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ORIGINAL PAPER

Spindle Epithelial Tumor with Thymus-Like Differentiation (SETTLE): A Next-Generation Sequencing Study

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Abstract
Spindle epithelial tumor with thymus-like differentiation (SETTLE) is a malignant biphasic neoplasm of the thyroid or neck with propensity for late metastasis. Unlike synovial sarcoma, its main morphologic mimic, SETTLE lacks synovial sarcoma-associated translocations. A single case of SETTLE has shown a *KRAS* mutation but to date no comprehensive next generation sequencing studies of this rare neoplasm have been undertaken. Herein, we subjected 5 well defined cases of SETTLE to direct sequence analysis of 592 genes and fusion gene analysis of 52 genes frequently rearranged in human cancers. We identified one case with two pathogenic variants in the *KMT2D* gene, one being in an intron splice site (c.674-1A>G) and the other being a frameshift variant (p.M2829fs). This same case also had a pathogenic nonsense variant in the *KMT2C* gene (p.R1237*). A second case of SETTLE carried a pathogenic *NRAS* missense variant, Q61R. No other molecular alterations, microsatellite instability, gene fusions or amplifications were identified.

Keywords SETTLE · Next-generation sequencing · Molecular diagnostics · Thyroid

Introduction

Spindle epithelial tumor with thymus-like differentiation (SETTLE) is an extremely rare, morphologically biphasic malignant tumor of the thyroid or neck, which typically occurs in young patients and has a propensity for late metastasis [1, 2]. The molecular pathogenesis of SETTLE has not been studied in depth, although it is known that they lack synovial sarcoma-associated molecular events, and a single example with a *KRAS* mutation has been reported [2, 3].

We studied a series of well-characterized SETTLEs by next generation sequencing (NGS) with the goals of identifying specific genetic events and/or "actionable" mutations.

Materials and Methods

Approval for this study was granted by the Institutional Review Boards of all participating institutions. Five morphologically and immunohistochemically typical cases of SETTLE (Case 1 from UAB; Cases 2–5 from the files of ALF; Case 4 and Case 5 were previously published as "Case 2" and "Case 7", respectively, in Folpe et al. [2]) were included in this study. All cases were reviewed and diagnosis confirmed by an expert soft tissue pathologist (ALF).

Immunohistochemical Studies

Methods and results of immunohistochemistry performed on Cases 4 and 5 can be found in Folpe et al. [2]. Case 1 underwent a battery of immunohistochemical stains on either a Ventana Benchmark XT (Tucson, AZ) or Dako Autostainer (Dako Autostainer Link 48, Carpinteria, CA) (Table 1 for antibody details).

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Table 1 Details and conditions of immunohistochemistry stains used on Case 1

Antibody	Autostainer	Manufacturer	Details
Pan-cytokeratin (AE1/AE3)	Ventana Benchmark XT	Ventana	Polyclonal, predilute
CK5/6	Dako Autostainer	Dako	Close D5/16 B4; 1:200
CK7	Ventana Benchmark XT	Ventana	Clone SP52,predilute
CK20	Ventana Benchmark XT	Ventana	Clone SP33, predilute
p63	Ventana Benchmark XT	Ventana	Clone 4A4, predilute
MOC31	Dako Autostainer	Dako	Clone MOC31, 1:25
EMA	Ventana Benchmark XT	Cell Marque (Rocklin, CA)	Clone E29, predilute
INI-1	Dako Autostainer	Cell Marque	Clone MRQ-27, 1:50
Calcitonin	Ventana Benchmark XT	Ventana	Polyclonal, predilute
Calretinin	Ventana Benchmark XT	Ventana	Polyclonal, predilute
Mesothelin	Dako Autostainer	LabVision	Clone 5B2, 1:40
WT-1	Dako Autostainer	Dako	Clone 6F-H2, predilute
D2-40	Dako Autostainer	Dako	Clone D2-40, 1:50
CD34	Dako Autostainer	Dako	Clone QBEnd10, predilute
STAT6	Dako Autostainer	Abcam	Clone YE361, 1:800
C-KIT	Ventana Benchmark XT	LabVision	Polyclonal, 1:400
S100	Ventana Benchmark XT	Dako	Polyclonal, predilute
Myogenin	Dako Autostainer	Dako	Clone F5D, predilute
CD5	Ventana Benchmark XT	Ventana	Clone 4C7, predilute
TdT	Ventana Benchmark XT	Cell Marque	Polyclonal, predilute
Alpha feto-protein	Ventana Benchmark XT	Ventana	Clone C3, predilute
Glypican 3	Dako Autostainer	Cell Marque	Clone 1G12, 1:200
PLAP	Ventana Benchmark XT	Cell Marque	Clone NB10, predilute

