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# Epithelioid sarcoma is associated with a high percentage of *SMARCB1* deletions

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## Abstract

*SMARCB1* gene alterations were first described in highly malignant rhabdoid tumors of the kidney, brain (atypical teratoid/rhabdoid tumor) and soft tissue. An increasing number of tumors have now shown loss of SMARCB1 protein expression by immunohistochemistry including the majority of epithelioid sarcomas. However, investigations of *SMARCB1* gene alterations in epithelioid sarcoma have produced conflicting results. The aim of this study was to evaluate *SMARCB1* status using Sanger sequencing of the coding region and multiplex ligation dependent probe amplification, a rapid and sensitive method for detecting intragenic deletions and duplications, which has not been used in previous studies. Twenty-one epithelioid sarcomas of both classical and proximal type were selected for *SMARCB1* gene testing and SMARCB1 immunohistochemistry. Nineteen of 21 (90%) epithelioid sarcomas were SMARCB1 negative by immunohistochemistry. Twelve of the 19 (63%) had adequate DNA recovery for evaluation. Ten of 12 (83%) tumors showed homozygous deletions of the gene. Two cases showed heterozygous deletions and polymorphisms, but no sequence mutations. These results confirm the high frequency of *SMARCB1* deletions in epithelioid sarcoma and show that multiplex ligation dependent probe amplification is a reliable method for detection of deletions in these cases which can be performed on formalin-fixed, paraffin-embedded tissue. Given the high percentage of *SMARCB1* alterations in epithelioid sarcoma, these findings argue against using *SMARCB1* gene deletion as a tool in distinguishing them from malignant rhabdoid tumors.



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**Disclosure/Conflicts of Interest**

The authors have no conflicts of interest.

**Keywords**

Epithelioid sarcoma; malignant rhabdoid tumor; *SMARCB1*

## Introduction

Malignant rhabdoid tumor is a highly aggressive childhood neoplasm that occurs in the kidney, liver, soft tissue and central nervous system. Classically, these tumors exhibit varying numbers of rhabdoid cells with eccentric nuclei containing prominent nucleoli and abundant cytoplasm with eosinophilic globular cytoplasmic inclusions. The diagnosis of malignant rhabdoid tumor was revolutionized by the discovery of *SMARCB1* alterations in these tumors with subsequent loss of protein expression detectable by immunohistochemistry (1–4). Comprehensive genomic analysis using high density single nucleotide polymorphism based oligonucleotide arrays, multiplex ligation dependent probe amplification and coding sequence analysis has shown that 98% of malignant rhabdoid tumor, atypical teratoid/rhabdoid tumor and extra renal rhabdoid tumors have bi-allelic *SMARCB1* alterations with few additional karyotypic abnormalities (5, 6).

It is becoming increasingly clear that loss of SMARCB1 protein expression is not exclusively limited to malignant rhabdoid tumor, as an increasing number of tumors have been described which are also negative for SMARCB1 by immunohistochemistry. Soft tissue tumors comprise the majority of cases, and include epithelioid sarcoma, schwannomas associated with germline *SMARCB1* abnormalities, and a subset of cases diagnosed as epithelioid malignant peripheral nerve sheath tumors, pediatric undifferentiated sarcomas, synovial sarcomas, myoepithelial carcinomas and extraskeletal myxoid chondrosarcomas (6–12). While the percentage of negative cases varies by tumor type, both proximal and classical type epithelioid sarcomas have consistently shown loss of staining in the majority of cases (8).

Classical and proximal type epithelioid sarcomas occur most often in adolescents and young adults as compared to early childhood for malignant rhabdoid tumor. Classical type epithelioid sarcoma is composed of plump epithelioid to spindled cells and usually exhibits a nodular growth pattern with hyalinized collagen and central necrosis, at times simulating a benign necrobiotic process. Proximal type epithelioid sarcoma is most often seen in the pelvis, perineal region and genital tract. Histologically, it is characterized by large cells with epithelioid cytomorphology, marked cytologic atypia with frequent rhabdoid features and lack of a granuloma-like pattern (13).

