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Utility of a Monoclonal ERG/FLI1 Antibody for Immunohistochemical Discrimination of Ewing's Family Tumors

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Abstract

Ewing family tumors (EFTs) and prostate carcinomas (PCa) are characterized by rearrangement of ETS genes, most commonly *FLI1* (EFTs) and *ERG* (PCa). Previously, we characterized an antibody against ERG (EPR3864) for detecting *ERG*-rearranged PCa. EPR3864 also cross reacts with FLI1, thus, here we evaluated the utility of EPR3864 for discriminating EFTs from other small round blue cell tumors (SRBCTs) by immunohistochemistry. Of 57 evaluable EFTs, 47 (82%) demonstrated at least moderate, diffuse, nuclear ERG/FLI1 staining (including 89% and 100% of cases with confirmed *EWSR1:FLI1* and *EWSR1:ERG* fusions, respectively), of which 1, 3 and 43 showed negative, cytoplasmic or membranous CD99 staining, respectively. Amongst other SRBCTs (n=61 cases, 6 types), at least moderate, diffuse, nuclear EPR3864 staining was seen in all precursor-B-lymphoblastic lymphomas/leukemias and subsets of Burkitt's lymphomas (10%) and synovial sarcomas (45%). In summary, EPR3864 may have utility for detecting *EWSR1:FLI1* and *EWSR1:ERG* rearranged EFTs, in addition to PCa.

Keywords

EPR3864; EWSR1:FLI1; EWSR1:ERG; Ewing's tumor

Introduction

Ewing family tumors (EFTs), which encompass Ewing sarcomas/peripheral nueroectodermal tumors, are characterized by chromosomal rearrangements fusing *EWSR1* to members of the ETS transcription factor family. Although most commonly fused to the

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Conflict of interest:
The University of Michigan has been issued a patent on the detection of ETS gene fusions in prostate cancer, on which A.M.C. and S.A.T. are listed as co-inventors. The University of Michigan licensed the diagnostic field of use to Gen-Probe, Inc, which sublicensed rights to Ventana Medical Systems, Inc. Neither company played a role in data collection, interpretation or analysis, and did not participate in the study design, review of the manuscript or the decision to submit for publication. N.P. has served as a consultant for Ventana Medical Systems. A.M.C. has served as consultant to Gen-Probe, Inc. and Ventana Medical Systems. S.A.T. has received honoraria and served as a consultant to Ventana Medical Systems.

ETS gene *FLI1* (~90%) through t(11;22)(q24;q12), *EWSR1* can also fuse to *ERG* (~5–10%) and rarely *ETV1*, *FEV*, *ETV4* and *ETV5*[1–4]. EFTs and other small round blue cell tumors, including neuroblastomas, rhabdomyosarcomas, synovial sarcomas (poorly differentiated and monophasic variants), lymphoblastic lymphomas/leukemias, desmoplastic small round cell tumors and nephroblastomas (Wilm's tumors), can be morphologically indistinguishable and definitive diagnosis commonly involves immunohistochemistry, typically against CD99 and FLI1, and molecular tests[2,5–15].

CD99, also known as *MIC2*, encodes an integral membrane glycoprotein and shows diffuse membranous staining in >90% EFTs by immunohistochemistry using a variety of monoclonal antibodies (including 12E7, HBA71 and O13)[13,16–19]. Additionally, less specific cytoplasmic staining can also be observed. However, CD99 is not specific for EFTs, as it also stains lymphoblastic lymphomas/leukemias[19,20], anaplastic large cell lymphomas[21], synovial sarcomas[22,23], some rhabdomyosarcomas[24,25], as well as a variety of other tumors[26–30].