Molecular Studies

Next Generation Sequencing

All cases underwent direct sequence analysis of 592 genes (list of genes in Supplement 1) performed on genomic DNA isolated from formalin fixed paraffin embedded (FFPE) tumor samples using the Illumina NextSeq platform (Illumina, Inc., San Diego, CA). Matched normal tissue was not sequenced. A custom-designed SureSelect XT assay was used to enrich 592 whole-gene targets (Agilent Technologies, Santa Clara, CA). All variants were detected with > 99% confidence based on allele frequency and amplicon coverage, with an average sequencing depth of coverage of > 500 and an analytic sensitivity of 5%. Prior to molecular testing, tumor enrichment was achieved by harvesting targeted tissue using manual microdissection techniques. Genetic variants identified were interpreted by board-certified molecular geneticists and categorized as 'pathogenic,' 'presumed pathogenic,' 'variant of unknown significance,' 'presumed benign,' or 'benign,' according to the American College of Medical Genetics and Genomics (ACMG) standards. When assessing mutation frequencies of individual genes, 'pathogenic,' and 'presumed pathogenic' were counted as mutations while 'benign', 'presumed benign' variants and 'variants of unknown significance' were excluded.

Fusion Gene Analysis

Fusion gene analysis of 52 targeted genes commonly rearranged in tumors (list of genes in Supplement 2) was performed as follows. Anchored multiplex PCR was performed for targeted RNA sequencing using the ArcherDx fusion assay (Archer FusionPlex Solid Tumor panel). The formalin-fixed paraffin-embedded tumor samples were microdissected to enrich the sample to ≥ 20% tumor nuclei, and mRNA was isolated and reverse transcribed into complementary DNA (cDNA). Unidirectional gene-specific primers were used to enrich for target regions, followed by next-generation sequencing (Illumina MiSeq platform). Reads and contigs that were matched to a database of known fusions and other oncogenic isoforms (Quiver database, ArcherDx), as well as those novel isoforms or fusions with high reads (> 10% of total reads) and high confidence after bioinformatic filtering, were analyzed. Samples with < 4000 unique RNA reads were reported as indeterminate and excluded from analysis, and all the analyzed fusions were in-frame and were predicted to have kinase domains preserved. Fusions among

the > 11,000 fusions known to be found in normal tissues were excluded. The detection sensitivity of the assay allows for detection of a fusion that is present in at least 10% of the cells in the samples tested.

Reverse Transcriptase Polymerase Chain Reaction Studies

Reverse transcriptase polymerase chain reaction (RT-PCR) for *SS18*/*SSX1* and *SS18*/*SSX2* gene fusions was performed on Cases 4 and 5 as previously described and reported [2].

Microsatellite Instability Testing by NGS

Microsatellite instability (MSI) was examined using over 7000 target microsatellite loci and compared to the reference genome hg19 from the University of California, Santa Cruz (UCSC) Genome Browser database. The number of microsatellite loci that were altered by somatic insertion or deletion was counted for each sample. Only insertions or deletions that increased or decreased the number of repeats were considered. Genomic variants in the microsatellite loci were detected using the same depth and frequency criteria as used for mutation detection. MSI-NGS results were compared with results from over 2000 matching clinical cases analyzed with traditional PCR-based methods [4]. The threshold to determine MSI by NGS was determined to be 46 or more loci with insertions or deletions to generate a sensitivity of > 95% and specificity of > 99% [4].

