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Secondary *EWSR1* Gene Abnormalities in *SMARCB1*-Deficient Tumors with 22q11-12 Regional Deletions: Potential Pitfalls in Interpreting *EWSR1* FISH Results

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Abstract

SMARCB1 inactivation occurs in a variety of tumors, being caused by various genetic mechanisms. Since *SMARCB1* and *EWSR1* genes are located close to each other on chromosome 22, larger *SMARCB1* deletions may encompass the *EWSR1* locus. Herein, we report four cases with *SMARCB1*-deletions showing concurrent *EWSR1* gene abnormalities by FISH, which lead initially to misinterpretations as *EWSR1*-rearranged tumors. Our study group included various morphologies: a poorly differentiated chordoma, an extrarenal rhabdoid tumor, a myoepithelial carcinoma, and a proximal-type epithelioid sarcoma. All cases showed loss of *SMARCB1* (INI1) by immunohistochemistry (IHC) and displayed characteristic histologic features for the diagnoses. The *SMARCB1* FISH revealed homozygous or heterozygous deletions in three and one case, respectively. The co-hybridized *EWSR1* probes demonstrated either unbalanced split signals or heterozygous deletion in two cases each. The former suggested *bona fide* rearrangement, while the latter resembled an unbalanced translocation. However, all the FISH patterns were quite complex and distinct from the simple and uniform split signals seen in typical *EWSR1* rearrangements. We conclude that in the context of 22q11-12 regional alterations present in *SMARCB1*-deleted tumors, simultaneous *EWSR1* involvement may be misinterpreted as equivalent to *EWSR1* rearrangement. A detailed clinicopathologic correlation and supplementing the *EWSR1* FISH assay with complementary methodology is mandatory for correct diagnosis.

INTRODUCTION

SMARCB1 (also known as INI1, BAF47, or hSNF5) is a core subunit of the switch/sucrose nonfermenting (SWI/SNF) complexes, which consume ATP to modulate chromatin remodeling (Wilson and Roberts, 2011; Masliah-Planchon et al., 2015). The association between SWI/SNF complexes and neoplasia was first recognized nearly two decades ago,

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with the discovery of *SMARCB1* truncating mutations and deletions in malignant rhabdoid tumors at different locations (brain, kidney, soft tissue) (Versteeg et al., 1998; Biegel et al., 2002). Subsequently, *SMARCB1* loss has been implicated in the pathogenesis of most epithelioid sarcomas and renal medullary carcinomas (Hollmann and Hornick, 2011; Agaimy, 2014). Loss of *SMARCB1* protein expression was further described in a variety of epithelial and epithelioid mesenchymal tumors, such as myoepithelial carcinoma, extraskeletal myxoid chondrosarcoma, epithelioid malignant nerve sheath tumor, chordoma and a subset of GI and sinonasal carcinoma (Hollmann and Hornick, 2011; Agaimy, 2014). Remarkably, by whole genome sequencing, 19.6% of all human tumors harbor mutations in various SWI/SNF subunits, a similar rate to *TP53* mutation (Kadoch et al., 2013).

Biallelic disruption of the *SMARCB1* gene is the molecular hallmark of *SMARCB1*-deficient tumors and stems from a variety of mechanisms, including inactivating mutations, intragenic or whole-gene deletions, copy number-neutral loss of heterozygosity (LOH), and, infrequently, translocations (Jackson et al., 2009; Calderaro et al., 2015; Jamshidi et al., 2016). The incidence of homozygous or heterozygous *SMARCB1* deletions varies depending on the tumor type and anatomic location of *SMARCB1*-deficient tumor. Furthermore, the involved region in 22q11-12 shows great diversity and complexity (Biegel et al., 2002; Jackson et al., 2009; Sullivan et al., 2013; Le Loarer et al., 2014). Among malignant rhabdoid tumors, brain tumors often exhibit large deletions or LOH of the whole chromosome arm 22q, while soft tissue counterparts usually harbor regional deletions across 22q11.22-11.23, and the renal tumors have either complex copy number deviation or copy number-neutral LOH (Jackson et al., 2009). Aside from 22q genomic abnormalities, malignant rhabdoid tumors are characterized by relatively simple karyotypes, containing only few other copy number variations or mutations (Rousseau-Merck et al., 1999; Jackson et al., 2009; Lee et al., 2012). In contrast, epithelioid sarcomas show a complex cytogenetic profile, with frequent aneuploidies, numerous structural aberrations, a high mutation burden and copy number changes (Lualdi et al., 2004; Jamshidi et al., 2016).

Since the *SMARCB1* and *EWSR1* genes are located only 5.5 Mb from each other in chromosome bands 22q11.23 and 22q12.2, respectively, secondary *EWSR1* regional deletions may occur in *SMARCB1*-deleted tumors. In our previous study, we detected heterozygous telomeric *EWSR1* deletions in 25% (9/36) of *SMARCB1*-deleted epithelioid sarcomas by fluorescence in situ hybridization (FISH) (Le Loarer et al., 2014). Herein, we describe four *SMARCB1*-deleted tumors associated with concurrent *EWSR1* regional abnormalities that had been interpreted as primary gene rearrangements by outside cytogenetic reports, and therefore mis-diagnosed as *EWSR1*-rearranged tumors, to better illustrate the importance of clinicopathologic correlation and comprehensive interpretation of FISH findings.

