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High Sensitivity of FISH Analysis in Detecting Homozygous *SMARCB1* Deletions in Poorly Differentiated Chordoma: A Clinicopathologic and Molecular Study of 9 Cases

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Abstract

Poorly differentiated chordomas (PDCs) represent a rare subset of notochordal neoplasms, affecting primarily children and associated with an aggressive outcome. In contrast to conventional chordomas, PDC show solid growth and increased cellularity, cytologic atypia, and mitotic activity. Recent studies have shown that PDCs are characterized by recurrent deletions encompassing the *SMARCB1* locus, resulting in consistent loss of nuclear *SMARCB1* expression. Thus PDC joined the expanding family of *SMARCB1*-deficient tumors characterized by various *SMARCB1* structural abnormalities, ranging from large homozygous deletions to small intragenic mutations. In the present study we investigate the *SMARCB1* abnormalities in a group of 9 well characterized PDCs and to establish the sensitivity of FISH method in detecting these changes in the clinical setting. We further assessed the pathologic features and clinical behavior of this cohort managed at our referral center over a 20-year period. The mean age at diagnosis was 10 years-of-age. All except one case occurred in the cranial region. All demonstrated strong nuclear expression of brachyury and loss of *SMARCB1* expression. FISH identified homozygous *SMARCB1* deletions in all except one case; additionally 2 cases revealed a heterozygous *EWSR1* locus co-deletion. Clinical follow-up information was available in 5 patients. Two patients presented with distant metastases at initial diagnosis. Two of the 3 remaining patients with primary disease failed both locally and distantly after multimodality therapy. We conclude that PDC are highly aggressive tumors and the dominant mechanism of loss of *SMARCB1* expression is through large, homozygous *SMARCB1* deletions that can be readily detected by FISH.

Keywords

Poorly differentiated chordoma; Chordoma; Brachyury; *SMARCB1*; *INI1*

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1. INTRODUCTION

Chordomas are relatively common, malignant bone neoplasms arising from remnants of the notochord along the craniovertebral axis, commonly involving the clivus and sacrum.¹ Chordomas make up the 4th most frequent primary malignant bone tumor after osteosarcoma, chondrosarcoma, and Ewing sarcoma. Most chordomas occur in adults with a peak incidence in the 6th and 7th decades, with only a small subset, < 5%, arising in patients younger than 20 years of age.^{2, 3} Pediatric chordomas show a predilection for the cranial region and may exhibit increased cellularity, solid growth, cytologic atypia, and increased mitotic activity.⁴ Based on these histologic features, which are in sharp contrast with the low cellularity, physaliphorous cytology and myxoid matrix of typical chordomas, pediatric chordomas have been classified as “poorly differentiated chordoma”, to also emphasize their aggressive biologic behavior and a high incidence of metastases.^{4, 5}

Additionally, recent studies have shown that poorly differentiated chordomas (PDCs) are associated with recurrent deletions encompassing the *SMARCB1* locus, resulting in consistent loss of nuclear INI1/SMARCB1 expression and implicating this abnormality as the leading pathogenetic mechanism in these tumors.^{5–10} SMARCB1 (also known as INI1, hsnf5, or BAF47) is an ATP-dependent core subunit of the switch/sucrose non-fermenting complexes.^{11, 12} *SMARCB1* functions as a classic tumor suppressor gene, homozygous loss of function mutations/deletions having been described initially as the hallmark of atypical teratoid/malignant rhabdoid tumors (AT/MRT) at different sites (brain, soft tissue, and kidney).^{13, 14} Subsequently, loss of SMARCB1 function was identified in a variety of other tumors, such as epithelioid sarcomas, renal medullary carcinomas, myoepithelial carcinomas, epithelioid malignant peripheral nerve sheath tumors, sinonasal carcinomas, and a subset of GI tumors.^{15–18}

In this study, we sought to examine *SMARCB1* abnormalities in a large series of PDCs diagnosed and managed at a single tertiary cancer institution and to establish the sensitivity of FISH method in detecting these changes as an adjunct for diagnosis.

2. PATIENTS AND METHODS

2.1 Clinical Data

The study was approved by the Institutional Review Board of Memorial Sloan Kettering Cancer Center, New York. Cases of poorly dedifferentiated chordoma (PDC) were identified from our institutional and consultation files of the senior authors (CRA, MKR). The following clinical data was retrieved: age at diagnosis, gender, anatomic site, tumor size, history of initial metastasis before therapy, modality of therapy (surgical resection, radiotherapy, and chemotherapy), local recurrence, distant recurrence, vital status (alive or died of disease) and survival time.

