

# SMARCB1 protein and mRNA loss is not caused by promoter and histone hypermethylation in epithelioid sarcoma

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**About 10% of epithelioid sarcomas have biallelic mutation of the *SMARCB1* (SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily b, member 1) gene resulting in a lack of this nuclear protein. It has been suggested that *SMARCB1* may be silenced by epigenetic changes in the remaining 90% of tumors. Thus, we hypothesized that the promoter of *SMARCB1* is hypermethylated. We also examined *SMARCB1* mRNA level to determine if a post-translational change was possible. Thirty-six cases of epithelioid sarcomas were studied. Immunohistochemistry and mutation analysis of the *SMARCB1* gene were performed to select appropriate cases. Methylation status was assessed by methylation-specific PCR. Laser capture microdissection of tumor cells followed by real-time PCR was applied to examine the expression of *SMARCB1* mRNA. Of 36 epithelioid sarcomas, 31 (86%) displayed a lack of SMARCB1 nuclear protein. In all, 4 (13%) of 31 SMARCB1-negative cases harbored biallelic deletion while 9 (33%) cases showed single-allelic deletion. One (4%) frameshift deletion of exon 3 and one point mutation of exon 7 were also found. In 16 (59%) cases, both alleles were intact. Altogether, 25/31 (81%) SMARCB1-negative cases had at least one intact allele. None of these cases demonstrated promoter hypermethylation. Low levels of *SMARCB1* mRNA were found in all cases with tumor tissue extracted RNA (because of the minimal normal cell contamination) but no mRNA could be detected in laser dissected cases (containing only tumor cells). *Enhancer of zeste homolog 2* (*EZH2*) overexpression was not characteristic of epithelioid sarcoma. Thus, loss of SMARCB1 expression in epithelioid sarcoma is caused neither by DNA hypermethylation nor by post-translational modifications. Most likely it is the microRNA destruction of *SMARCB1* mRNA but further investigations are needed to elucidate this issue.**

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The *SMARCB1* (SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily b, member 1; also known as *INI1*, *BAF47* and *hSNF5*) gene, located on chromosomal position 22q11.23, encodes a core subunit of the SWI/SNF

ATP-dependent chromatin remodeling complex.<sup>1</sup> Such observations show these complexes regulate gene expression, at least in part, by inducing a nucleosome conformation that is more approachable to the transcriptional machinery.<sup>2</sup> Recently, inactivation of both alleles of the *SMARCB1* gene was identified in the majority of malignant rhabdoid tumors, suggesting that loss-of-function mutations of the gene contributed to the oncogenesis of this type of tumor.<sup>3</sup> *SMARCB1* abnormalities also have been found in other malignant neoplasms including epithelioid sarcoma, epithelioid

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malignant peripheral nerve sheath tumor, myo-epithelial carcinoma, extraskeletal myxoid chondrosarcoma and renal medullary carcinoma.<sup>4</sup>

Epithelioid sarcoma, described by Enzinger,<sup>5</sup> is a rare and distinctive soft tissue neoplasm typically arising in the distal extremities of young adults.<sup>6</sup> Many studies reported the absence of nuclear staining of SMARCB1 in approximately 90% of tumors.<sup>7–10</sup> Recent studies of molecular analysis of the *SMARCB1* gene revealed only 10% of epithelioid sarcoma cases had both absent SMARCB1 expression and mutation of *SMARCB1* that suppress expression. In cases without DNA alterations, either epigenetic changes or post-transcriptional modification could prevent expression of SMARCB1.<sup>10</sup> In addition to point mutations and deletions, transcriptional repression by hypermethylation of promoter sequences is an alternative mechanism of inactivation of tumor-suppressor genes in cancer.<sup>11,12</sup> Further mechanisms of epigenetic silencing of gene expression include post-translational modification of histones. The trimethylation of histone-3 on lysine-27 (H3K27me3) is a well-known histone mark associated with gene silencing. H3K27me3 is mediated by the methyltransferase enhancer of zeste homolog 2 (EZH2), which is the enzymatically active component of the polycomb repressor complex 2 (PRC2).<sup>13</sup> EZH2 is abnormally overexpressed in a wide range of tumors as compared with corresponding normal tissues, its level of expression being correlated with cancer aggressiveness.<sup>14–16</sup> Recently, studies have shown that *EZH2* aberrations are strongly associated with many mesenchymal tumors (synovial sarcoma, Ewing sarcoma and alveolar rhabdomyosarcoma).<sup>17</sup> In our study, we carried out an epigenetic investigation to detect the methylation profile in *SMARCB1* promoter areas of 25 epithelioid sarcoma cases. Immunohistochemical studies were also carried out to detect the EZH2 protein expression of 36 epithelioid sarcomas.