Regardless of the classical or proximal type designation, these tumors express epithelial and mesenchymal markers with loss of SMARCB1, reminiscent of malignant rhabdoid tumor (8, 13, 14). However, expression of CD34 in approximately half of all epithelioid sarcomas may aid in their distinction from malignant rhabdoid tumor which are CD34 negative (14, 15). While many malignant rhabdoid tumors and epithelioid sarcomas can be distinguished based upon anatomic location, histologic features, patient demographics and CD34 immunoreactivity, this is not true in all instances. Particularly challenging are tumors in older children and adolescents in axial locations, which show overlapping features of

malignant rhabdoid tumor and proximal epithelioid sarcoma. While some may argue that proximal epithelioid sarcoma and malignant rhabdoid tumor represent different manifestations of the same tumor, differential CD34 expression and the rarity of epithelioid sarcoma in adolescents and young adults with germline *SMARCB1* mutations support these tumors as distinct entities (6, 14, 15). Furthermore, malignant rhabdoid tumors most often show isolated 22q abnormalities whereas many proximal and classical epithelioid sarcomas have chromosome 22 abnormalities in addition to a more complex karyotype including abnormalities on chromosomes 6, 7, 8, 14, 18, 20 and 21 (5, 16–18).

Investigations of *SMARCB1* gene alterations in epithelioid sarcoma have shown conflicting results. Previously reported data from 3 studies which cumulatively included 65 SMARCB1 negative tumors by immunohistochemistry showed that the percentage of cases with homozygous *SMARCB1* deletions ranged from 5–71% (19–21). Of note, these investigators employed a range of techniques including combinations of fluorescence in situ hybridization, array comparative genomic hybridization, and quantitative polymerase chain reaction. In addition to chromosome 22 deletions, there are also 3 reports of *SMARCB1* inactivating mutations in epithelioid sarcoma, including 2 frameshift and 1 nonsense mutations (20, 22).

None of the previous studies used multiplex ligation dependent probe amplification, which allows for multiple specific nucleic acid sequences to be amplified simultaneously using a single polymerase chain reaction. Multiplex ligation dependent probe amplification for the nine exons of *SMARCB1* as well as probes proximal and distal on 22q is currently used as a clinical test for the diagnosis of malignant rhabdoid tumor at our institution. The advantage of this method is that it is rapid, with an overnight turn around time. It is also the most sensitive method for detecting 0, 1, 2 or 3 copies of a given target sequence in a DNA specimen (5). Commercial probe mixes (MRC-Holland, Amsterdam, the Netherlands) allow for the interrogation of 45–50 sequences simultaneously. In most cases, formalin-fixed, paraffin-embedded tissue can be used in this assay.

The aim of this study was to better establish the percent and spectrum of *SMARCB1* gene abnormalities in epithelioid sarcoma, using sequencing to identify mutations, and multiplex ligation dependent probe amplification for the detection of small deletions and duplication within the *SMARCB1* gene (5, 23). The *SMARCB1* results for epithelioid sarcoma were compared to previously published data on malignant rhabdoid tumor.

## Materials and Methods

### Cases

This study was conducted under the auspices of The Children’s Hospital of Philadelphia and Mayo Clinic Institutional Review Boards approval for the use of human tissues. Twenty-one formalin-fixed, paraffin-embedded epithelioid sarcoma cases diagnosed between 1994 and 2008 were selected from the files of the Department of Pathology and Laboratory Medicine, Mayo Clinic and used for *SMARCB1* gene testing and immunohistochemistry. Routine hematoxylin and eosin staining, SMARCB1 immunohistochemistry and pathology reports were reviewed for each tumor to confirm the diagnosis and ensure adequate tissue for

examination. Patient demographic and SMARCB1 staining information are shown in Table 1.

### Immunohistochemical staining and scoring

SMARCB1 antibody (BAF47, BDBioscience 612110) staining, deparaffinization, epitope retrieval with pH 9 buffer and immunohistochemistry at an antibody concentration of 1:100 were performed on the Leica Bond-Max Autostainer using the Bond Polymer Refine Detection System (Leica Microsystems, Bannockburn, IL). Nuclear staining for SMARCB1 was evaluated as positive (normal) or negative. Non-neoplastic tissue and infiltrating lymphocytes on the slide served as internal positive controls.

### *SMARCB1* gene evaluation

Cases were selected for DNA analysis if they were SMARCB1 negative by immunohistochemistry, and if there were available scrolls of formalin-fixed, paraffin-embedded tissue composed of at least 50% tumor based upon review of corresponding hematoxylin and eosin stained slides. DNA was extracted from scrolls using the RecoverAll Total Nucleic Acid Isolation Kit for Formalin-Fixed, Paraffin-Embedded Tissues (Applied Biosystems/Ambion, Austin, TX) according to the manufacturer's protocol. DNA was then quantitated using a Nanodrop ND-1000 UV-Vis Spectrophotometer (Nanodrop Technologies, Wilmington, DE). Multiplex ligation dependent probe amplification was performed with genomic DNA using the SALSA MLPA P258 (SMARCB1) kit (MRC-Holland) according to previously published methods based on the manufacturer's protocol (5). Briefly, the kit contains 2 probes for each of the 9 exons of *SMARCB1*, probes for 9 other genes on chromosome 22, and 14 control probes from other chromosomes. The samples were processed and data analyzed as previously described (24).