2.2 Immunohistochemistry (IHC)

IHC for brachyury was reviewed in all cases. IHC for SMARCB1 was performed on 4-μm-whole sections of formalin-fixed, paraffin embedded tissue blocks. The primary antibody used was a mouse monoclonal anti-BAF47 antibody (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) as previously described.¹⁰

2.3 Fluorescence in situ hybridization (FISH)

FISH directed at chromosome 22q11-12 region was performed on cases with tissue available. FISH was performed on interphase nuclei using paraffin embedded 4- μ m-whole sections. FISH was performed by applying custom probes using bacterial artificial chromosomes (BACs), covering or flanking the *SMARCB1* and *EWSR1* genes (Supplementary Table 1). BAC clones were obtained from BACPAC sources of Children's Hospital of Oakland Research Institute (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. Two hundred successive nuclei were examined using a Zeiss fluorescence microscope (Zeiss Axioplan, Oberkochen, Germany), controlled by Isis 5 software (Metasystems, Newton, MA). Normal copy number pattern was defined when two copies of the *SMARCB1* gene were identified, with a 1:1 ratio to the control probe (i.e., telomeric-*EWSR1* or 22q11). Heterozygous deletion was defined as only one copy of the gene of interest being present compared to the reference control probe on 22q (ratio 2:1). Homozygous deletion of *SMARCB1* was interpreted when both copies of the gene were lost, compared to the control probes, either telomeric-*EWSR1* or 22q11. A monosomy pattern (or large deletion) was defined if one allele copy of both the gene of interest and control were lost, with a ratio of 1:1. In cases where both *SMARCB1* and *EWSR1* signals were lost concurrently, two additional control probes were used as reference on 22q11 as previously described.¹⁷

3 RESULTS

3.1 Clinicopathologic features

A summary of the clinical characteristics is presented in Table 1. Nine cases of poorly differentiated chordoma (PDC) were identified. One case was previously described in the study by Huang et al.¹⁰ There were 7 females and 2 males with ages ranging from 2 to 25 years (mean: 10), including 7 (78%) pediatric and 2 (22%) young adult patients. The anatomic sites comprised clivus (n=3), cervical spine (n=3), clivus and cervical spine (n=1), dura mater (n=1) and sacrum (n=1). Tumor size could be assessed in 6 patients, ranging from 3 to 7.5 cm (mean: 5.7). The MRI images reviewed showed lytic, destructive bone lesions. Two of the 5 patients with adequate clinical information presented at diagnosis with metastases to the lung (n=2) and pericardium (n=1). Five patients received surgery and radiation to the primary tumor site and additional systemic therapy, 4 of whom were enrolled on a clinical trial with an EZH2 inhibitor (Tazemetostat).

The microscopic appearances displayed a wide spectrum of growth patterns, including tumor cells arranged in solid nests or acinar-type formation (Fig 1). The cytomorphology varied from epithelioid with abundant eosinophilic to clear cytoplasm (Fig 2), rhabdoid with dense paranuclear inclusions (Fig 2), ovoid to short spindled-cells suggestive of sarcomatoid

growth (Fig 1). Scattered cells with empty cytoplasmic vacuoles, reminiscent of signet-ring or blister cells, were also detected in a subset of cases (Fig 1). Most cases showed mild to moderate nuclear pleomorphism, with irregular nuclear contours, often vesicular chromatin and visible nucleoli (Fig 1). Geographic areas of necrosis were present in all cases (Fig 2). Mitotic activity was low to intermediate in all cases with up to 5 MF/10 HPFs.

Immunohistochemically, all cases showed reactivity, often diffuse for various epithelial markers, including pan-cytokeratin AE1:AE3, CAM5.2 and EMA (Fig 3). Furthermore, all cases (n = 6) tested demonstrated strong nuclear expression for brachyury and all 9 cases showed loss of nuclear SMARCB1 expression (Fig. 3). FISH studies showed that all except one case of 8 evaluated harbored homozygous *SMARCB1* deletions (Fig. 4). The deletions varied in size, with 2 cases showing that *EWSR1* probe was heterozygously co-deleted with *SMARCB1*, in keeping with large deletions at chromosome 22q locus (Fig. 4). One case showed normal pattern, with two copies of the *SMARCB1*. Results are summarized in Table 2.