Fluorescent In Situ Hybridization Studies

Case 1 underwent fluorescence in situ hybridization (FISH) analysis for *SS18* gene rearrangement. This was performed using a Vysis LSI *SS18* Dual Color Break-Apart Probe (Previously called LSI SYT) (Abbott Molecular, Des Plaines, IL), as previously described [2].

Results

The five cases occurred in patients 10–73 years old (mean age: 38.4), in 3 females and 2 males. Two cases involved the thyroid, two the neck, and one case (Case 1) was a 6.7 cm superior mediastinal mass positioned between the superior vena cava and the aortic arch.

Histologic and Immunophenotypic Findings

Histologically, cases 1, 3, 4 and 5 were essentially identical, with only Case 2 showing an architectural deviation from the rest (see below). All showed an unencapsulated to partially pseudoencapsulated, blue spindle cell appearance on low power (Fig. 1a). The tumors were made up of predominantly spindled cells arranged in short fascicles and focal herringbone and storiform areas. Hypercellular areas showed spindled cells that were tightly packed together, but in less cellular areas the spindled cells exhibited reticular and sieve-like architectures (Fig. 1b–d). Cleft-like spaces filled with a gray-blue mucoid substance were also identified (Fig. 1c, e). These clefts on occasion opened up into larger cystic spaces filled with similar mucoid material. Occasional intraluminal glomeruloid proliferations as well as foci with a palisaded and stellate reticulum-like appearance were noted (Fig. 1f). Scattered, admixed lymphocytes were present in all cases. Focal necrosis was seen in only Case 3. Mitotic figures were rare (no more than 1–2 per 10 high power fields). Lymphovascular space invasion was seen only in Case 1.

Case 2 deviated from the other cases in that it showed a more lobulated and jigsaw-like type of architecture with prominent microcystic growth pattern.

The neoplastic cells from case 1 showed diffuse and strong expression of pan-cytokeratin (AE1/AE3), CK5/6 and p63. There was patchy but strong expression of D2-40, and more limited focal and weak expression of MOC-31 and epithelial membrane antigen. Case 1 was negative for cytokeratin 7, cytokeratin 20, calcitonin, calretinin, mesothelin, WT1, CD5, CD34, STAT-6, S100 protein, myogenin, C-KIT (CD117), alpha-fetoprotein, placental alkaline phosphatase (PLAP), and glypican-3. Nuclear INI-1 expression was retained, and the admixed lymphocytes did not express TdT.

Immunohistochemical results from cases 4 and 5 can be found in Folpe et al. [2]. Immunohistochemical data were not available for cases 2 and 3.

Molecular Findings

Four pathogenic mutations were detected across 2 cases (Table 2). Case 1 had two pathogenic mutations in *KMT2D*, one in an intron splice site (c.674-1A>G) and the other being a frameshift variant (p.M2829fs). Case 1 also had a pathogenic nonsense variant in the *KMT2C* gene (p.R1237*). Case 2 had a pathogenic *NRAS* mutation (p.Q61R). No gene fusions, DNA microsatellite instability, or gene amplifications were detected. Cases 1, 4 and 5 were negative for *SS18* rearrangement.

Treatment and Clinical Course for Case 1

An intensity modulated radiotherapy plan was designed to deliver 54 Gy at 2 Gy per fraction to the area near the great vessels. The patient tolerated the treatment without significant acute toxicity. It is early but so far there is no subacute toxicity noted. Patient is currently without evidence of recurrence 9 months post resection.