MATERIALS AND METHODS

Case Collection

We identified three cases from the consultation files of the senior author (CRA) with a presumed *EWSR1* gene rearrangement, which were sent for further classification or in an attempt to identify the *EWSR1* fusion partner. An additional case with similar *EWSR1*

alteration was retrieved from the archive of MSKCC Surgical Pathology. The slides and correspondent immunohistochemical stains were reviewed to validate the diagnosis according to the current WHO criteria (Fletcher et al., 2013). In addition, potential fusion partners of *EWSR1* were also investigated by FISH according to the initial diagnosis rendered, such as *PBX1*, *ZNF444*, *POU5F1*, *PBX3* and *KLF17* for myoepithelial tumors, (Huang et al., 2015) *FLII* and *ERG* for Ewing sarcoma, (Fletcher et al., 2013) and *NR4A3* for extraskeletal myxoid chondrosarcoma (Agaram et al., 2014). The study was approved by the Institutional Review Board.

Immunohistochemistry

IHC staining, including INI1 (BAF47), was performed on whole sections of formalin-fixed, paraffin-embedded tissue blocks. The primary antibody used was a mouse monoclonal anti-BAF47 antibody (23, 1:30; BD bioscience, San Jose, CA). Antigen retrieval, antibody incubation, and chromogen counterstaining were performed in a BenchMark ULTRA automated immunostainer (Ventana, Tucson, AZ).

Fluorescence in situ hybridization

FISH for *SMARCB1* deletion and *EWSR1* break-apart assay was applied on formalin-fixed and paraffin-embedded 4 μ m-sections in all cases. FISH was performed by applying custom probes using bacterial artificial chromosomes (BACs), covering or flanking the *SMARCB1* or *EWSR1* genes (Fig. 1A and Supporting Information Table 1). The BAC clones were obtained from BACPAC sources of Children's Hospital of Oakland Research Institute (CHORI) (Oakland, CA)(<http://bacpac.chori.org>). DNA from individual BACs was isolated according to the manufacturer's instructions, labeled with different fluorochromes in a nick translation reaction, denatured, and hybridized to pretreated slides. Slides were then incubated, washed, and mounted with DAPI in an antifade solution, as previously described (Huang et al., 2015). The genomic location of each BAC set was verified by hybridizing them to normal metaphase chromosomes. Two hundred successive nuclei were examined using a Zeiss fluorescence microscope (Zeiss Axioplan, Oberkochen, Germany), controlled by Isis 5 software (Metasystems, Newton, MA). A positive score was interpreted when at least 20% of the nuclei showed a deleted or break-apart signal. Nuclei with incomplete set of signals were omitted from the score.

RESULTS

A *SMARCB1*-Deficient Clival Tumor with *EWSR1* Regional Abnormalities

Case no. 1 was a 3-year-old girl presenting with a large clivus tumor, who subsequently developed distant metastases (Table 1). The histology revealed interconnecting solid nests of large polygonal cells, separated by thin fibrovascular septae infiltrated by lymphocytes and plasma cells. The tumor cells showed distinctive cell borders, abundant eosinophilic cytoplasm, focal nuclear atypia and coarse chromatin with prominent nucleoli (Fig. 1B). Variable rhabdoid cells were noted, but no cytoplasmic vacuoles, physaliferous cells, or myxoid matrix was present. The mitotic activity was <1/10 HPFs. Immunohistochemically, the tumor cells expressed cytokeratin and S100 protein, with complete loss of *SMARCB1* immunoexpression. T-brachyury was reported as negative on the initial evaluation. Under the

suspicion of a myoepithelial carcinoma, *EWSR1* FISH was performed and initially reported as positive for rearrangement. We received the case for further molecular evaluation, in an attempt to determine the *EWSR1* fusion partner. However, all known *EWSR1* gene partners in the setting of myoepithelial tumor were negative by FISH, including: *PBX1*, *ZNF444*, *POU5F1*, *PBX3* and *KLF17*. The FISH assay displayed homozygous *SMARCB1* deletion associated with a heterogeneous and complex *EWSR1* split pattern (Fig. 1D). The latter showed *EWSR1* split signals of smaller size and lower intensity, compared to the normal copies, suggesting a more complex *EWSR1* regional deletion rather than a simple, balanced rearrangement. The T-brachyury immunostain performed in our lab was positive and, corroborated with the homozygous *SMARCB1* FISH deletions, was consistent with a diagnosis of poorly differentiated chordoma, rather than an *EWSR1*-rearranged myoepithelial carcinoma (Fig. 1C) (Hoch et al., 2006).