3.2 Follow-up data

Adequate therapeutic and follow-up information (Table 1) was available for 5 of the 9 patients, ranging from 14 to 76 months (median 23 months). Two patients presented with distant metastases at initial diagnosis. Two of the 3 remaining patients presenting with primary disease, failed both locally and distantly after surgical resection and radiation, developing local and distant (ribs, humerus and lung) recurrences. One additional patient (Case 6) without adequate therapeutic and follow-up information was reported to have developed distant recurrence. The metastatic rate was 87% (5/6) in the entire cohort and 75% (3/4) in the localized group. All 5 patients are alive with a median survival of 23 months; 4 alive with disease and 1 patient without evidence of disease.

4 DISCUSSION

Poorly differentiated chordoma (PDC) is a rare tumor frequently occurring in the pediatric age group and involving the cranial region.^{5, 7–9, 19} Reported cases of PDC have been associated with aggressive biologic behavior and a high incidence of distant metastasis.^{5, 8, 9} Recently, recurrent deletions at the *SMARCB1* locus, resulting in loss of nuclear INI1/SMARCB1 expression, have been described as the leading pathogenetic mechanism in PDC.⁵ Based on these reports, we carried out a clinicopathologic and molecular analysis of PDCs from our institutional and consultation files, all diagnosed in the last 2 decades. Our main goal was to evaluate the incidence and structural abnormalities of *SMARCB1* deletions in PDC and to establish if FISH methodology is the appropriate tool to investigate this genetic mechanism in the clinical setting. Second, we sought to evaluate the clinical, pathologic and survival findings in a homogeneous cohort of PDC diagnosed and managed at a single tertiary center. Similar to other reported series, our cases occurred predominately in children (7/9, 78%) and had a predilection for the cranial region (8/9, 89%).^{8, 9} As previously suggested, PDCs followed an aggressive clinical course with disease progression even after multimodality therapy.^{5, 7–9} In our series, 80% (4/5) of patients with available follow-up developed local recurrences after surgical removal and radiotherapy and 83%

(5/6) of patients developed distant metastases. In a recent study by Hasselblatt et al.⁹ (including cases from Chavez et al. and Mobley et al.),^{5, 7} all patients had disease progression after therapy and 86% died of disease, with a median overall survival of 9 months. Similarly, the study by Renard et al.⁸ reported 2 PDC patients with *SMARCB1* deletions, one developing distant recurrence 8 months after therapy and the other succumbing to disease only a few months after diagnosis. In contrast to these studies, the 5 patients with follow-up information are still alive at a median survival of 23 months. This favorable outcome may be explained due to the fact that 4 of these 5 patients were enrolled on an on-going clinical trial using EZH2 inhibitor (Tazemetostat) after initial therapy.

Previous studies have suggested that conventional chordomas are clinically, histopathologically and molecularly distinct from PDCs.^{5, 9} PDCs occur predominately in the pediatric age-group, while conventional chordomas typically affect older adults. PDCs are clinically more aggressive, with higher rates of distant metastasis and mortality. Morphologically, PDCs lack the myxoid matrix of conventional chordomas and are characterized instead by increased cellularity, solid growth, cytologic atypia, and increased mitotic activity. Immunophenotypically, PDCs demonstrate loss of *SMARCB1* expression, while conventional chordomas show nuclear retention of this marker.^{5, 9} Molecularly, PDCs harbor recurrent deletions encompassing the *SMARCB1* region, which are not detected in conventional chordomas.⁹ PDCs should not be confused with dedifferentiated chordomas, which represent conventional chordomas that have undergone progression to undifferentiated spindle cell sarcoma histology and lack *SMARCB1* gene abnormalities. A pathologic diagnosis of PDC should be considered in a child or young adult patient with a cranial region lesion, typically involving the clivus. Differential diagnosis would include other *SMARCB1*-deficient tumors, such as the AT/RT and epithelioid sarcoma. AT/RT is an aggressive, poorly differentiated pediatric tumor which typically involves the brain, but may secondarily invade the skull base and clivus. AT/RT is the prototypical lesion characterized by loss of function mutations involving the *SMARCB1* locus, resulting in loss of *SMARCB1* nuclear expression. Intragenic mutations and large deletions in the *SMARCB1* locus can be detected by FISH in AT/RT.²⁰ In contrast to AT/RT, PDCs express brachyury, a transcription factor required for differentiation and development of the notochord during embryogenesis.^{5, 9} Brachyury, a sensitive and specific marker for chordomas, is negative in AT/RT.^{5, 6, 9}