The *EWSR1:FLI1* gene fusions results in the fusion of the N-terminus of EWSR1 to the C-terminus of FLI1, which preserves the ETS DNA binding domain, and transforms NIH 3T3 cells[31,32]. FLI1 is normally expressed in endothelial and hematopoietic cells[5], and consistent with its role as a transcription factor, both FLI1 and the EWSR1:FLI1 product show nuclear localization[5,33]. Both polyclonal and monoclonal antibodies against FLI1 have been shown to have diagnostic utility in EFTs, with staining of 63–89% (median 81%)[5,6,9,10,34–36] and 75–100% (median 91%)[7–9,37,38] of EFTs, respectively. In addition to EFTs, both monoclonal and polyclonal antibodies against FLI1 have been reported to also stain vascular tumors, lymphoblastic lymphomas and Merkel cell carcinomas, as well as a fraction of other small round blue cell tumors including poorly differentiated synovial sarcomas, and other non-Hodgkin lymphomas[5–7,9,20,35,37,39]. Polyclonal antibodies against FLI1 have also been reported to stain at least some olfactory neuroblastomas, desmoplastic small round cell tumors, and a variety of carcinomas (but not prostate carcinomas)[6,35]. Similarly, monoclonal antibodies against FLI1 have been reported to stain haemangiopericytomas, neuroendocrine carcinomas, melanomas, lung adenocarcinoma, and a variety of normal tissues, including prostate, breast, and colon epithelium[7,9]. In the only head to head comparison we are aware of, Mhawech-Fauceglia *et al.* reported that monoclonal antibodies against FLI1 were more sensitive for EFTs, while polyclonal antibodies were more specific, consistent with other published studies (summarized above)[9].

Like EFTs, prostate carcinoma is characterized by chromosomal rearrangements involving ETS transcription factor family members, which are fused to the 5′ untranslated regions of androgen regulated genes and occur in approximately half of prostate carcinomas[40–42]. Fusions involving *ERG* (most commonly *TMPRSS2:ERG*) represent approximately 90% of all ETS fusions in prostate carcinoma, with less frequent fusions involving *ETV1*, *ETV4*, *ETV5* and one reported case involving *FLI1*[40–44]. Recently we and others have demonstrated the utility of a novel rabbit monoclonal antibody raised against the c-terminus of ERG (clone EPR3864), which demonstrates high sensitivity and specificity (>95%) for the detection of *ERG* rearranged prostate carcinoma[45–53]. As Mohamed *et al.* recently demonstrated that EPR3864 also reacts with exogenous FLI1 by Western blotting[54], we hypothesized that EPR3864 may also have utility in the discrimination of EFTs from other small round blue cell tumors. Thus, here we characterized EPR3864 staining of ERG/FLI1 by immunohistochemistry in the discrimination of EFTs.

Methods

EFTs and SRBCTs tissues

A tissue microarray was constructed using formalin-fixed paraffin-embedded blocks from 105 EFT cases (each case represented by triplicate cores) from 85 patients, which includes a mixture of primary diagnostic specimens, primary samples post chemotherapy, recurrences and metastases (Table 1), and includes multiple cases from 16 patients (range 2–4 cases). Single sections from one EFT case each from two additional patients, who did not have samples on the tissue microarray, were also evaluated and are included in the results. The tissue microarray also contained cores representing normal ovary, spleen, lung, spinal cord, colon, kidney, tonsil, liver and testes.

Single formalin-fixed paraffin-embedded sections from 61 other SRBCTs, which include 11 nephroblastomas (Wilm's tumors), 11 neuroblastomas, 7 rhabdomyosarcomas (4 alveolar, 2 embryonal, and 1 indeterminate, favor alveolar), 10 Burkitt's lymphomas, 4 desmoplastic small round cell tumors, 11 monophasic synovial sarcomas and 7 precursor-B-lymphoblastic lymphomas/leukemias, were also evaluated for ERG/FLI staining.

EFTs and small round blue cell tumors included a mixture of primary diagnostic specimens, primary samples post chemotherapy, recurrences and metastases. All cases were diagnosed at the University of Michigan Health Systems, with EFTs diagnosed based on characteristic morphology and immunohistochemistry staining, with some cases undergoing molecular confirmation (cytogenetics, fluorescence in situ hybridization and/or reverse transcription-PCR) as part of the diagnostic workup. Cases were also assessed for *EWSR1* breakapart by fluorescence in situ hybridization and reverse transcription PCR for *EWSR1:FLI1* and *EWSR1:ERG* if not performed as part of the diagnostic workup. Cases were considered molecularly confirmed (for *EWSR1:FLI1* or *EWSR1:ERG*) if two of the three tests (cytogenetics, fluorescence in situ hybridization and reverse transcription-PCR) were concordant. All tissues were obtained with prior Institutional Review Board approval.