## Materials and methods

### Tissue Samples

For this retrospective study, formalin-fixed, paraffin-embedded tumor samples were collected from 36 patients diagnosed with epithelioid sarcoma (World Health Organization classification) from the archives of the Semmelweis University First Department of Pathology ( $n=17$ ); National Institute of Oncology, Hungary ( $n=6$ ); the University of Chicago Medical Center ( $n=9$ ); Department of Laboratory Medicine and Pathology, Mayo Clinic ( $n=3$ ) and from Scott and White Healthcare Temple Clinic, Texas ( $n=1$ ). Beside H&E slides, immunohistochemistry was performed for cytokeratin (AE1/AE3), EMA, vimentin, CD34, S100 and SMARCB1 to confirm the diagnosis of epithelioid sarcoma. Main clinical data are summarized in Table 1. The

possibility of malignant rhabdoid tumor was excluded by fluorescence *in situ* hybridization (FISH) and direct sequencing, described later.

### Immunohistochemistry

Immunohistochemical staining was performed with Bond Max™ Autostainer (Leica Biosystems Newcastle, Newcastle, UK). Tissue sections of 3  $\mu$ m thickness were cut from the blocks, after deparaffinization in xylene and retrieval using either the Bond Epitope Retrieval Solution 1 (pH~6) or the Bond Epitope Retrieval Solution 2 (pH~9) (Leica Microsystems, Wetzlar, Germany) at 99–100 °C for 20–30 min, were immunostained using a purified mouse anti-BAF47 (SMARCB1) antibody (clone 25; 1:50; BD Transduction Laboratories, San Diego, CA, USA), monoclonal mouse anti-human cytokeratin (clones AE1/AE3; 1:150; Dako Cytomation, Carpinteria, CA, USA), monoclonal mouse anti-human epithelial membrane antigen (clone E29; 1:200; Dako), monoclonal mouse anti-human vimentin, (clone V9; 1:600; Dako), monoclonal mouse anti-human CD34 class II (clone QBEnd 10; 1:300; Dako), polyclonal rabbit anti-S100 (1:1500; Dako) and purified mouse anti-EZH2 antibody (clone 11, 1:25, BD Biosciences, USA). Sections were incubated with the primary antibody for 25 min, followed by using the peroxidase/DAB Bond Polymer Refine Detection System (Leica Microsystems) for visualization.

For SMARCB1, the tumors were scored as having nuclear expression intact or absent. To assess the immunohistochemical labeling of EZH2, we modified the scoring system described by Pacheo *et al.*<sup>18</sup> The intensity of the nuclear staining was evaluated as follow: score 0: no visible staining; score 1: weak; score 2: moderate; score 3: intense. To evaluate the extension of the positive cells was based on the percentage of the cell with positive nuclear stain against the background (score 0: no visible staining; score 1: 1–50%; score 2: 51–75%; score 3: above 75%). Each score contained at least 100 tumor cells to be evaluated. If >30% of the tumor cells carried stronger nuclear intensity, we used the higher nuclear score. The sum of these two scores defined the final score of each sample; if it is above 3, the case was considered as EZH2 positive.

### Fluorescence *In Situ* Hybridization

FISH analysis was carried out on all SMARCB1-negative tumors. In all, 4.5  $\mu$ l of BCR/ABL Dual Color Translocation Probe Set, consisting of *BCR*- (SpectrumGreen-labeled, green) and *ABL*- (SpectrumOrange-labeled, orange) specific probes (Abbott Molecular, Illinois, USA), was applied and covered by coverslips sealed with rubber cement. *BCR* DNA probe covers the whole 22q.11.2 chromosomal region including both *BCR* (22q11.23) and

**Table 1** Clinical data and immunohistochemical results (SMARCB1, CD34 and EZH2) of 36 patients with epithelioid sarcoma

No	Sex	Age (years)	Site	Subtype	SMARCB1	CD34	EZH2 score
1	M	42	Lymph node metastasis	Proximal	–	+	2
2	M	69	Right inguinal region	Proximal	–	–	2
3	F	30	Left hand	Proximal	–	–	3
4	M	84	Left thigh	Proximal	–	+	3
5	F	62	Greater omentum	Proximal	–	–	6
6	M	29	Left lower limb	Distal	–	+	3
7	F	49	Left lower limb	Distal	–	–	2
8	F	30	Left inguinal region	Proximal	–	+	4
9	F	36	Right thigh	Distal	–	+	3
10	F	40	Joint	Proximal	–	+	4
11	M	29	Scalp	Distal	–	–	4
12	M	34	Right inguinal region	Proximal	–	+	4
13	F	70	Vagina	Proximal	–	+	4
14	F	87	Right hand	Distal	–	–	2
15	F	50	Perineum	Proximal	–	+	3
16	F	43	Left thumb	Distal	–	+	3
17	M	83	Right ring finger	Distal	–	–	3
18	M	29	Left forearm	Distal	–	–	2
19	M	48	Pleural metastasis	Proximal	–	+	4
20	M	23	Right forearm	Distal	–	–	0
21	M	17	Left forearm	Distal	–	+	0
22	F	18	Right forearm	Distal	–	+	2
23	M	42	Left thumb	Distal	–	+	0
24	F	12	Right hand	Distal	–	+	0
25	M	12	Scalp metastasis	Distal	–	+	2
26	F	23	Lymph node metastasis	Distal	–	+	5
27	M	31	Lymph node metastasis	Distal	–	–	3
28	M	25	Left calf	Distal	–	+	3
29	F	82	Left femoral region	Distal	–	–	3
30	F	68	Vulvar region	Proximal	–	–	6
31	M	28	Right inguinal region	Proximal	–	+	3
32	M	48	Lymph node metastasis	Proximal	+	+	2
33	M	63	Left hip	Proximal	+	+	6
34	M	34	Lymph node metastasis	Proximal	+	+	5
35	M	63	Nose	Distal	+	+	3
36	M	52	Left arm	Distal	+	+	3