Epithelioid sarcoma (ES) is a rare and aggressive soft tissue neoplasm, both subtypes (proximal and distal) exhibiting *SMARCB1* deletions. The study by Le Loarer et al.¹⁷ identified 90% (36/40) of ES as having large, homozygous *SMARCB1* deletions detected by FISH. All (15) cases of distal ES showed homozygous deletions, while 84% (21/25) of cases of proximal ES harboring homozygous deletions. One proximal ES exhibited heterozygous deletion of *SMARCB1*, while remaining 3 cases had a normal *SMARCB1* pattern by FISH resolution.

SMARCB1 is 5.5 Mb away from the *EWSR1* gene on 22q11.23-12.2. In the setting of *SMARCB1*-deficient tumors harboring large deletions covering the *SMARCB1* locus, secondary abnormalities of the *EWSR1* gene locus can occur, including unbalanced rearrangements or deletions.^{5, 10, 17, 19} Thus, diagnostic pitfalls are encountered when a

FISH analysis for *EWSR1* gene abnormality is interpreted in isolation, without appreciation of the larger chromosomal deletion that may encompass this region. Thus, false positive FISH results for *EWSR1* gene rearrangements can occur in the setting of *SMARCB1* gene abnormalities/deletions, triggering misdiagnosis of a *EWSR1*-fusion positive tumor such as Ewing sarcoma or myoepithelial carcinoma. In fact, 2 of our PDCs exhibited heterozygous co-deletions of *EWSR1* locus in the background of homozygous *SMARCB1* deletions. One of these was previously reported,¹⁰ clival tumor, which had been misdiagnosed as myoepithelial carcinoma due to the positive FISH result for *EWSR1* gene rearrangement. On re-evaluation, complex *EWSR1* regional deletions rather than a balanced rearrangement were identified, which together with positive brachyury immunoexpression supported a diagnosis of PDC with co-deletions of *SMARCB1* and *EWSR1*. Furthermore, our previous study of *SMARCB1*-deleted epithelioid sarcomas showed that 25% of cases were associated with heterozygous telomeric *EWSR1* deletions.¹⁷ Similarly, co-deletions in *SMARCB1* and *EWSR1* genes can also occur in malignant rhabdoid tumor and myoepithelial carcinoma.¹⁰

PDC joins the expanding family of *SMARCB1*-deficient tumors that includes atypical teratoid/malignant rhabdoid tumors, epithelioid sarcomas, renal medullary carcinomas, myoepithelial carcinomas, epithelioid malignant peripheral nerve sheath tumors, sinonasal carcinomas, and a subset of GI tumors. Our study specifically showed loss of *SMARCB1* expression in 100% of PDCs and 87.5% (7/8) of evaluated PDC cases demonstrated homozygous deletions by FISH. One case exhibited intact copies of *SMARCB1* with loss of *SMARCB1* expression. The loss of *SMARCB1* expression in this case might be attributed either to intragenic mutations (beyond the FISH resolution) or to epigenetic silencing of *SMARCB1* by microRNA (miRNA) activation. In this regard, a recent study found upregulation of miR-671-5p and miR-193a-5p in PDC with loss of *SMARCB1* expression.²¹ Prior studies on epithelioid sarcomas have also identified epigenetic silencing of *SMARCB1* by functionally active miRNAs (miR-206, miR-381 and miR-671-5),^{22, 23} suggesting an alternative mechanism for loss of *SMARCB1* expression in cases lacking genetic evidence of *SMARCB1* abnormalities.