Immunohistochemistry for ERG/FLI1 on the tissue microarray and single sections of EFTs and other SRBCTs was performed as described, using a ready-to-use, pre-diluted monoclonal antibody raised against ERG, clone EPR3864 (Ventana Medical Systems, Tucson, AZ)[45,53,55]. Staining of vessels was used as a positive control and cores or sections without staining of vessels were excluded from further analysis. Nuclear ERG/FLI1 staining intensity was scored as 0 (absent), 1+ (weak), 2+ (moderate) or 3+ (strong). Unless otherwise indicated, staining was diffuse (>80% of tumor). Immunohistochemistry for CD99 was performed on the tissue microarray using the rabbit monoclonal antibody EPR3097 (BioCare Medical, catalog #CME392), at 1:200 dilution for 30 min with Envision+ horseradish peroxidase detection. Epitope retrieval was performed using 10mM citrate buffer (pH 6) in a microwave for 10 min. Immunohistochemistry for CD99 was performed previously on the single sections of EFTs during the diagnostic workup and were re-reviewed. Staining for CD99 was scored as negative, cytoplasmic, or membranous. Unless otherwise indicated, staining was diffuse. EFT presence and viability, and ERG/FLI1 and CD99 staining, were evaluated by S.A.T, J.N.S. and L.P.K., with discrepancies resolved by D.R.L.

Analysis

EFT cases where no viable tumor was present in any of the three cores were excluded from further analysis. In cases where variable expression in two or more cores was observed, the greatest staining in any core was reported as the overall score and the variable expression was noted. Association between ERG/FLI1 and CD99 staining was evaluated using a two-tailed Fisher's exact test using GraphPad Prism v. 5 (GraphPad Software).

Results

55 EFT cases from 47 patients had at least one core with viable tumor and were evaluable for FLI1/ERG and CD99 staining (from a tissue microarray with 105 cases from 85 patients). Single formalin-fixed paraffin-embedded sections from 2 additional EFTs (from patients not represented on the tissue microarray) were evaluable for ERG/FLI1 staining as well as CD99 staining performed at diagnosis. Thus, in total, our final evaluable cohort consisted of 57 EFT cases from 49 patients, as summarized in Table 1. ERG/FLI1 staining was scored as strong (3+), moderate (2+), weak (1+) or negative (0), while CD99 staining was scored as membranous or cytoplasmic (both positive) or negative. Examples of ERG/FLI1 and CD99 staining are shown in Figure 1. Amongst control cores of normal tissue on the tissue microarray, normal spleen and tonsil showed 3+ ERG/FLI1 staining, while normal ovary, lung, spinal cord, colon, kidney, liver and testes were negative (0+).

Of the 55 evaluable cases on the tissue microarray, 54 (98%) showed homogenous ERG/FLI1 staining between evaluable cores, and thus all cases were scored based on the highest staining intensity. The primary case from patient #8 showed 2 cores with 3+ ERG/FLI1 staining, and one core with 1+ staining, while a metastatic lesion from this patient showed 3 cores with 3+ ERG/FLI1 staining (Figure 2). Of 6 additional patients with more than one evaluable case on the tissue microarray, 2 showed different ERG/FLI1 staining intensity between cases. Patient #3 had three evaluable metastatic cases, with two showing 3+ERG/FLI staining in all three cores each, while one showed 2+ ERG/FLI1 staining in all three cores. The primary case from patient #28 showed negative ERG/FLI1 staining in all three cores, while a recurrence showed 2+ staining in all three cores (Figure 2).

All evaluable cases on the tissue microarray showed homogenous CD99 staining within evaluable cores, and one patient had two cases with discordant CD99 staining. Patient #6 had one case (a lung metastasis) showing membranous CD99 expression in one evaluable core, while a separate case (a femur metastasis) showed negative CD99 staining (Figure 2).