A Pediatric Round Cell Sarcoma with *INI1* Loss and *EWSR1* Genes Abnormalities

Case no. 2 was a round cell sarcoma of the abdominal wall in a 5-year-old boy. Microscopically, the tumor was composed of compact sheets of small round cells, with scant cytoplasm, and eccentric nuclei with open chromatin and prominent nucleoli (Fig. 1E). Necrosis, brisk mitotic activity (>50/10 HPF), and abundant karyorrhexis was noted. The tumor was negative for CD99, cytokeratin, S100, desmin, myogenin, CD45, TdT and synaptophysin, and showed complete *INI1* loss (Fig. 1F). The outside FISH analysis showed a positive *EWSR1* rearrangement, and a diagnosis of an atypical Ewing sarcoma with loss of *SMARCB1* expression was entertained. The case was sent for further molecular characterization and diagnosis confirmation. Our FISH results failed to show abnormalities in *FLII* and *ERG* genes. The combined *SMARCB1* and *EWSR1* FISH assay demonstrated a homozygous *SMARCB1* deletion, accompanied by loss of the telomeric *EWSR1* signal on one allele and a split *EWSR1* signal on the other allele (Fig. 1G). The split-apart *EWSR1* signals were smaller in size, as observed in Case no. 1. The overall histologic and molecular findings fit the diagnosis of an extrarenal malignant rhabdoid tumor (Hollmann and Hornick, 2011; Fletcher et al., 2013).

A Meningeal Myoepithelial Neoplasm with *SMARCB1* Deletions and Heterogeneous *EWSR1* Alterations

Case no. 3 was a large dural-based tumor occurring in a 16-year-old girl. Morphologically, the tumor consisted of cords and small nests embedded in a hyalinized stroma (Fig. 2A), but solid sheets with central necrosis were also seen. The tumor cells ranged from round, ovoid, to spindle, without rhabdoid features. Frequent mitotic figures (>10/10 HPFs), moderate nuclear pleomorphism and distinct nucleoli were noted. The tumor showed focal immunoreactivity for EMA, cytokeratin and S100 (Fig. 2B), but negativity for estrogen receptor and T-brachyury. *SMARCB1* expression was lost. The initial FISH examination reported *EWSR1* rearrangement and a presumed diagnosis of myoepithelial carcinoma was rendered. Our combined *SMARCB1* and *EWSR1* assay revealed homozygous *SMARCB1* deletion with heterogeneous alterations of the *EWSR1* locus (Fig 2C, 2D). Most tumor cells exhibited deletion of the telomeric *EWSR1* region (loss of green signals) on one allele, while the other harbored a 22q11 deletion extending to the centromeric part of *EWSR1* region (residual tiny yellow signals)(Fig. 2C). This FISH pattern was misleading for an

unbalanced *EWSR1* translocation with telomeric deletion (chromosome 22 monosomy with break-apart signals), as the other allele abnormalities with small yellow signals were easy to miss. Overall findings were in keeping with a myoepithelial carcinoma with homozygous *SMARCB1* deletion and secondary *EWSR1* genetic abnormalities. This case illustrates the complexity and heterogeneity of 22q11-12 regional alterations in *SMARCB1*-deficient myoepithelial tumors.

A High-Grade Myxoid Sarcoma Showing Epithelioid/Rhabdoid Cell Features and Heterozygous *SMARCB1* and *EWSR1* Deletions

Case no. 4 was a left groin tumor in a 21-year-old male patient. The tumor showed solid areas with necrosis alternating with myxoid regions where the tumor cells floated singly or in cords (Fig. 2E). The neoplastic cells showed an epithelioid to rhabdoid morphology, with large vesicular nuclei and macronucleoli. Intracytoplasmic hyaline inclusions, occasional binucleation, and brisk mitotic activity (>20/10 HPFs) were noted. By IHC, the tumor was positive for EMA, but negative for cytokeratin, CD34, desmin, and S100. The tumor showed complete loss of *SMARCB1*. The original *EWSR1* FISH reported one residual copy of normal *EWSR1* signals and a diagnosis of extraskeletal myxoid chondrosarcoma was suspected. Our *NR4A3* FISH testing was negative for rearrangement. The combined *SMARCB1* and *EWSR1* FISH assay revealed heterozygous *SMARCB1* deletion extending to the telomeric-*EWSR1* region (small residual green signals) (Fig. 2F). These heterozygous deletions resulted in a misinterpretation as an unbalanced *EWSR1* translocation with centromeric loss, even though the 5'-end of *EWSR1* gene is always represented in the *EWSR1* chimeric fusion transcript. Corroborating all the genetic findings of *SMARCB1* deletion and absence of *NR4A3* abnormalities the diagnosis was reclassified as a proximal-type epithelioid sarcoma (Hollmann and Hornick, 2011; Fletcher et al., 2013).

DISCUSSION

FISH analysis on paraffin-embedded tissue sections has emerged as a reliable and widely applied clinical assay for detection of gene rearrangements, deletions, and amplifications (Speicher and Carter, 2005; Bayani and Squire, 2007). In the setting of *SMARCB1*-deficient tumors, FISH can potentially be used to investigate *SMARCB1* gene deletions as the size range of the genomic regions (100 kb–18 Mb) involved are often within the resolution of FISH probes (50 kb–2 Mb) (Jackson et al., 2009; Lee et al., 2012; Le Loarer et al., 2014). Although FISH analysis is not suitable to identify other *SMARCB1* molecular alterations, such as mutations, small deletions, or LOH, it remains a reliable test in *SMARCB1*-deficient tumors where large homozygous or heterozygous 22q11-12 deletions predominate. For example, homozygous *SMARCB1* deletions are present in the overwhelming majority of both proximal and distal epithelioid sarcomas (83–90%) (Sullivan et al., 2013; Le Loarer et al., 2014). In malignant rhabdoid tumors, the genetic abnormalities are equally divided between homozygous *SMARCB1* deletions and *SMARCB1* missense mutations with heterozygous deletion or LOH (Jackson et al., 2009). Despite limited case numbers, *SMARCB1*-deficient chordoma and sinonasal rhabdoid/basaloid carcinoma appear to have a comparable high rate of homozygous or heterozygous *SMARCB1* deletion (Mobley et al., 2010; Bishop et al., 2014). At least a small subset of extraskeletal myxoid chondrosarcoma

