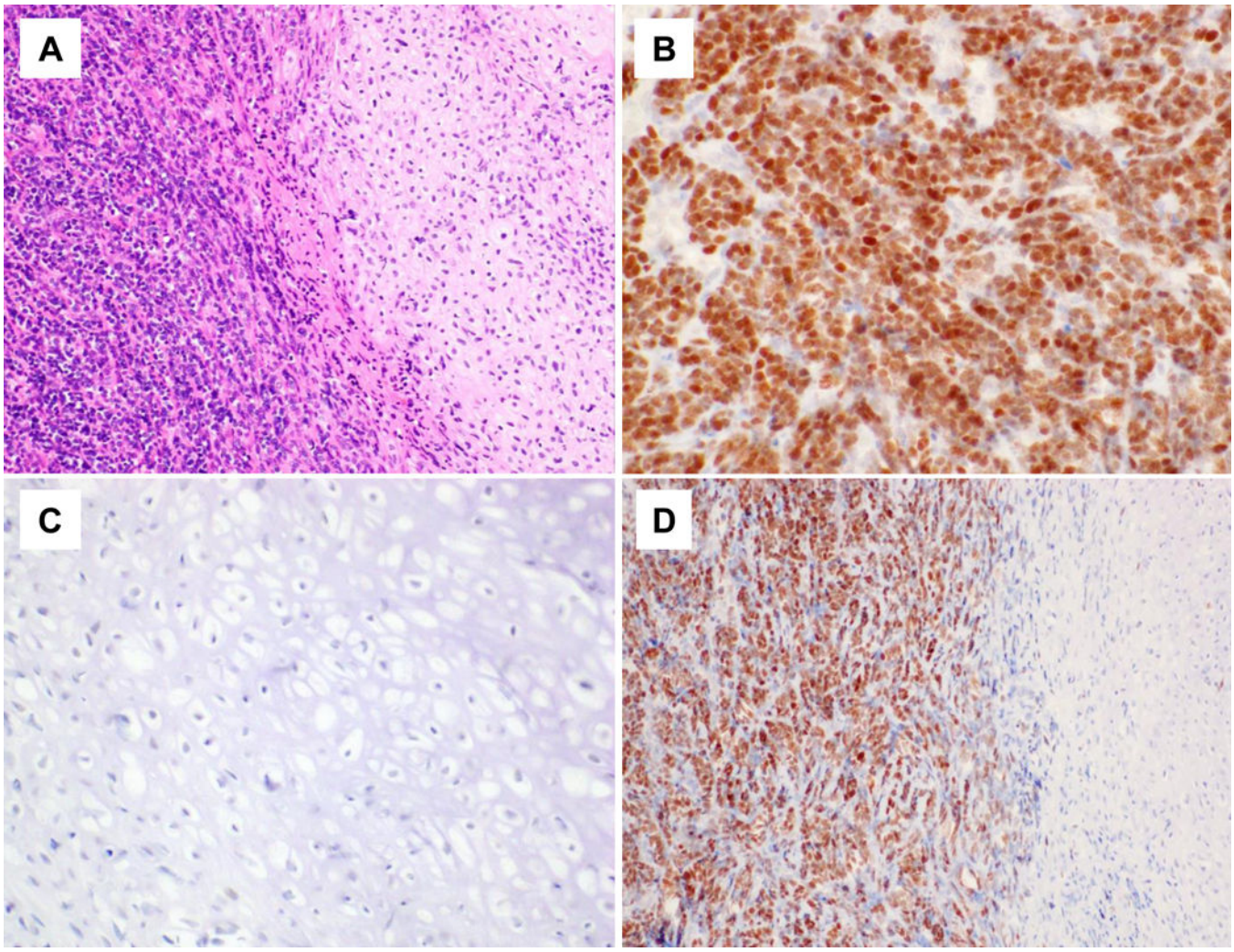

Figure 4.

All 12 mesenchymal chondrosarcomas tested were positive for NKX3–1. **A**. shows the classic biphasic histology of mesenchymal chondrosarcoma. Only the primitive small round cell component showed NKX3–1 reactivity (**B**), whereas the cartilaginous component stained negative (**C**) or only faintly in a few cells. The staining interface between the two components was relatively sharp (**D**).