Of the 57 total evaluable EFT cases, 6 (11%) demonstrated negative (0) ERG/FLI1 staining, 4 (7%) demonstrated weak (1+) staining, 13 (23%) demonstrated moderate (2+) staining, and 34 (60%) demonstrated strong (3+) staining (Figure 2). All EFTs with positive ERG/FLI1 staining showed diffuse nuclear ERG/FLI1 expression. Of the 47 (82%) EFTs with at least moderate (2+) ERG/FLI1 staining, 1 (2%) showed negative CD99 staining, 3 (6%) showed cytoplasmic staining, and 43 (91%) showed membranous staining. Of the remaining 10 (18%) EFTs with negative to weak (0–1+) ERG/FLI1 staining, 3 (30%) showed negative CD99 staining, 2 (20%) showed cytoplasmic staining, and 5 (50%) showed membranous staining (Figure 2). Overall, at least moderate (2+) ERG/FLI1 staining and membranous CD99 staining were significantly associated, (43 of 57 evaluable cases, p=0.005, Fisher's exact test), and 52 of 57 (91%) of cases showed either at least moderate (2+) ERG/FLI1 staining or membranous CD99 staining.

Of the 57 cases, 45 (79%) had evaluable molecular data (See **Methods**). Of evaluable cases, 35 (78%) harbored *EWSR1:FLI1* fusions, 4 (9%) harbored *EWSR1:ERG* fusions, and 6 (13%) lacked evidence of *EWSR1* rearrangements. Amongst the 35 cases with *EWSR1:FLI1* fusions, 31 (89%) showed at least moderate ERG/FLI1 staining, and 30 (86%) showed membranous CD99 staining. All 4 cases with *EWSR1:ERG* fusions showed at least moderate ERG/FLI staining and membranous CD99 staining. Lastly, amongst the 6 cases without evidence of *EWSR1* rearrangement, 2 (33%) showed at least moderate ERG/FLI1 staining and 4 (67%) showed membranous CD99 staining. Importantly, these results confirm the ability of EPR3864 to detect the products of both *EWSR1:FLI1* and *EWSR1:ERG* gene fusions.

In addition to EFTs, we also evaluated ERG/FLI1 staining using single sections from 61 other SRBCTs (Figure 3). Amongst other SRBCTs, at least 2+ *focal* nuclear staining was observed in 0 of 11 (0%) nephroblastomas (Wilm's tumors), 0 of 11 (0%) neuroblastomas, 0 of 7 (0%) alveolar/embryonal rhabdomyosarcomas, 0 of 4 (0%) desmoplastic small round cell tumors, 4 of 10 (40%) Burkitt's lymphomas, 9 of 11 (82%) synovial sarcomas (10 monophasic, 1 poorly differentiated), and 7 of 7 (100%) precursor-B-lymphoblastic lymphomas/leukemias. Of all non EFTs stained for ERG/FLI1, at least 2+ *diffuse* nuclear staining was seen in 1 of 10 (10%) Burkitt's lymphomas, 5 of 11 synovial sarcomas (45%) and 7 of 7 (100%) of precursor-B-lymphoblastic lymphomas/leukemias. A heat map of ERG/FLI1 staining in all small round blue cell tumors is shown in Figure 3.

Discussion

The diagnosis of EFTs from other small round blue cell tumors often requires immunohistochemistry, in addition to morphology, cytogenetics and/or molecular techniques. CD99 shows high sensitivity for EFTs, although it is not entirely specific. A combination of CD99, FLI1, HNK1 and CAV1, show high specificity and sensitivity for EFTs and has been proposed as an immunohistochemistry panel for the differential diagnosis of SRBCTs[10]. Both polyclonal and monoclonal antibodies against FLI1 have been employed, each with described limitations.

Previously we identified EPR3864, a monoclonal antibody raised against ERG, as showing utility for the detection of gene fusions involving ERG in prostate cancer[53] (most commonly *TMPRSS2:ERG*), which occur in approximately half of all prostate cancers identified by prostate specific antigen screening[40–42]. More recently, Mohamed *et al.* showed that EPR3864 also detects FLI1[54], while another recently developed monoclonal antibody against ERG does not react with FLI1[54] and stains only 7% of EFTs[56]. FLI1 cross-reactivity of EPR3864 does not appear to be relevant in prostate cancer, given the >95% sensitivity and specificity of EPR3864 for detecting ERG-rearranged prostate cancer and the >99.99% reported specificity for cancer[45–48,50–53,55,57]. However, we hypothesized that this antibody may show utility for the discrimination of EFTs, which harbor both FLI1 and ERG rearrangements.