and myoepithelial carcinoma with loss of SMARCB1 expression show concurrent *SMARCB1* deletion, although none of the cases reported to date show co-existing *NR4A3* or *EWSR1* rearrangements, suggesting a mutually exclusive mechanism (Kohashi et al., 2008; Le Loarer et al., 2014). The absence of *SMARCB1* deletions by FISH in the settings of SMARCB1-deficient tumors harboring a high rate of *SMARCB1* deletions should raise concerns about the correct diagnosis. To illustrate this point, we have recently encountered a 28-year-old female African American presenting with disseminated disease involving lymph nodes, lung, liver, and kidney. The lymph node biopsy revealed malignant epithelioid and rhabdoid tumor cells with a nested arrangement in a fibromyxoid background (Fig. 3). The tumor was positive for cytokeratin and vimentin and had complete loss of SMARCB1 expression; thus an epithelioid sarcoma diagnosis was considered. By FISH examination no *SMARCB1* deletions were identified, which was unusual for an epithelioid sarcoma and alerted us to obtain additional clinical information, which revealed that the patient's sister had sickle cell trait. Thus a diagnosis of a renal medullary carcinoma was rendered, in which the preferred mechanism of SMARCB1 inactivation is more likely through a *SMARCB1* translocation or LOH (Swartz et al., 2002; Liu et al., 2013; Calderaro et al., 2015).

However, FISH assays entail certain limitations and pitfalls (Gozzetti and Le Beau, 2000) and their applicability is largely restricted by the narrow selection of commercially available labeled DNA probes. As no FISH probes are available for *SMARCB1*, a number of labs and studies use as a surrogate the anti-*BCR* telomeric probe, which targets a 645-kb long region (23,986–24,631 kb genomic position, spanning the *SMARCB1* gene, 24,129–24,176 kb) (Papp et al., 2013). However, this commercial probe covers a bigger target region (fourfold larger than our specific custom probe, 148 kb), including the centromeric end of the *SMARCB1* gene. Thus the smaller size or centromeric-centered *SMARCB1* deletions might result in partial or minimal loss of signal intensity, which can be easily missed by the low FISH resolution. Using this approach, Papp et al. reported a significantly lower percentage (15/31, 48%) of *SMARCB1* deletions in epithelioid sarcoma using the Vysis LSI *BCR* SpectrumGreen probes (BCR/ABL Dual Color Translocation Probe Set, Abbott Molecular, Illinois, USA) (Papp et al., 2013). An identical setback occurred in our Case no. 2 in which the anti-*BCR* FISH probe failed to identify a *SMARCB1* deletion, and thus a diagnosis of rhabdoid tumor was excluded. In contrast, our custom *SMARCB1*-specific strategy detected homozygous deletion in this case, which also explained the secondary nearby *EWSR1* gene abnormalities. Furthermore, another limitation of FISH assay is that it examines only the targeted region without providing information on a more global scale of the whole chromosome structure. In this regard, we have recently identified that 27% of Ewing sarcoma with *EWSR1-ERG* fusions show a false negative result by FISH using the *EWSR1* break-apart probes, due to a complex pattern of inversions and rearrangements (Chen et al., 2016).

In this study, we describe four cases of *SMARCB1*-deleted tumors harboring concurrent *EWSR1* regional alterations, mimicking either balanced or unbalanced *EWSR1* rearrangements. These four cases spanned a variety of diagnoses, including a poorly differentiated chordoma, myoepithelial carcinoma, extrarenal malignant rhabdoid tumor, and an epithelioid sarcoma. In two cases, the *SMARCB1* deletion involved the *EWSR1* gene, and the FISH result showed a split signal, although the size of the signals was smaller than

the ones seen in a typical balanced rearrangement. In the other two cases, FISH showed a heterozygous deletion of *EWSR1* FISH probes. Although our previous study showed one-fourth of *SMARCB1*-deleted epithelioid sarcoma contained synchronous deletion of telomeric *EWSR1* region, (Le Loarer et al., 2014) the prevalence of concurrent break-apart or heterozygous *EWSR1* abnormalities reminiscent of *EWSR1* rearrangement in *SMARCB1*-deleted tumors remains unclear. The single nucleotide polymorphism-based oligonucleotide array showed heterozygous 22q12.2 deletion in 8.5% (3/35) of malignant rhabdoid tumors (Jackson et al., 2009). Spectral karyotyping depicted complex chromosomal aberrations encompassing a t(12;22)(q12;q12) without *EWSR1* break-apart FISH signal in one of the six epithelioid sarcoma (Lualdi et al., 2004). Conversely, most of *EWSR1*-rearranged soft tissue sarcomas, such as Ewing sarcoma, clear cell sarcoma, desmoplastic small round cell tumor, and myxoid liposarcoma, are not associated with *SMARCB1* protein loss (Kohashi et al., 2008). In two studies, none of the *SMARCB1*-deficient myoepithelial carcinomas or extraskeletal myxoid chondrosarcomas had concurrent *EWSR1* rearrangement and *SMARCB1* deletion (Kohashi et al., 2008; Le Loarer et al., 2014).