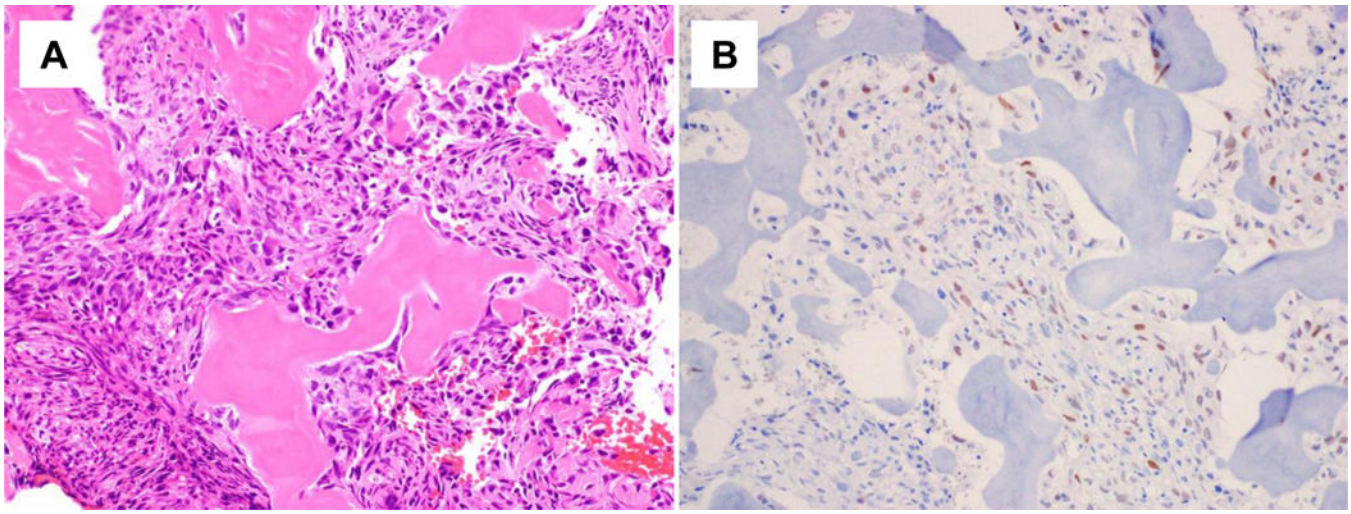

Figure 5.
One conventional osteoblastic osteosarcoma showed focal NKX3–1 staining in pleomorphic spindle cells.