Polymerase chain reaction-based mutation analysis was performed as reported previously using primers for exons 1–9 of the *SMARCB1* gene (1). Polymerase chain reaction products for individual exons were sequenced utilizing the BigDye Terminator v3.1 Cycle Sequencing Kit from Applied Biosystems as per the manufacturer's protocol. Sequencing products were analyzed on a 3730 DNA Analyzer (Applied Biosystems/Ambion) by The Children's Hospital of Philadelphia Nucleic Acid/Protein Core.

## Results

### Immunohistochemical staining in epithelioid sarcomas

Results of the SMARCB1 immunohistochemistry staining are shown in Table 1. Nineteen of 21 cases (90%) were negative for SMARCB1, which is similar to previously published data (8). Figure 1 shows examples of SMARCB1 negative and positive cases.

### *SMARCB1* gene alterations

Twelve of the 19 (63%) SMARCB1 negative cases had an adequate tumor percentage and adequate DNA recovery to perform multiplex ligation dependent probe amplification and sequence analysis. Results are shown in Table 2. Ten of the cases (83%) were characterized by homozygous deletions of at least 2 exons within the *SMARCB1* gene. Figure 2

demonstrates multiplex ligation dependent probe amplification results from case 15 with a homozygous deletion. The remaining 2 cases (16%) showed heterozygous deletions of all 9 exons. Polymerase chain reaction-based sequencing of the 2 cases with loss of only one copy of *SMARCB1* failed to show a mutation in the coding region of the gene, and thus the second inactivating event was not identified.

## Discussion

While approximately 98% of malignant rhabdoid tumors harbor bi-allelic *SMARCB1* gene abnormalities with few additional karyotypic abnormalities, there is conflicting data for epithelioid sarcoma (5, 19–22). The first major study to evaluate *SMARCB1* in epithelioid sarcoma was by Modena et al in 2005 (19). Of 7 (5 proximal and 2 classical) immunohistochemistry negative cases, 5 (71%) proximal epithelioid sarcomas demonstrated homozygous deletions of *SMARCB1* using bacterial artificial chromosome probes for dual-color fluorescence in situ hybridization. Two of the cases with homozygous deletions were also tested using comparative genomic hybridization, with one case showing a heterozygous deletion and the other failing to demonstrate any deletions. The discrepancy in the fluorescence in situ hybridization and comparative genomic hybridization results was likely due to the comparative genomic hybridization resolution of 2–4 Mb in this study. Semi-quantitative polymerase chain reaction of *SMARCB1* exon 1was performed on 4 of the cases with homozygous deletions and showed concordant results. No mutations were identified by sequencing.

In 2009 Kohashi et al. studied 39 immunohistochemistry-negative epithelioid sarcomas (19 proximal and 27 classical) (20). Deletions were identified by quantitative real-time polymerase chain reaction for exons 1 to 9 of the *SMARCB1* gene. Only 2 (5%) proximal epithelioid sarcoma cases showed homozygous deletions. Sequencing was performed for mutation analysis and 2 (5%) proximal epithelioid sarcomas showed homozygous frameshift mutations consisting of a one base pair deletion in exon 9 and a two base pair deletion in exon 3. Following this article, a correspondence was written by Flucke et al. describing a case of proximal epithelioid sarcoma with a c.769C>T mutation in exon 6 resulting in the generation of an in-frame stopcodon (22).

Most recently in 2011, Gasparini et al. evaluated 19 immunohistochemistry-negative epithelioid sarcomas (5 proximal and 14 classical) (21). As tabulated in the article, a total of 11 cases (58%, 4 proximal and 7 classical) were shown to harbor homozygous deletions of *SMARCB1* with 9 identified through fluorescence in situ hybridization and an additional 2 by quantitative polymerase chain reaction for exon 4. No mutations were identified through sequencing. This was the first series to demonstrate *SMARCB1* deletions in both proximal and classical epithelioid sarcoma.