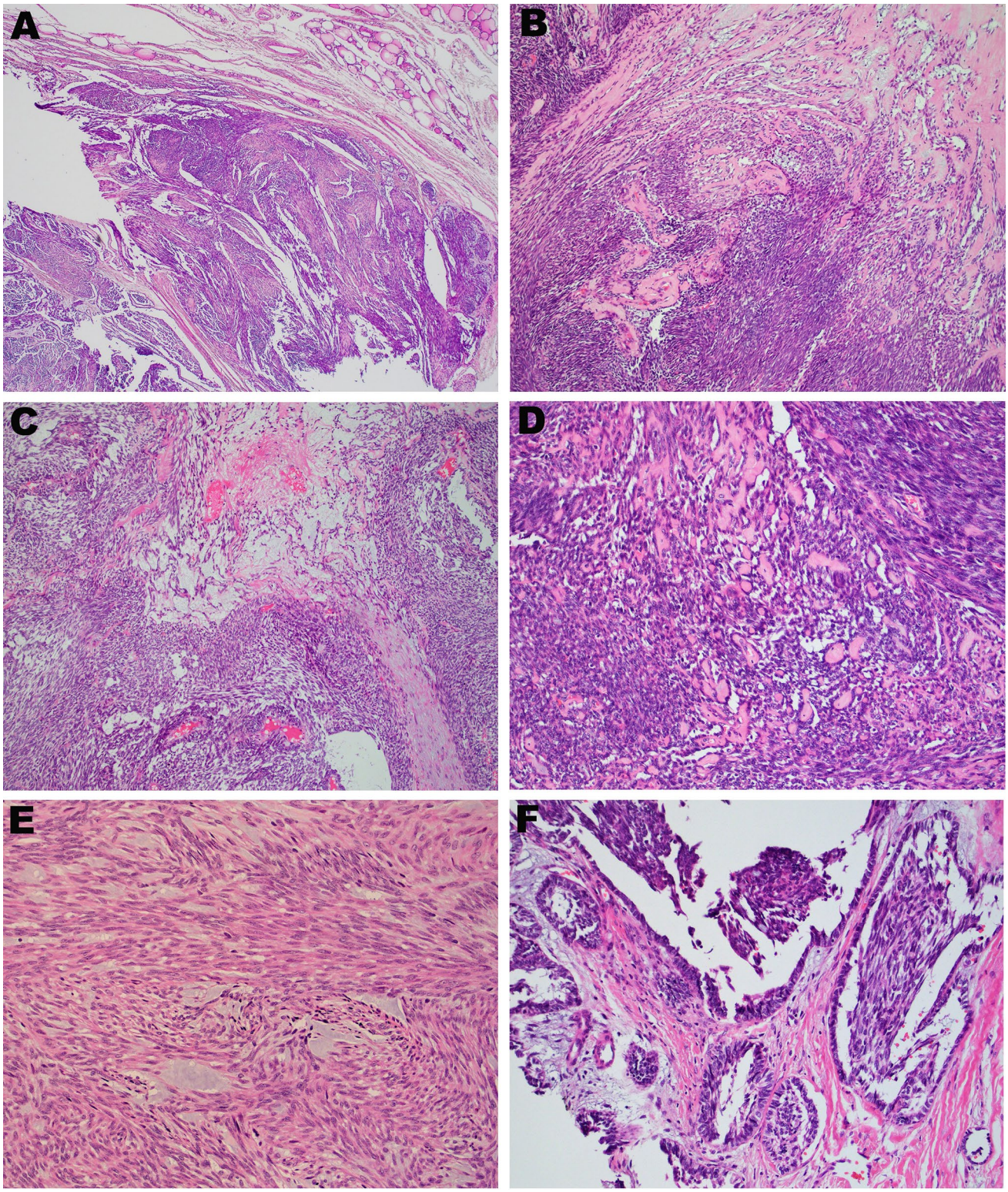

Fig. 1 **a** Thyroid SETTLE showing a blue spindle cell lesion with prominent clefts. Typical examples of SETTLE featured a hypercellular neoplasm of spindle cells growing in fascicles with areas of stromal hyalinization (**b**), reticular (**c**), pseudopapillary (**d**), fascicular (**e**), and palisading and glandular (**f**) architectures. Mucinous type material can be seen in **c** and **e**