Chordomas arising in children and adolescents account for less than 5% of all chordoma cases, have a predilection for the skull base and behave more aggressively, especially the subset exhibiting poorly differentiated features (Hoch et al., 2006; Ridenour et al., 2010). They often have loss of *SMARCB1* protein expression as a result of *SMARCB1* deletions (Mobley et al., 2010; Yadav et al., 2014). The co-expression of cytokeratin and S100 in Case no. 1 raised the differential diagnosis between myoepithelial carcinoma and chordoma. The initial negative result of T-brachyury immunostaining and FISH positivity for *EWSR1* rearrangement favored a myoepithelial carcinoma. However, our FISH findings of *SMARCB1* deletions encompassing the *EWSR1* locus, as well as the lack of rearrangements in all *EWSR1*-gene partners described in myoepithelial tumors, questioned the outside *EWSR1* gene break-apart result. Furthermore the repeat T-brachyury showed nuclear reactivity confirming a diagnosis of chordoma. Indeed, chromosomal 22q translocation or mitotic recombination with variable regional deletion, outside the hot spot *EWSR1* breakpoints, represents one of the main mechanisms for *SMARCB1* inactivation (Rousseau-Merck et al., 1999; Lualdi et al., 2004).

Rare round cell sarcomas harboring *SMARCB1* genetic alterations and consequent loss of expression have been previously described, having a predilection for young children and a favorable outcome (Kreiger et al., 2009). Their morphology includes undifferentiated round cells arranged in sheet-like pattern, with variably prominent nucleoli but lacking a classic rhabdoid phenotype. Jahromi et al. (2012) recently reported that 10/106 Ewing sarcomas had complete *SMARCB1* protein loss, most of them occurring in the soft tissue and displaying atypical features and even rhabdoid cells. *EWSR1* rearrangements were only demonstrated in 2/4 such cases using break-apart FISH strategy, but not confirmed by other methods. It is possible that some of the so-called “*EWSR1*-rearranged *SMARCB1*-deficient Ewing sarcomas” are equivalent to our Case no. 2, representing rhabdoid tumors with secondary *EWSR1* abnormalities due to *SMARCB1* deletions. Additional FISH or RT-PCR testing to assess *FLII* or *ERG* gene rearrangements would settle this dilemma as confirming the diagnosis of Ewing sarcoma.

Loss of SMARCB1 protein expression has been described in 10–40% of myoepithelial carcinoma, with pediatric cases showing a higher frequency (Hollmann and Hornick, 2011). Our previous study showed homozygous *SMARCB1* deletions in three of five myoepithelial carcinomas with SMARCB1 immunonegativity, but none of them harbored *EWSR1* gene rearrangement (Le Loarer et al., 2014). Even though Kohashi et al. (2008) reported *SMARCB1* deletions in 2/4 SMARCB1-deficient extraskeletal myxoid chondrosarcoma, none of them harbored *EWSR1-NR4A3* or *TAF2A-NR4A3* fusion transcript by RT-PCR. Furthermore, in our prior study, none of seven SMARCB1-deficient extraskeletal myxoid chondrosarcoma with *NR4A3* gene rearrangements showed *SMARCB1* abnormalities by FISH (Le Loarer et al., 2014). In this setting, we highly recommend evaluation of *NR4A3* gene rearrangement by FISH or RT-PCR to confirm the diagnosis of extraskeletal myxoid chondrosarcoma, as *EWSR1* gene abnormalities might be secondary to large *SMARCB1* deletions. Except for the above-mentioned Ewing sarcoma study (Jahromi et al., 2012), there are three additional case reports describing SMARCB1-deficient tumors with co-existing *EWSR1* rearrangements, including two myoepithelial carcinomas and one desmoplastic small round cell tumor (Machado et al., 2015; Soon and Petersson, 2015; Thway et al., 2015). The first myoepithelial carcinoma case was reported to have an unbalanced *EWSR1* translocation (one normal *EWSR1* pair signal and an isolated green telomeric signal), (Thway et al., 2015) similar to our Case no. 4, which suggests the loss of derivative chromosome 22 that contained the critical 5' end of *EWSR1* gene. The second myoepithelial carcinoma case showed rhabdoid cells and myxoid background, and despite the *EWSR1* rearrangement reported by FISH there was no identified fusion partner (Machado et al., 2015). The third case was a cytokeratin and desmin-negative desmoplastic small round cell tumor which by RT-PCR showed an *EWSR1-WTI* transcript, but by FISH had one pair of *EWSR1* break-apart signals, indicating monosomy 22 or allelic deletion (Soon and Petersson, 2015).