By immunohistochemistry with EPR3864, we show that 82% (47 of 57) of EFTs (including 89% of cases with confirmed *EWSR1:FLI1* fusions and 100% of cases with confirmed *EWSR1:ERG* fusions) show at least moderate nuclear staining of ERG/FLI1, which was always diffuse. This rate is comparable to those reported using other polyclonal and monoclonal antibodies against FLI1[5–10,34–38]. Additionally, at least moderate ERG/FLI1 staining and membranous CD99 staining were significantly associated in our study, with 91% of cases showed either at least moderate ERG/FLI1 staining or membranous CD99 staining.

Amongst 61 other SRBCTs, no Wilm's tumors, neuroblastomas, rhabdomyosarcomas or desmoplastic small round cell tumors showed at least focal moderate (2+) ERG/FLI1 staining. However, we observed at least focal, moderate ERG/FLI1 staining in 40% of Burkitt's lymphomas, 82% of monophasic synovial sarcomas and 100% of precursor-B-lymphoblastic lymphomas. Unlike EFTs, which always showed diffuse ERG/FLI1, heterogeneous absent-weak (0–1+), or weak-moderate (1–2+) staining was observed in 25% of desmoplastic small round cell tumors, 9% of Wilm's tumors and 30% of Burkitt's lymphomas, suggesting that only diffuse moderate-strong staining supports the diagnosis of EFT. In our study, the majority of monophasic synovial sarcomas and precursor-B-lymphoblastic lymphomas showed at least moderate nuclear ERG/FLI1 staining. Previous studies have reported occasional reactivity of FLI1 monoclonal and polyclonal antibodies

with poorly differentiated synovial sarcomas (more relevant to the differential diagnosis of EFTs). In our cohort, only one synovial sarcoma was poorly differentiated (which showed focal CD99 staining), while of the remaining 10 monophasic sarcomas, all 6 cases evaluated for CD99 staining were strongly positive. As we did not have available additional poorly differentiated synovial sarcomas for evaluation, additional studies will be required to characterize EPR3864 staining in that entity. Similarly, CD99 and FLI1 staining in precursor-B-lymphoblastic lymphomas/leukemias is well characterized and represents an important differential diagnostic consideration in EFT evaluation[58,59]. Differentiating EFTs from these entities, which may show cross-reactivity with all FLI1 antibodies, will likely continue to require a combination of morphology, immunohistochemistry and molecular studies.

While this study was in preparation, Minner *et al*. evaluated EPR3864 staining in 11,483 tumors and 72 normal tissue types and reported strong staining nearly exclusively in prostate carcinoma and vascular tumors, however they reported no staining in 17 evaluable PNETs[60]. Wang *et al*. also recently evaluated EPR3864 staining in 32 EFTs, including 22 with *EWSR1:FLI1*, 8 with *EWSR1:ERG* and 2 with *EWSR1-NFATC2*. They observed predominantly diffuse, moderate to strong staining in 7 of 8 *ERG* rearranged cases, but only 3 of 24 non *ERG* rearranged cases showed staining with EPR3864 (all very weak)[61]. Importantly, Minner *et al*. and Wang *et al*. used substantially different antibody dilutions (1:400 and 1:2000) compared to our current study which used ready-to-use, pre-diluted EPR3864 antibody (1:50–1:100). Our study also used the same pretreatment conditions and automated staining and detection instrumentation as we recently validated in prostate cancer biopsies[45] and use clinically at our institution.

Although the availability of molecular techniques has reduced the need for additional immunohistochemistry markers to identify EFTs, EPR3864 shows similar sensitivity to other polyclonal and monoclonal antibodies against FLI1, and has the advantage of being well characterized (in the context of prostate cancer) in its ability to detect ERG, with minimal background staining. Similarly, like other FLI1/ERG antibodies[5,9,56], EPR3864 is a highly sensitive vascular marker[53,60,62], supporting an additional area of diagnostic utility (S.A.T., D.R.L. and L.P.K., unpublished observations).

Additional studies will be needed to directly compare EPR3864 with other FLI1 antibodies, and we are not aware of whether studies have investigated whether other currently used antibodies against FLI1 also cross-react with ERG, however we hypothesize that this is unlikely given the lack of reported utility for detection of prostate cancer.