Taken together, the above studies demonstrate that *SMARCB1* deletions are far more common than gene mutations in epithelioid sarcoma; however, the exact number of cases with primary gene abnormalities varies dramatically between published reports. In the present study, 12 immunohistochemistry-negative epithelioid sarcomas (7 classical, 5 proximal) had at least 50% tumor in the sample and adequate DNA recovery for multiplex

ligation dependent probe amplification and sequencing. Multiplex ligation dependent probe amplification revealed that 10/12 (83%, 6 classical, 4 proximal) harbored homozygous deletions. This percentage is similar to what we reported for extrarenal rhabdoid tumors but higher than that seen in CNS atypical teratoid/rhabdoid tumor or renal malignant rhabdoid tumor (6). Of note, 2 of the 10 cases (20%) had a homozygous deletion spanning a small portion of the gene, either exons 4 to 5 or exons 6 to 9. These cases would likely have been missed using traditional fluorescence in situ hybridization techniques or limited quantitative polymerase chain reaction including only a subset of exons. Of the 2 remaining cases (17%, 1 classical, 1 proximal), multiplex ligation dependent probe amplification showed heterozygous deletions involving all exons of the *SMARCB1* gene. No mutations were identified through sequencing.

The finding of this high rate of bilallelic loss or inactivation of *SMARCB1* in a high percentage of both proximal and classical epithelioid sarcoma tumors tested is consistent with the two hit model of carcinogenesis associated with loss of function of a tumor suppressor gene, and correlates with the near universal biallelic loss or inactivation of *SMARCB1* seen in malignant rhabdoid tumor. Although 2 of our cases only showed heterozygous deletions, the loss of SMARCB1 protein expression suggests the presence of an undetected abnormality of *SMARCB1* in the other allele. Possible explanations for the failure to demonstrate this may be due to the high percentage of normal stromal cells in the samples, because a high percentage of normal cell contamination can mask a deletion using multiplex ligation dependent probe amplification. Despite the fact that hematoxylin and eosin stained sections of each tumor were evaluated for percentage of tumor present, increased proportions of normal cells could have been present in the tissue scrolls used for DNA extraction. As a result, the 2 cases with heterozygous deletions may represent false negative results in tumors with true homozygous deletions. One of the 2 cases had additional formalin-fixed, paraffin-embedded tissue available for fluorescence in situ hybridization testing, which would have allowed for evaluation of SMARCB1 copy number limited exclusively to the tumor tissue. However, the fluorescence in situ hybridization assay was unsuccessful. Alternatively, there may have been an intronic mutation present that would have been missed by only analyzing the exons and intron/exon boundaries.

It is important to note that the high percentage of cases with homozygous deletions, relatively low number of cases with intragenic deletions and subset of cases where only a single gene defect could be identified raises the possibility that loss of SMARCB1 protein expression may be a secondary event in epithelioid sarcoma. The driving force for chromosome 22 deletions seen in epithelioid sarcoma may actually result from selection of a nearby gene instead of *SMARCB1*. Additionally, an intronic or upstream change that affects expression of the SMARCB1 protein also cannot be excluded based upon our data.

The results from this study and others suggest that somatic *SMARCB1* status alone is not sufficient to differentiate malignant rhabdoid tumor from epithelioid sarcoma. As a result, the finding of bi-allelic mutations or deletions in *SMARCB1* should not be used as criteria to support reclassifying epithelioid sarcoma as malignant rhabdoid tumor. Being able to reliably differentiate epithelioid sarcoma and malignant rhabdoid tumor is important due to differences in treatment, prognosis and the association of malignant rhabdoid tumor with

germline mutations necessitating additional genetic testing and family counseling (6, 25–27). Despite intensive treatment for patients with malignant rhabdoid tumor, many patients die of widespread metastatic disease, especially those with germline mutations and second primary lesions. For example, one recent study limited to extra-renal non-cerebral rhabdoid tumors demonstrated a median time to recurrence of 5 months with only 1 of 26 (4%) patients alive and without evidence of disease at 7 years (28). Currently, many patients with soft tissue malignant rhabdoid tumor are enrolled in Children's Oncology Group protocol AREN0321 which employee a highly aggressive initial multimodality approach including surgery, radiation therapy and multiagent chemotherapy using vincristine, doxorubicin, cyclophospamide, carboplatin and etoposide, but results of patient outcome are unknown at this time.