Table 2 Summary of next-generation sequencing results of 5 cases of SETTLE

Case	Age	Sex	Location	Pathogenic genetic alterations
1	20	M	Mediastinum	*KMT2D* (c.674-1A>G and p.M2829fs) *KMT2C* (p.R1237*)
2	73	F	Neck	*NRAS* (p.Q61R)
3	40	M	Neck	ND
4	49	F	Thyroid	ND
5	10	F	Thyroid	ND

ND none detected

Treatment and clinical details of cases 2–5 were not available.

Discussion

Spindle epithelial tumor with thymus-like differentiation (SETTLE) is a very rare low grade malignant neoplasm originally described by Chan and Rosai typically arising in young adults [1, 2]. Forty two cases have been previously reported in the literature [5, 6] with a mean age at diagnosis of 19 (range of 2–59 years) and a 1.4:1 male:female ratio. SETTLE usually arises in the thyroid but cases arising in the lateral neck [2, 6] exist and herein we describe a case arising in the superior mediastinum (Case 1). SETTLE is a slow growing neoplasm with metastasis in approximately 26% of cases [5], typically to the lungs, kidneys and liver [7], often occurring years after initial diagnosis. Treatment is mainly surgery and radiation with some cases treated with chemotherapy [5].

To date, other than a single case showing a *KRAS* mutation at codon 13 and 15 (c.37G>A, p.Gly13Ser and c.43G>A, p.Gly15Ser) but lacking a *TP53* gene mutation [3], another case showing diploid DNA content by flow cytometry [8], and the study by Folpe et al. [2] showing a lack of synovial sarcoma specific translocations, there are no comprehensive molecular studies of SETTLE. Therefore, to our knowledge, this is the first study of a series of histologically and immunophenotypically confirmed cases of SETTLE that were subjected to genomic analysis using a NGS platform.

A novel finding was encountered in Case 1, which showed *KMT2D* (c.674-1A>G and p.M2829fs) and *KMT2C* (p.R1237*) mutations. The products of the *KMT2C* (lysine methyltransferase 2C; Mixed-Lineage Leukemia Protein-3) and *KMT2D* (lysine methyltransferase 2D; Mixed-Lineage Leukemia 2) genes are part of the ASC-2/NCOA6 (ASCOM) complex, which possess histone methylation activity and is a transcriptional regulator of the beta-globin and estrogen receptor genes [9]. The *KMT2D* c.674-1A>G mutation disrupts an intron splice site, while the p.R1237* in *KMT2C* is a nonsense mutation in exon 23. Germline mutations in the *KMT2D* gene cause Kabuki syndrome, an autosomal dominant syndrome of abnormal facies, cardiac and skeletal anomalies, immunological defects and mild to moderate mental retardation [10]. As far as somatic mutations is concerned, one study described *KMT2D* as the most frequently mutated cancer-related gene in a cohort of pheochromocytomas [11] and another found *KMT2D* mutations to be common in intraocular medulloepitheliomas [12]. The *KMT2D* gene mutations identified in Case 1 have not been reported before. The *KMT2C* (p.R1237*) mutation identified in Case 1 has been found in just a single case of lung adenocarcinoma [13]. Nevertheless, the significance and specificity of these *KMT2D* and *KMT2C* mutations for SETTLE is likely low given that these genes are not uncommonly mutated in a variety of human malignancies. It is unclear if these mutations have any role in driving the development of these tumors or are in fact just "bystander" genomic events. See Supplement 3 for the frequency and tissue of origin distribution of pathologic mutations in *KMT2D* and *KMT2C* found at Caris Life Sciences (Phoenix, AZ).