In summary, we demonstrate that EPR3864 shows utility for detecting EFTs harboring both *EWSR1:FLI1* and *EWSR1:ERG* gene fusions. By immunohistochemistry, EPR3864 detection of ERG/FLI1 shows high sensitivity for EFTs (>80% with diffuse moderate-strong nuclear staining) and complements CD99 staining. EPR3864 also stains a substantial proportion of Burkitt's lymphomas, monophasic synovial sarcomas and precursor-B-lymphoblastic lymphomas/leukemias, and in cases where these entities remain in the differential diagnosis based on morphology, molecular confirmation of EFTs will likely be required. Our results suggest that EPR3864, which has demonstrated utility in the diagnosis and molecular subtyping of prostate cancer (which also harbor ETS gene fusions), may also have utility in the diagnosis of EFTs from other SRBCTs.

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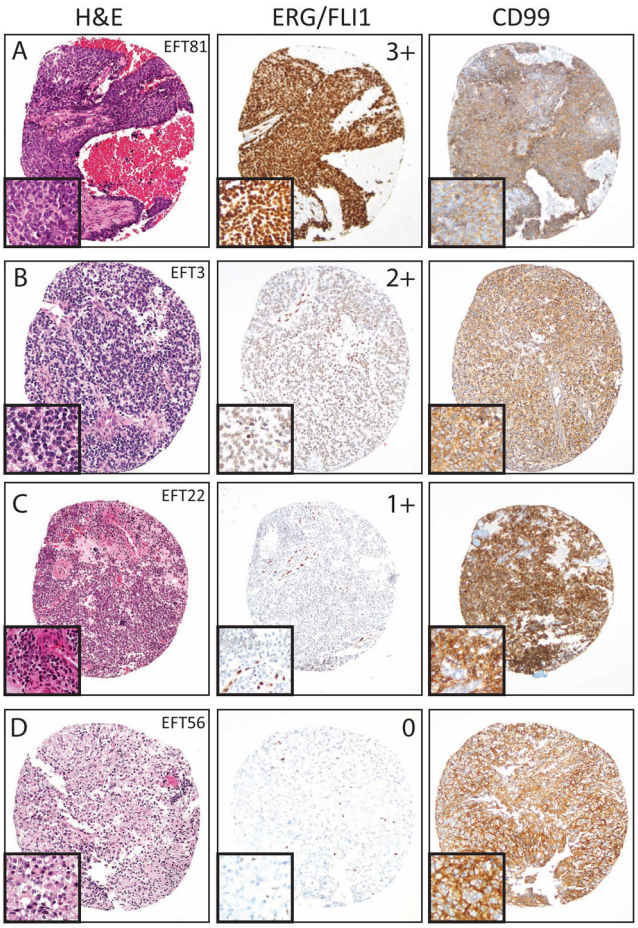

Figure 1. ERG/FLI1 staining in Ewing family tumors (EFTs)

EFTs were evaluated for ERG/FLI1 and CD99 staining by immunohistochemistry. ERG/FLI1 staining (diffuse nuclear) was scored as negative (0), weak (1+), moderate (2+), or strong (3+), and CD99 staining was scored as negative, cytoplasmic or membranous. Representative hematoxylin and eosin (H&E left panels), ERG/FLI1 (middle panel) and CD99 (right panel) staining from cases showing (**A**) 3+, (**B**) 2+, (**C**) 1+ and (**D**) 0 ERG/FLI1 staining and membranous CD99 staining are shown. All images are 10x original magnification with 20x insets.