In summary, this study illustrates a recurring pitfall in interpreting the *EWSR1* FISH results in the setting of SMARCB1 deficient and *SMARCB1*-deleted tumors. We describe four index cases, spanning four different diagnoses, which were misinterpreted mainly due to the positive FISH results for *EWSR1* gene rearrangement. Despite the fact that all cases demonstrated SMARCB1 loss of expression, the possibility of a primary *SMARCB1* genetic inactivation event was dismissed once the *EWSR1* abnormality was found. Our results emphasize that a subset of *SMARCB1*-deleted tumors contain secondary *EWSR1* regional alterations by FISH, including break-apart and heterozygous deletions; both of these *EWSR1* FISH abnormalities patterns having the potential for misdiagnosis. The key feature in recognizing this abnormal *EWSR1* pattern includes the presence of smaller size signals of lower intensity by FISH compared to the normal cells. Our results corroborated with a careful literature review further emphasizes that most *EWSR1* rearranged tumors do not show concurrent *SMARCB1* gene inactivations, and most likely represent independent mechanisms of pathogenesis. Our recommendation for a case showing loss of SMARCB1 expression by IHC and found concurrently to display rearrangements of the *EWSR1* gene region by FISH, is that additional genetic investigation should be applied, including complementary molecular methods to exclude a primary *SMARCB1* gene abnormality. Additional FISH testing for confirming rearrangements in the potential *EWSR1* gene

partners or applying complementary methods (RT-PCR, NGS) should be recommended in challenging cases, and close clinicopathologic correlation with the FISH results should be carried out to avoid these pitfalls.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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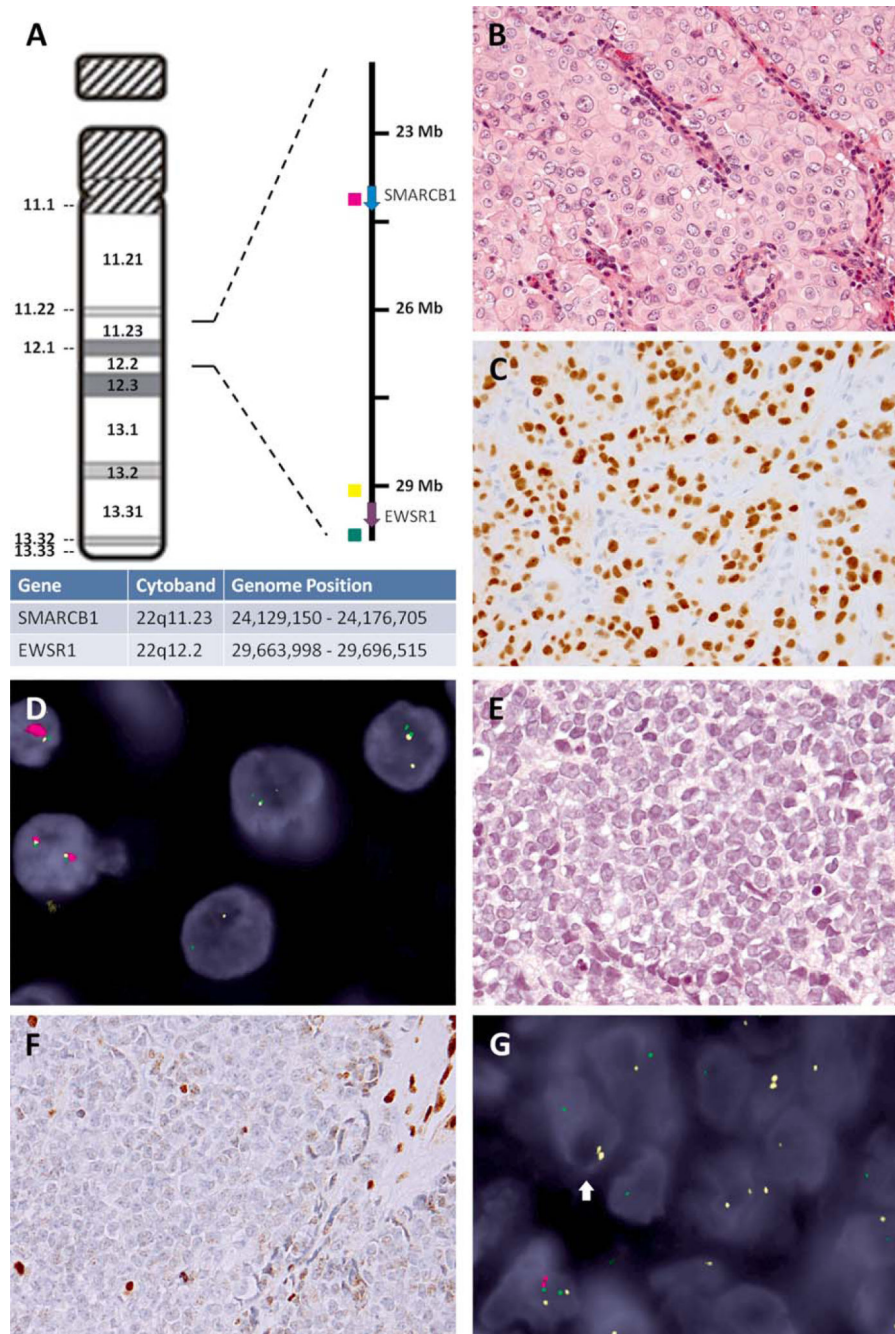