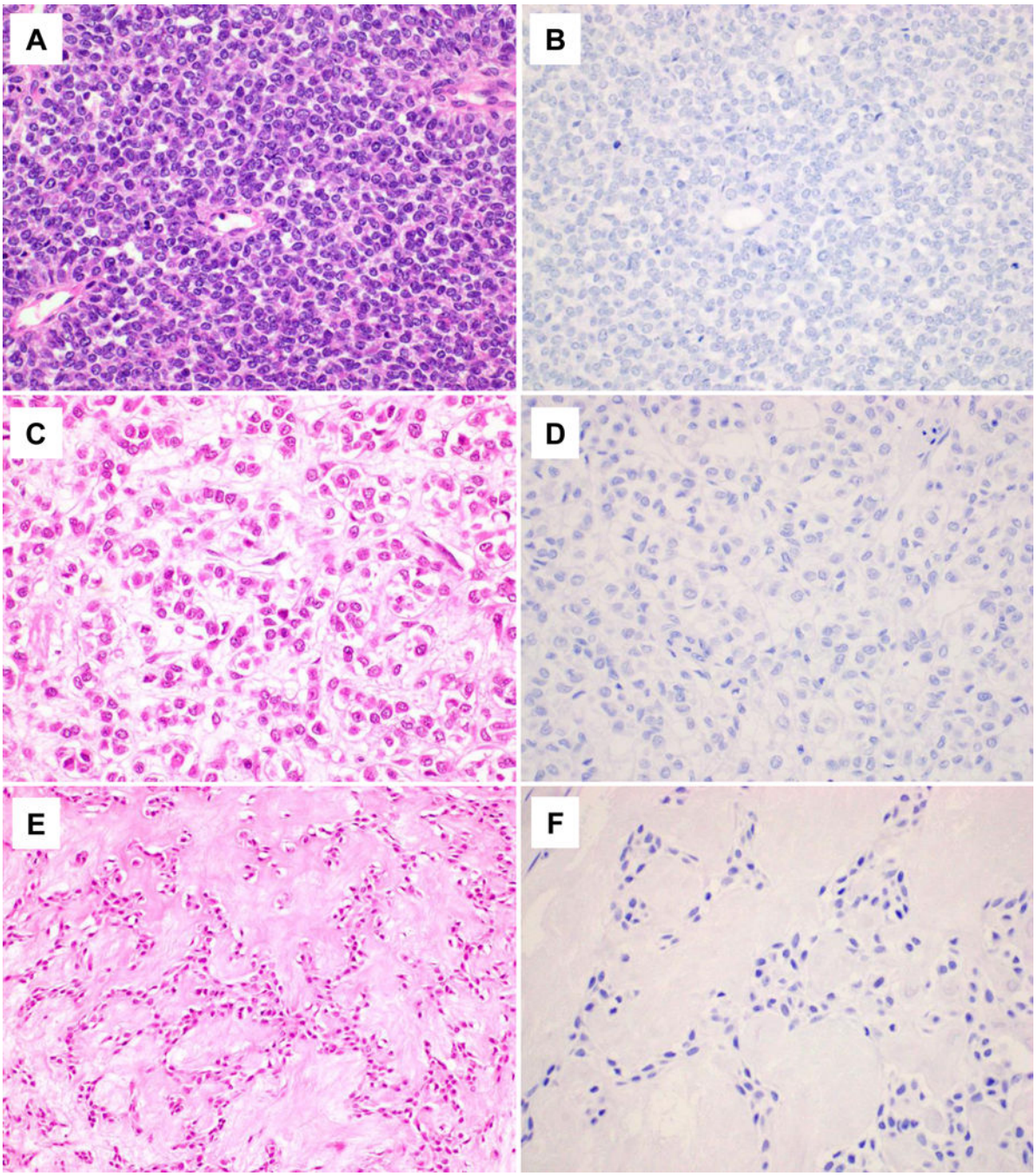

Figure 6.

NKX3–1 staining was negative in most mimics of *EWSR1-NFATC2* sarcoma; including all Ewing sarcomas (**A**, HE; **B**, NKX3–1), myoepithelial tumors (**C**, HE; **D**, NKX3–1; this case harbored the *EWSR1-POU5F1* fusion), and OFMTs (**E**, HE; **F**, NKX3–1).

Table 1.

Clinicopathological summary and NKX3–1 status of *EWSR1-NFATC2* sarcoma

Case	Age/Sex	Primary site	Cord/nest/trabecula pattern	NKX3–1 immunohistochemistry
1	39/M	Femur	Yes	Strong, 40%
2	46/M	Femur	Yes	Negative (0%)
3	27/M	Retroperitoneum	Yes	Moderate, 60%
4	31/F	Scapula	Yes	Negative (<5%)
5	36/M	Neck	Yes	Strong, 90%
6	51/M	Forearm	Yes	Weak, 60%
7	78/F	Tibia	No	Moderate, 20%
8	38/M	Thigh	Yes	Strong, 60%
9	44/M	Thigh	Yes	Strong, 90%
10	36/F	Fibula	Yes	Strong, 80%
11	62/F	Femur	Yes	Strong, 80%

Table 2.

Summary of NKX3–1 immunostaining results in tested tumors.

Tumor type	Positivity	Extent	Intensity
***EWSR1-NFATC2* sarcoma**	9/11 (82%)	F2, D7	W1, M2, S6
***FUS-NFATC2* sarcoma**	0/1 (0%)	—	—
Ewing sarcoma	0/20 (0%)	—	—
Myoepithelial tumor	0/20 (0%)	—	—
Ossifying fibromyxoid tumor	0/11 (0%)	—	—
***CIC*-rearranged sarcoma**	0/10 (0%)	—	—
***BCOR-CCNB3* sarcoma**	0/10 (0%)	—	—
Poorly-differentiated synovial sarcoma	0/10 (0%)	—	—
Extraskeletal myxoid chondrosarcoma	0/8 (0%)	—	
Sclerosing epithelioid fibrosarcoma	0/8 (0%)	—	—
Alveolar rhabdomyosarcoma	0/5 (0%)	—	—
Spindle cell/sclerosing rhabdomyosarcoma	0/5 (0%)	—	—
Desmoplastic small round cell tumor	0/5 (0%)	—	—
Small cell carcinoma	0/5 (0%)	—	—
Mesenchymal chondrosarcoma	12/12 (100%)	F5, D7(*)	W1, M7, S4
Osteosarcoma (including 5 small-cell type)	1/30 (3%)	F1	M1
Conventional chondrosarcoma	0/5 (0%)	—	—
Adamantinoma	0/3 (0%)	—	—

Reactivity was defined as positive if at least 5% of tumor cells were stained. Staining characteristics are indicated as follows: F, focal (5–50%); D, diffuse (>50%); W, weak; M, moderate; S, strong.

*In mesenchymal chondrosarcoma, positive tumor fraction was evaluated in primitive cell component only, excluding cartilaginous area.