In contrast, the overall 5-year survival rates for classical epithelioid sarcoma range from 50–85% and 10-yr survival rates range from 42–55% (18). The propensity of epithelioid sarcoma to grow along fascial planes, tendons and nerves often results in initial resections with positive margins and a protracted clinical course including multiple local recurrences and metastasis (26). Adequate treatment for epithelioid sarcoma requires early radical local excision or amputation with regional lymph node dissection. The specific regimen for radiation therapy and adjuvant chemotherapy is unclear, but is often similar to treatment plans for other adult sarcomas (29, 30).

The survival rate for proximal epithelioid sarcoma is challenging to define due to difficulties in separating these lesions from malignant rhabdoid tumor in many studies and the fact that many of the reported adverse prognostic features for classical epithelioid sarcoma are often seen in proximal epithelioid sarcoma, including non-distal extremity location, large tumor size, increased tumor depth and inadequate initial excision (26, 27, 31). Until recently, many used *SMARCB1* deletions or absence of SMARCB1 protein expression by immunohistochemistry as evidence for the diagnosis of malignant rhabdoid tumor, especially in children. However, studies of firmly diagnosed epithelioid sarcoma, including those in older patients, have shown *SMARCB1* abnormalities as well, suggesting that *SMARCB1* status alone cannot reliably distinguish epithelioid sarcoma from malignant rhabdoid tumor (19–21).

Our study demonstrates that the majority of both proximal and classical epithelioid sarcoma have homozygous deletions of *SMARCB1*, which can be identified with multiplex ligation dependent probe amplification. Given the high percentage of *SMARCB1* alterations in epithelioid sarcoma, these findings argue against using *SMARCB1* gene deletion to distinguish epithelioid sarcoma from malignant rhabdoid tumor (20). Moreover, these findings suggest an underlying genetic relationship between epithelioid sarcoma and malignant rhabdoid tumor. Further study is warranted to explore why these two tumors which so closely share phenotypic and genetic features behave in clinically distinctive ways.

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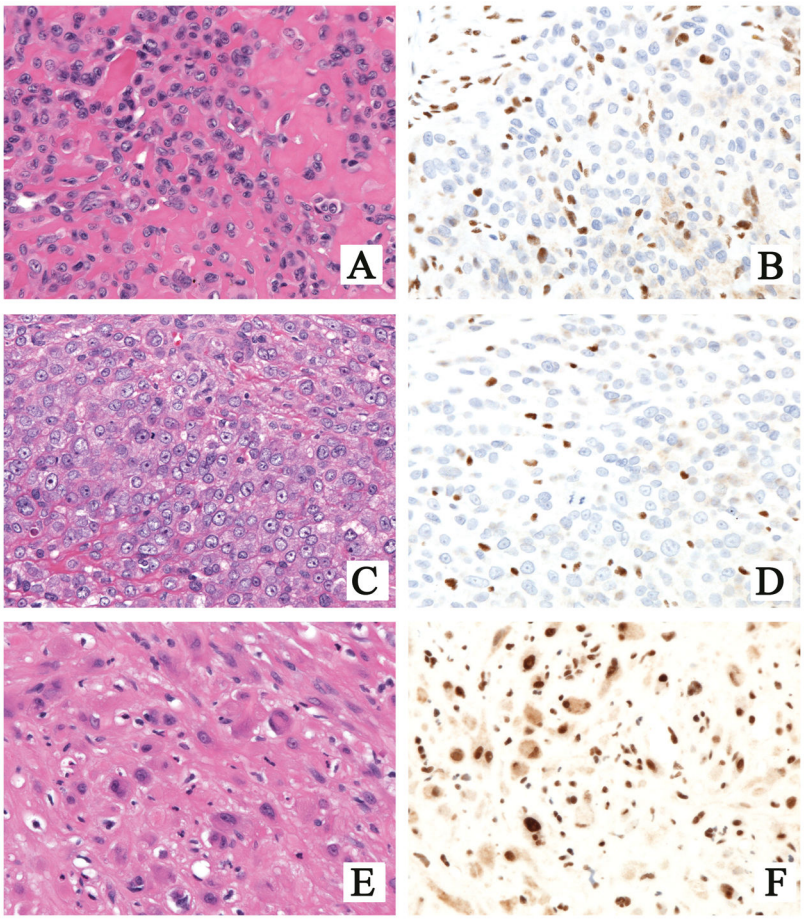