Figure 1. Secondary break-apart *EWSR1* patterns in *SMARCB1*-deleted tumors. A: *SMARCB1* and *EWSR1* genomic positions on chromosome 22 ideogram, with correspondent fluorochrome-labeled DNA probes used in the 3-color FISH assays (red: intragenic *SMARCB1*, yellow: centromeric *EWSR1*, green: telomeric *EWSR1*). B: Poorly differentiated chordoma showing compact sheets of epithelioid cells with moderate nuclear pleomorphism (Case 1, 400x) and (C) strong T-brachyury nuclear immunoreactivity (400x). D: The 3-color FISH assay showed homozygous *SMARCB1* deletion (loss of both red signals) and *EWSR1* break-apart pattern (yellow and green split signals with lower intensities). E: Extrarenal rhabdoid tumor

resembling Ewing sarcoma, characterized by solid sheets of small round cells with prominent nucleoli (Case 2, 400x) and (F) loss of SMARCB1 expression (internal positive control, inflammatory and endothelial cells; 400x). G: The 3-color FISH revealed a homozygous *SMARCB1* deletion (loss of both red signals) and coexistent *EWSR1* regional abnormalities (the arrow): one intact yellow signal (telomeric *EWSR1* deletion) and one split pair of smaller-sized yellow and green signals (*EWSR1* regional break and deletion). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

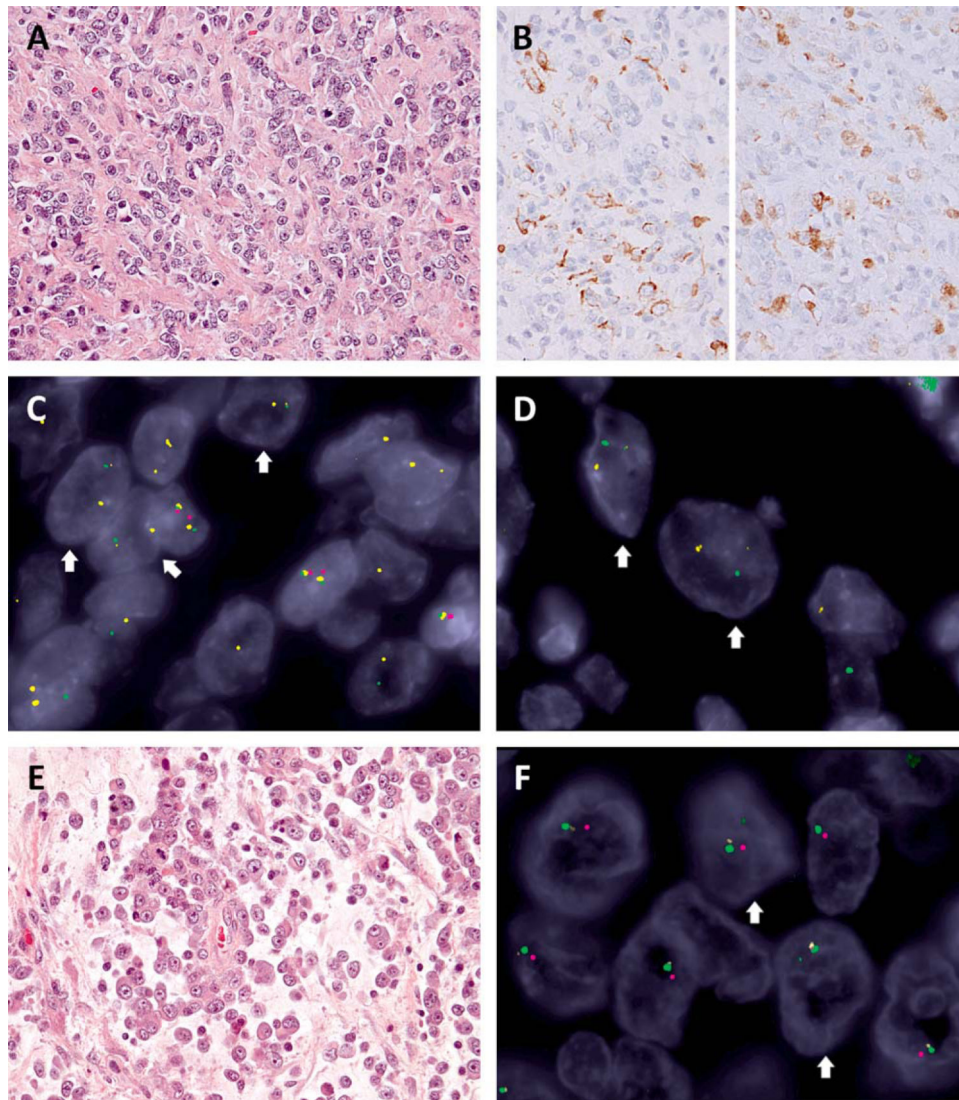


Figure 2.