Additionally, a pathogenic *NRAS* missense mutation, Q61R, was found in Case 2. Interestingly this case showed a more pronounced microcystic architecture compared to the other cases in this series which showed more prominent fascicular growth patterns. *NRAS* is an oncogene and a member of the (GTPase) ras family, which includes *KRAS* and *HRAS*. This alteration has been detected in multiple cancers including melanoma (15%), colorectal cancer (4%), acute myeloid leukemia (10%), and bladder cancer (2%). Germline mutations in *NRAS* have been associated with Noonan syndrome, autoimmune lymphoproliferative syndrome and juvenile myelomonocytic leukemia [14]. The histologic description of a single reported case of SETTLE with a *KRAS* mutation [3] appeared to resemble our cases with dominant fascicular growth patterns (Cases 1, 3, 4, 5). We did not, however, identify disease-specific genomic events or "actionable" molecular targets in 5 cases of well-defined SETTLE.

Histologically, SETTLE is typically a biphasic neoplasm composed predominantly of monomorphic spindled cells arranged in fascicular, reticular, myxoid and hyalinized patterns with variable epithelial formations arranged in tubulocystic, papillary and glomeruloid architectures [1, 2]. Cases exhibiting a preponderance of spindle cells, so-called predominant monophasic variants, have been reported [15, 16].

The main histologic differential diagnosis of SETTLE is with synovial sarcoma [2]. The histomorphologic and immunophenotypic overlap between these two entities is undeniable and in some cases the main discriminating and defining feature will be the lack of synovial sarcoma-associated translocations/fusion gene products in SETTLE lesions [2], as confirmed in our series. If molecular study

is not available, diffuse expression of cytokeratins would favor SETTLE, as synovial sarcoma typically only focally expresses keratins [2].

Other differential diagnoses of SETTLE for the surgical pathologist to consider include ectopic thymoma, spindle cell variant of medullary carcinoma, intrathyroid thymic carcinoma (carcinoma showing thymus-like elements-CASTLE) and ectopic hamartomatous thymoma. Ectopic thymoma typically will show a more puzzle-like architecture with less complex epithelial components and admixed TdT positive lymphocytes [1]. Spindle cell variant of medullary carcinoma can be identified on the basis of its TTF1 and calcitonin expression, as well as by the typical presence of amyloid [17], features that are lacking in SETTLE. Intrathyroid thymic carcinoma is histologically identical to a mediastinal thymic carcinoma and as such is often a squamous carcinoma with lymphocyte-rich stroma. CASTLE shows more obvious mitotic activity and is often CD5 positive, unlike SETTLE [1, 2, 18]. Finally, ectopic hamartomatous thymoma typically occurs in the lower neck soft tissues and features spindle cell and epithelial components but unlike SETTLE the epithelial components in ectopic hamartomatous thymoma show more prominent anastomosing syringoma-like networks with more squamoid features, often with a myoepithelial component and admixed adipocytes [1].

While the definitive cell of origin/line of differentiation for SETTLE is not known, a branchial pouch origin has been postulated [1, 5]. Ultrastructurally, SETTLE contains desmosomes and intracellular tonofilaments, variable basal lamina and microvillous borders in the epithelial cells, but no neurosecretory granules [1, 19].

In summary, this is the first study describing NGS findings on a series of well-characterized SETTLE cases with detection of a novel mutation in the *KMT2D* gene. The monotonous cytologic appearance of these tumors, a feature often present in translocation-associated neoplasms, suggests that a yet unidentified translocation or other recurrent genomic alteration might be present in SETTLE lesions. While we did not identify any recurrent genetic alterations in our cases of SETTLE, this study is useful in identifying many genes that are probably not involved in the pathogenesis of SETTLE (see Supplements 1 and 2), thus providing a starting point for future studies of SETTLE. This study further confirmed that SETTLE lacks *SS18* and *RET* gene alterations, which are useful in the distinction from synovial sarcoma and the spindle cell variant of medullary carcinoma, respectively. Thus, additional immunohistochemical and molecular studies of SETTLE are warranted to help unravel the underlying driving genetic mechanisms and line of differentiation of this rare neoplasm.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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