**Figure 1.**

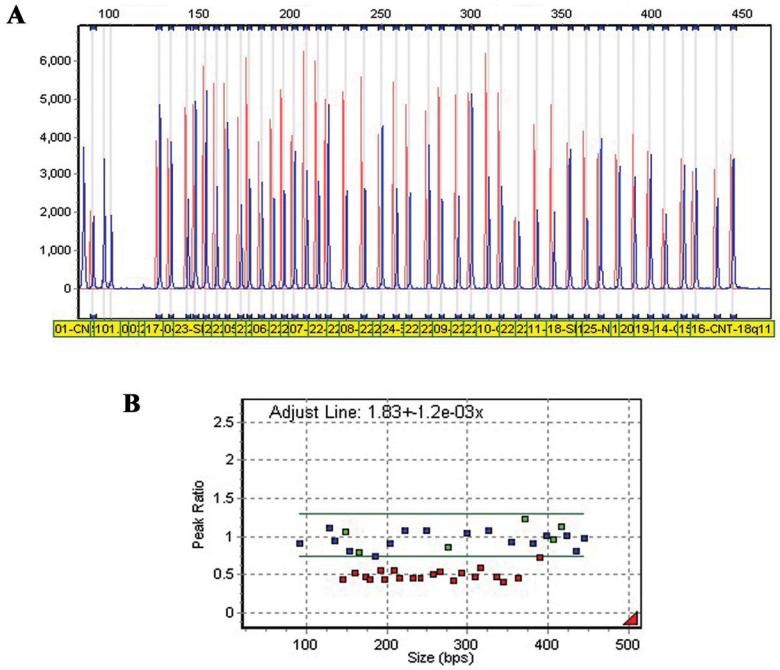


**Figure 2.**

**Table 1**

Epithelioid Sarcoma: Patient Demographics and SMARCB1 Staining

| Case | Diagnosis | Sex | Age at Diagnosis (years) | Location | SMARCB1 |
|---|---|---|---|---|---|
| 1 | Classical | Female | 40 | Hand | Negative |
| 2 | Classical | Male | 18 | Leg | Negative |
| 3 | Proximal | Male | 17 | Perineum | Negative |
| 4 | Classical | Female | 40 | Hand | Negative |
| 5 | Proximal | Male | 45 | Unknown | Negative |
| 6 | Proximal | Male | 37 | Unknown | Negative |
| 7 | Classical | Male | 42 | Arm | Negative |
| 8 | Classical | Female | 40 | Hand | Negative |
| 9 | Classical | Male | 58 | Arm | Negative |
| 10 | Proximal | Male | 49 | Groin | Negative |
| 11 | Proximal | Male | 37 | Inguinal | Negative |
| 12 | Classical | Male | 32 | Shoulder | Negative |
| 13 | Proximal | Male | 20 | Scrotum | Negative |
| 14 | Classical | Female | 24 | Thigh | Negative |
| 15 | Classical | Female | 25 | Hand | Negative |
| 16 | Classical | Male | 38 | Forearm | Negative |
| 17 | Classical | Male | 18 | Shin | Negative |
| 18 | Classical | Male | 56 | Arm | Negative |
| 19 | Classical | Male | 22 | Forearm | Negative |
| 20 | Proximal | Male | 48 | Buttock | Positive |
| 21 | Classical | Female | 72 | Foot | Positive |

## Table 2

### SMARCB1

Gene Alterations in 12 Epithelioid Sarcomas

| Case Number | Subtype | Multiplex Ligation Dependent Probe Amplification | Sequencing |
|---|---|---|---|
| 2 | Classical | Homozygous deletion, Exons 1–9 | Not done |
| 3 | Proximal | Homozygous deletion, Exons 1–9 | Not done |
| 5 | Proximal | Homozygous deletion, Exons 1–9 | Not done |
| 6 | Proximal | Heterozygous deletion, Exons 1–9 | Intron 5 polymorphisms: c.628+66G>C c.628+118C>T |
| 7 | Classical | Homozygous deletion, Exons 1–9 | Not done |
| 9 | Classical | Homozygous deletion, Exons 1–9 | Not done |
| 10 | Proximal | Homozygous deletion, Exons 4–5 | Not done |
| 11 | Proximal | Homozygous deletion, Exons 6–9 | Not done |
| 12 | Classical | Heterozygous deletion, Exons 1–9 | Intron 5 polymorphisms: c.628+66G>C c.628+118C>T Exon 7 polymorphism: c.897G>A Intron 7 polymorphism: c.986+56_986+57dupAA |
| 15 | Classical | Homozygous deletion, Exons 1–9 | Not done |
| 17 | Classical | Homozygous deletion, Exons 1–9 | Not done |
| 18 | Classical | Homozygous deletion, Exons 1–9 | Not done |