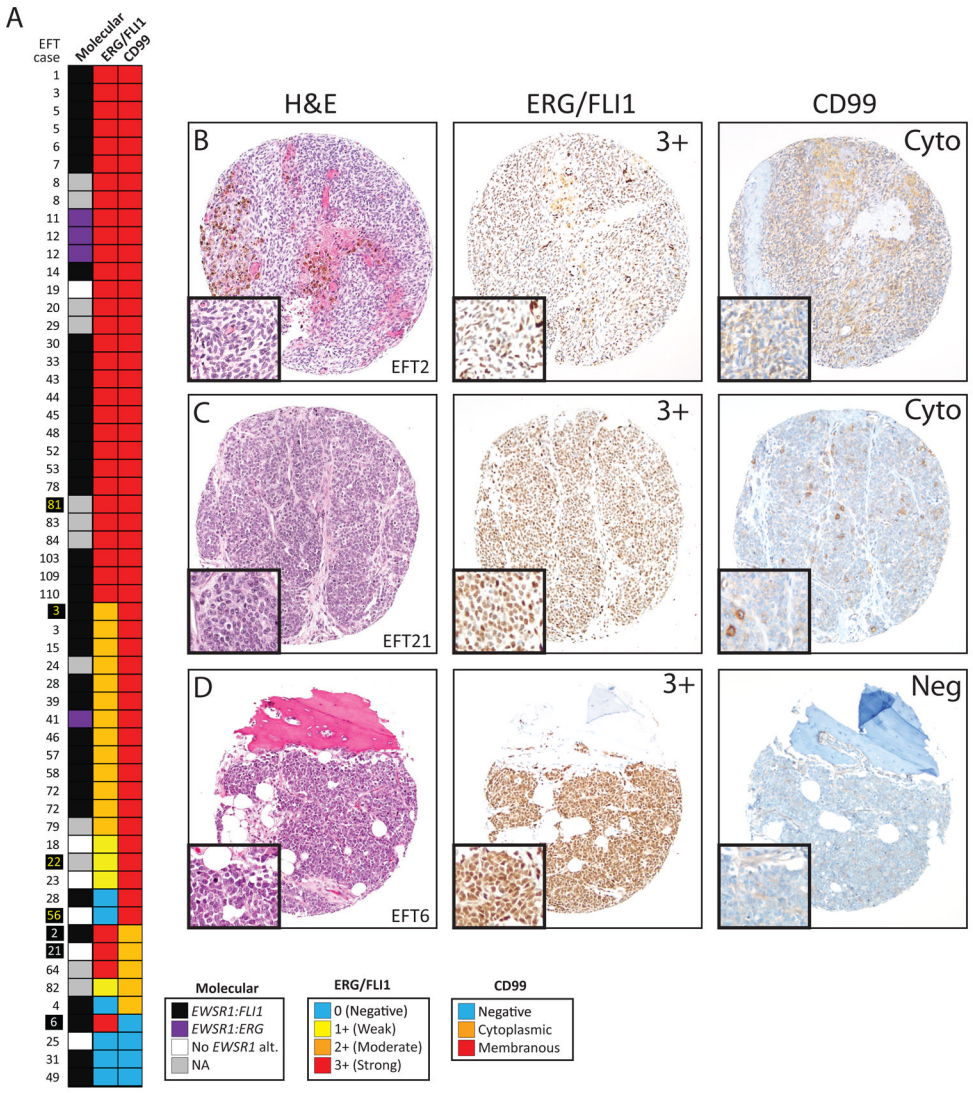

Figure 2. ERG/FLI1 and CD99 staining in Ewing family tumors (EFTs)

A. Heat map of molecular status and ERG/FLI1 and CD99 staining for 57 evaluable EFT cases. Cases with confirmed *EWSR1:FLI1* (black) or *EWSR1:ERG* (purple) rearrangements are indicated, along with cases without evidence of an *EWSR1* rearrangement (white) or those not assessed (gray). ERG/FLI1 staining (diffuse nuclear) and CD99 staining were scored as in Figure 1 (indicated in the legend). Cases shown in Figure 1 are indicated by yellow names. **B–D**. Representative hematoxylin and eosin (H&E left panels), ERG/FLI1 (middle panel) and CD99 (right panel) cores from cases showing 3+ ERG/FLI1 expression and (**B&C**) cytoplasmic or negative (**D**) CD99 staining are shown. Cases shown are indicated by white names in **A**. All images are 10x original magnification with 20x insets.

