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Original article

Immunohistochemical analysis of NKX2.2, ETV4, and BCOR in a large series of genetically confirmed Ewing sarcoma family of tumors

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ABSTRACT

Ewing sarcoma is an aggressive neoplasm of pediatric and adolescent patients. Immunohistochemistry (IHC) can be used to support the morphologic diagnosis of Ewing sarcoma family of tumors (ESFT) in a convincing clinical/radiological context. Although neither NKX2.2 nor CD99 alone are entirely specific, when combined, the diagnostic specificity is high. The aim of the present study was to investigate the IHC expression of NKX2.2, ETV4 and BCOR in a large series of genetically confirmed ESFT. The results for CD99 and CAV-1 immunoreactivity, and the histological and fusion gene subtypes were retrieved from our previous study. NKX2.2 demonstrated moderate or strong nuclear positivity in 91.2% of the tumors. The staining intensity was heterogeneous. Many of the ESFT with negative NKX2.2 immunoreactivity were in bone. Strong/moderate ETV4 nuclear expression was detected in two small round cell tumors, both were negative for NKX2.2. No relationships could be found between expression of NKX2.2 and the histological subgroups or ESFT gene fusion subtypes. BCOR was negative in all ESFT. In conclusion, NKX2.2, ETV4 and BCOR IHC may be helpful in daily practice for distinguishing ESFT from *CIC* or *BCOR*-associated sarcomas, especially in hospitals without access to molecular assays. In addition, the combination of strong CD99 membranous positivity and nuclear NKX2.2 positivity seems to be very reliable for ESFT diagnosis in an appropriate clinicoradiological setting. So far no antibody is entirely specific for ESFT diagnosis, and the IHC or molecular results in round cell tumors of bone may be strongly influenced by decalcification processes.

1. Introduction

Ewing sarcoma is an aggressive tumor that arises frequently in the long or flat bones, and less commonly in the soft tissues, of pediatric and adolescent patients [1–7]. Three histological variants (conventional/classic, primitive neuroectodermal tumor/PNET, and atypical) have previously been described in a large series of Ewing sarcoma family of tumors (ESFT) published by Llombart-Bosch et al. [4]. ESFT are almost always characterized by reciprocal translocations between *EWSR1* and genes of the *ETS* family of transcription factors, although they may occasionally reveal uncommon gene fusions (*EWSR1/non-ETS* or *FUS* rearrangement instead of *EWSR1*) [3–7].

At present, the demonstration of such translocations represent the "gold standard" in the diagnosis of ESFT, although the results depend strongly on fixation, age of tumor sample and, especially in bone tumors, decalcification [4–6]. Nevertheless, molecular studies in ESFT are not available in all Cancer Centers and are also not exempt from giving false positive or negative results. For instance, the *EWSR1* rearrangement is not exclusive to ESFT [1,2,4–7]. Furthermore, oncologists have only a short time in which to receive the definitive diagnosis of the biopsy, especially if neoadjuvant chemotherapy or further surgical procedures are indicated, and such molecular studies usually take longer than hematoxylin/eosin (H & E) and immunohistochemistry (IHC) analysis [4–7].

On the other hand, IHC can be used to support the morphologic diagnosis of ESFT in an otherwise convincing clinical/radiological context and specifically the widespread strong membranous CD99 positivity appears to be very sensitive in ESFT diagnosis [3–7]. CAV-1 and

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FLI-1 have also been tested in ESFT, both have been shown to be less sensitive than CD99 [1–7], although in our previously published study all CD99-negative ESFT were positive for CAV-1 [4]. It is well know that CD99, CAV-1 and FLI-1 may also be expressed in other round cell sarcomas of bone and soft tissue, thus lacking complete specificity in the diagnosis of ESFT [1,2,4–32].

In 2012, Yoshida et al. introduced NKX2.2, a well-recognized target of *EWSR1-FLI1*, as a diagnostically useful immunohistochemical marker of ESFT because it was expressed in 93% of their ESFTs and in only 11% of non-ESFT tumors tested [7]. Two years later Shibuya et al. reported a sensitivity of 80% and specificity of 84% for NKX2.2 expression in ESFT, and further demonstrated that the combination of NKX2.2 and CD99 improved the specificity to 98% with exceptional expression of both markers in only two non-ESFT tested [31]. In 2016, Hung et al. confirmed the utility of NKX2.2 by reproducing a high sensitivity (93%) and specificity (88%) for ESFT [33]. Despite the high sensitivity and specificity, all 3 studies agreed that NKX2.2 expression is not unique to ESFT; the non-ESFT tumors positive for NKX2.2 included mesenchymal chondrosarcomas (MCS), olfactory neuroblastomas, melanoma, small cell carcinomas [7,31,33], and a small subset of other round cell tumors such as *CIC*-rearranged sarcomas [33] or *BCOR-CCNB3* positive sarcomas [34]. In these previous studies on NKX2.2 expression in ESFT, at most only 50 ESFTs were tested [7,31,33]. Furthermore, ETV4 and BCOR, two recently introduced antibodies reported to support the diagnosis of *CIC*-rearranged sarcomas and *BCOR*-associated sarcomas respectively, have not previously been tested extensively in a large series of genetically confirmed ESFT [1,2,4,31–38,41]. *CIC*-rearranged sarcomas and *BCOR*-associated sarcomas represent at present two important differential diagnoses of ESFT arising in soft tissue or bone [3,5,32–41]. The purpose of the present study was to investigate the IHC expression of NKX2.2, ETV4, and BCOR in a large series of genetically confirmed ESFT, and assess their utility in combination with the currently used antibodies.