The study by Hasselblatt et al⁹ showed that *SMARCB1* deletions were identified in 4/7 (57%) PDC cases by FISH, but only in 2/7 (29%) cases by multiplex ligation-dependent probe amplification (MLPA), suggesting that FISH is more sensitive method than MLPA in the context of large homozygous deletions of PDC. In addition, the study by Sápi et al.²³ showed that FISH method detected the presence of deletions in 95% of epithelioid sarcoma cases, while MLPA only in 73% of cases. These findings further confirm our prior results showing high sensitivity of FISH studies to detect homozygous deletions in epithelioid sarcomas compared to MLPA.¹⁷ In contrast to PDC and epithelioid sarcomas in which most *SMARCB1* genetic abnormalities represent large, homozygous deletions, the spectrum of alterations in rhabdoid tumors are relatively evenly distributed between whole gene deletions, intragenic deletions and duplications, nonsense mutations and frameshift mutations.

In summary, our results reveal that the dominant mechanism of *SMARCB1* loss of expression in PDC is through large, homozygous deletions at the 22q11 locus which can be detected by FISH in the clinical setting. In addition, our study further documents the high

likelihood of regional recurrence and distant metastasis attaching to this tumor type even after multimodality therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Histologic spectrum of PDC

Tumors showed a variable growth pattern, ranging from solid (A), nested (B), pseudoalveolar (C) and pseudosarcomatoid (vague fascicles)(D). Rare cells with intra-cytoplasmic vacuoles, reminiscent of signet ring cells, are noted in A.

Figure 2. Cytomorphologic spectrum in PDC

Most tumors showed an epithelioid phenotype, with variable degree of nuclear pleomorphism (A), distinctive rhabdoid cells with dense eosinophilic cytoplasm and eccentric nuclei (B), or abundant clear cytoplasm, reminiscent of clear cell carcinoma (C). Geographic areas of necrosis were invariably present in all cases (D).

Figure 3. Immunoprofile of PDC

Tumor cells showed diffuse reactivity for AE1:AE3 (A), EMA (B), nuclear expression of Brachyury (C) and showed loss of SMARCB1 (INI1) expression (D).

Figure 4. FISH showing *SMARCB1* homozygous deletion associated with a heterozygous co-deletion of *EWSR1*

Custom BAC probes showed lack of both *SMARCB1* signals (red) with retention of one of reference probe (green, telomeric-*EWSR1*). Lower portion of the image shows 2 normal cells with 2 pairs of red and green signals each (with a small, normal gap in between).

Table 1

Clinical features and follow-up information of poorly differentiated chordomas

Case #	Age/Sex	Site	Size (CM)	Initial metastasis (site)	Therapy ^a	Recurrence (site)	Follow-up duration	Vital status
1	2/F	Clivus	4	Y (Lung)	S, RT, CT	LR	28 mos	Alive (AWD)
2	8/F	Cervical spine	3.8	N	S, RT, CT	LR, DR (rib, humerus, lung)	23 mos	Alive (AWD)
3	21/F	Cervical spine	7.5	N	S, RT, CT	LR, DR (rib)	14 mos	Alive (AWD)
4	17/M	Cervical spine	7	N	S, RT, CT	NR	76 mos	Alive (NED)
5	25/F	Sacrum	5.8	Yes (Lung/pericardium)	CT, RT, S	LR	23 mos	Alive (AWD)
6	3/F	Clivus	NA	N	NA	DR	NA	NA
7	7/F	Dura mater	5.9	NA	NA	NA	NA	NA
8	3/M	Clivus/cervical spine	NA	NA	NA	NA	NA	NA
9	4/F	Clivus	NA	NA	NA	NA	NA	NA

S – surgery; RT – radiotherapy; CT – chemotherapy; LR – local recurrence; DR – distant recurrence; NR – no recurrence; AWD – alive with disease; NED – no evidence of disease; NA – not available; Y – yes; N – no

^aTherapy in bold was the first modality administered

Table 2

Immunohistochemistry (IHC) and Fluorescence in situ hybridization (FISH) of PDC

Case #	Brachyury (IHC)	SMARCB1 (IHC)	SMARCB1 (FISH)	EWSR1 abnormality
1	+	Loss	Homozygous deletion	-
2	+	Loss	Homozygous deletion	Heterozygous deletion
3	+	Loss	Intact	-
4	ND	Loss	Homozygous deletion	-
5	+	Loss	ND	-
6	+	Loss	Homozygous deletion	Heterozygous deletion
7	+	Loss	Homozygous deletion	-
8	ND	Loss	Homozygous deletion	-
9	ND	Loss	Homozygous deletion	-

ND – not done; (+) – positive; (–) – negative