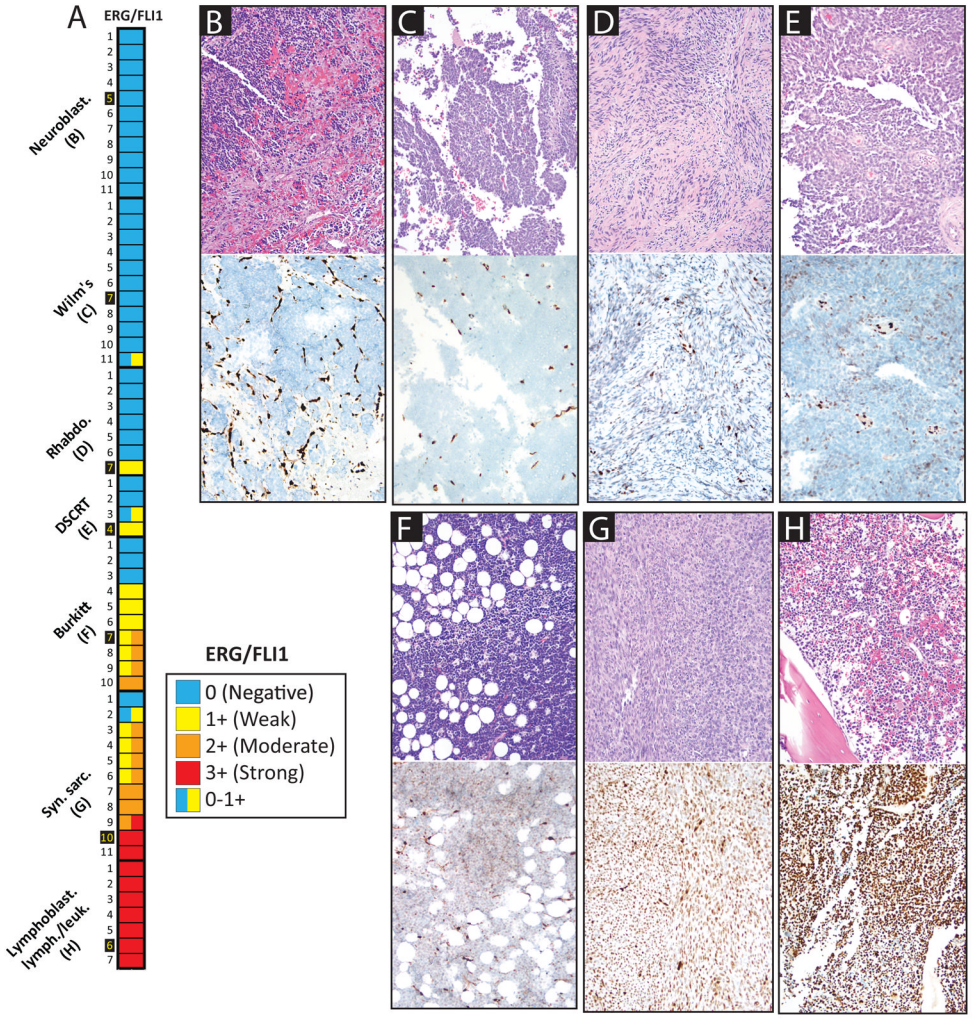

Figure 3. ERG/FLI1 staining in small round blue cell tumor (SRBCT) mimickers of Ewing family tumors (EFTs)

Heat map of ERG/FLI1 staining (nuclear) in 61 non-EFT SRBCTs., ERG/FLI1 staining was scored as in Figures 1 & 2. In cases with heterogeneous staining, the variable intensity is indicated by multiple colors in the heat map cell. Cases shown are indicated by yellow names. **B–H.** Hematoxylin and eosin (H&E, top panels) and ERG/FLI1 (bottom panels) staining for representative cases are shown (20x original magnification).

Table 1

Demographics of patients (and cases) with EFTs evaluable for ERG and CD99 staining

Parameter[1]	***Total number*** **(n)**	**Median (IQR)**
Age at diagnosis:	49	18 (13–30)
Parameter[1]	***Total number*** **(n)**	**Number of patients (%)**
Sex:	49	
Male		29 (59%)
Female		20 (41%)
Stage:	57	
Primary		37 (65%)
Primary; s/p chemo		4 (7%)
Recurrence		6 (11%)
Metastasis		10 (18%)
Location:	57	
Osseous; axial		18 (32%)
Osseous; extra-axial		19 (33%)
Extra-osseous; axial		18 (32%)
Extra-osseous; extra-axial		2 (4%)
Molecular Confirmation[2]:	49	
EWSR1:FLI1		29 (59%)
EWSR1:ERG		3 (6%)
No *EWSR1* rearrangement		6 (12%)
NA		11 (22%)

[1]Total number of patients with at least one evaluable core used for age at diagnosis and sex. Total number of cases with at least one evaluable core used for stage and location.

[2]Patients who had at least one case confirmed by two of three molecular tests (FISH for *EWSR1* breakapart, cytogenetics [t(11;22) or t(21;22)] and RT-PCR for *EWSR1:FLI1* or *EWSR1:ERG).* Those without available molecular data (NA) are indicated.