Heterozygous *EWSR1* deletion in *SMARCB1*-deleted tumors. A: Myoepithelial carcinoma composed of relatively monotonous epithelioid to round cells arranged in cords in a collagenous stroma (Case 3, 400x) showing (B) focal reactivity for cytokeratin (left, 400x) and S100 (right, 400x). The 3-color FISH revealed homozygous *SMARCB1* deletions (lack of both red signals) with heterogeneous *EWSR1* abnormalities, including (C) heterozygous deletions (arrows: one allele retained the paired yellow-and-green signals but with diminished yellow intensity, indicating partial deletion of centromeric part, while the other allele showed loss of green signal, consistent with deletion of telomeric part) and (D) break-apart (arrows: yellow-and-green split signals of smaller size, in keeping with concurrent deletions of centromeric and telomeric parts outside the *EWSR1* gene locus). E: Proximal-type epithelioid sarcoma with myxoid changes and rhabdoid morphology (Case 4, 400x). F: The 3-color FISH showed heterozygous *SMARCB1* (one retained red signal) and *EWSR1* regional deletions (arrows: the involved allele containing a tiny residual green signal in

keeping with a larger deletion across centromeric and telomeric part). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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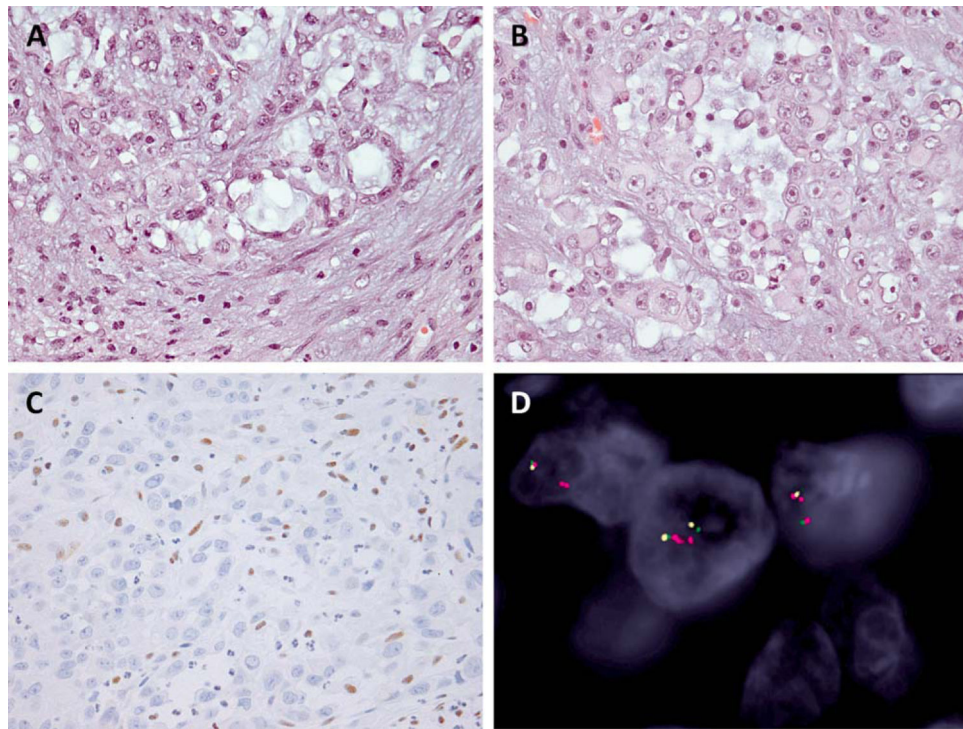


Figure 3. Metastatic renal medullary carcinoma. A,B: The lymph node biopsy showed epithelioid cells with light eosinophilic cytoplasm and scattered rhabdoid cells in a variably fibromyxoid stroma (200x, 400x). C: The SMARCB1 IHC was lost. D: The three-color FISH found two to four copies of intact *SMARCB1* gene. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Clinicopathologic and Genetic Summary of *SMARCB1*-Deleted Tumors with *EWSR1* FISH Alterations

TABLE 1

Case	Age (yrs)/Sex	Location	Initial diagnosis	Final diagnosis	<i>SMARCB1</i> deletion	<i>EWSR1</i> break apart FISH result	Other pertinent results
1	3/F	Clivus	Myoepithelial carcinoma	Poorly differentiated chordoma	Homozygous	Break-apart with deletion	T-brachyury IHC positive
2	5/M	Abdominal wall	Ewing sarcoma	Malignant rhabdoid tumor	Homozygous	Break-apart with deletion	<i>FLI1</i> , <i>ERG</i> FISH negative
3	16/F	Dura mater	Myoepithelial carcinoma	Myoepithelial carcinoma	Homozygous	Heterozygous deletion and break-apart	<i>POU5F1</i> , <i>PBX1/3</i> , <i>KLF17</i> FISH negative
4	21/M	Groin	Extraskeleral myxoid chondrosarcoma	Epithelioid sarcoma	Heterozygous	Heterozygous deletion	<i>NR4A5</i> FISH negative

FISH, fluorescence in situ hybridization; IHC, immunohistochemistry..