2. Material and methods

IHC studies for NKX2.2, ETV4, and BCOR were performed on 4-µm-thick formalin-fixed paraffin-embedded tissue microarray (TMA) sections. A total of 366 genetically confirmed ESFT (336 arising in bone and 30 in soft tissue) including in 12 TMAs were analyzed. Genetic confirmation was performed by *EWSR1* FISH analysis and/or RT-PCR assays as previously detailed [4]. Each TMA included two representative cores for each case (1 mm of diameter). Source, dilutions, and pretreatment conditions of each antibody are summarized in Table 1. Nuclear positivity was considering as informative for the three antibodies. NKX2.2 and BCOR positivity was defined as weak, moderate or strong nuclear immunoreactivity in at least 5% of tumor cells. The extent of NKX2.2 positivity was classified as focal (5% to 50%) or diffuse (> 50%).

ETV4 immunoreactivity was defined as positive when at least 30% nuclear staining was present with at least moderate intensity as recommended by Yoshida et al. [41]. In the case of an ETV4 IHC positive result a molecular analysis (RT-PCR) to search for a *CIC/DUX4* or *CIC/FOXO4* gene fusion was performed. Total RNA was extracted using the conventional protocol [42]. RNA was reverse-transcribed to cDNA using a high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA, USA). The PCR amplification was performed using AmpliTaq Gold 360 MasterMix (Applied Biosystems). The corresponding primer sets were applied to search for a *CIC/DUX4* or *CIC/FOXO4* gene fusion as previously reported [42]. Sequencing of the PCR products was performed [42].

All IHC sections were evaluated independently by three pathologists (IM, AY and ALLB). The scores by all observers were recorded, and in cases of disagreement, the score was determined by consensus. The staining conditions of NKX2.2, ETV4, and BCOR were previously validated at the National Cancer Center Hospital, Tokyo, by using sufficient number of ESFTs, *CIC*-rearranged sarcomas, and *BCOR*-associated sarcomas. *CIC*-rearranged sarcoma and *BCOR-CCNB3* sarcoma were used as positive controls for ETV4 and BCOR, respectively.

The results for CD99 and CAV-1 immunoreactivity, the histological and fusion gene subtypes were retrieved from the database of all the cases previously analyzed and published by Llombart-Bosch et al. [4] in the framework of the Prothets and EuroBoNet projects. All the cases included in the present study had also been analyzed in our previous study. Approval for data acquisition and analysis was obtained from the local Institutional Review Board at participating institutions.

3. Results

The results are summarized in Table 2. Among the 366 ESFT, the following histological subtypes were present: conventional Ewing sarcoma 237 (64.2%), PNET 51 (13.9%), atypical Ewing sarcoma 80 (21.8%). The atypical variant including those tumors with large/spindle and/or clear cells, vascular/hemangioendothelial-like, sclerosing or adamantinoma-like pattern. Of the 366 ESFT, 284 were evaluable for NKX2.2 immunostaining. The non-informative cases were due to tissue artifact, extensive necrosis and/or fibrosis, or absence of tumor cells in the core of each corresponding TMA section. In total, 91.2% of the tumors revealed moderate or strong nuclear positivity for NKX2.2 (Figs. 1 A–F, 2 A–F). Staining intensity was heterogeneous. Twenty-five ESFT were negative for NKX2.2 (22 arising in bone and 3 in soft tissue). Seven atypical ESFT lacked NKX2.2 immunoreactivity. Four ESFT with *EWSR1/ERG* fusion and 1 ESFT with *EWSR1/FEV* were included in the cohorts, and all were positive for NKX2.2.

Previous evaluation of CD99 and CAV-1 in the whole sections as well as in the TMA sections showed that CD99 was expressed in 99% of ESFT and CAV-1 in 95.2%. The four CD99-negative cases that expressed CAV-1 were in bone (data retrieved from previous publication) [4]. In the present study, almost all the negative NKX2.2 tumors also arose in bone (Fig. 1F) except for 3 cases arising in soft tissue.

The combined immunoreactivity results including CD99, CAV-1, NKX2.2 and ETV4 are listed in Table 3. BCOR was negative in 305 ESFT with informative IHC results.

Strong or moderate ETV4 nuclear expression in more than 30% of tumor cells was detected in only 2 tumors out of 291 informative cases. Both tumors revealed atypical morphology (oval/spindle focal cell formation, nuclear membrane irregularities and conspicuous nucleolus). Interestingly, both ETV4 positive cases were negative for NKX2.2 (Table 4 and Figs. 3 A–F and 4 A–F). No relationships could be found between NKX2.2 expression and the histological subgroups or

Table 1
Antibodies used for the immunohistochemical analysis.

Antibodies	Source	Clone	Dilution	System	Pretreatment condition
NKX2.2	Developmental Studies Hybrid- oma Bank, Iowa City, IA, USA	74.5A5	1:250	EnVision (Dako)	Autoclaved in citrate buffer
ETV4	Santa Cruz Biotechnology, Santa Cruz, CA, USA	16	1:50	EnVision with LINKER (Dako)	Autoclaved in Targeted Retrieval Solution pH9 (Dako)
BCOR	Santa Cruz Biotechnology, Santa Cruz, CA, USA	C-10	1:200	EnVision (Dako)	Autoclaved in citrate buffer