EZH2 score: see Materials and methods section.

*SMARCB1* (22q11.23) genes, therefore lack of one or two *BCR* signals indicate the deletion of *SMARCB1* gene too. The sample and probe were denatured and hybridized using the ThermoBrite Denaturation/Hybridization System (Abbott Molecular) by programming 5 min of denaturation at 73 °C, followed by overnight hybridization at 37 °C. Post-hybridization wash was performed in 0.4 × SSC/0.1% NP-40 (73 °C, 2 min) followed by a wash in 2 × SSC/0.1% NP-40 (room temperature, 2 min). The slides were air dried in the dark, then counterstained with 10 μl of 4',6'-diamidino-2-phenylindole from Vector Laboratories (Burlingame, CA, USA). The FISH signals were visualized by using filter sets and a Nikon Eclipse E600 epifluorescence microscope. Image analysis was performed by using Lucia Cytogenetics image acquisition system (Laboratory Imaging, Republic of Czech).

#### DNA and RNA Extraction

Genomic DNA (gDNA) and total RNA, for mutational analysis, promoter methylation study and gene

expression profile, respectively, were isolated from paraffin-embedded materials in the *SMARCB1* expression lacking cases by using RecoverAll Total Nucleic Acid Isolation Kit (Ambion, Austin, TX, USA), according to the respective manufacturers' instructions. The concentration and integrity of nucleic acids were measured with NanoDrop 1000 (NanoDrop Technologies, Houston, TX, USA). In all, 500–1000 ng of RNA were reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) to prepare first-strand complementary DNA.

#### *SMARCB1* Mutational Analysis

The sequences of the applied primers for exons 2–9 of the *SMARCB1* gene (GenBank accession nos. Y17119–126) were the same as previously described K Kohashi *et al.*<sup>19</sup> For the exon 1 (GenBank accession nos. Y17118) a new primer pair were designed: *SMARCB1*ex1F (5'-TCCTGATCCCTCGCA GCC-3') and *SMARCB1*ex1R (5'-CCCGATGAATGG AGACGC-3'). Using the ABI Prism 310 sequence

analyzer (Applied Biosystems), PCR products for individual exons were analyzed by direct sequencing. The sequence of individual exons was compared with the reported sequence in GenBank.

### Methylation-Specific PCR

DNA (500–1000 ng) was bisulfited using the Cells-to-CpG Bisulfite Conversion Kit (Applied Biosystems) according to the specified protocol of the company. DNA extracted from peripheral blood mononuclear cells and amplified with Repli-g kit (Qiagen, Hilden, Germany), totally unmethylated product was served as negative control for the methylation-specific PCR and as a template DNA for the methylation with CpG (M.SssI) Methyltransferase (New England Biolabs, Ipswich, MA, USA) as a methylated-positive control. Primers for methylation-specific PCR are designed to anneal specifically to CpG-rich sequences and, thus, selectively amplify either methylated DNA or unmethylated DNA in separate PCRs were shown in Table 2. Approximately 15 ng of the bisulfite-converted gDNA was used for PCR, but the functionally analyzable amounts were much less because of the compromised nature of formalin-fixed, paraffin-embedded samples and degradation during bisulfite conversion. Each of 45 PCR cycles included a denaturation step at 95 °C for 30 s, followed by annealing at 51–58 °C for 30 s and extension at 72 °C for 30 s. PCR products were analyzed by electrophoresis using a 2% agarose gel and visualized after staining with ethidium bromide. If an amplicon for both UM- and M-primer or for M-primer alone could be seen, the gene promoter could be considered methylated.

### Laser Capture Microdissection

Laser capture microdissection was carried out with PALM laser microdissector (PALM, Bernried, Germany). Tissue specimens of 5 μm thickness from seven paraffin-embedded blocks were cut onto PALM membrane mounted glass slides. To avoid detachment of paraffin sections we irradiate with UV light at 254 nm for 30 min as it was advised by the manual. To facilitate the identification of the SMARCB1-negative tumor cells a rarely used immuno-laser capture microdissection strategy was applied. To ensure RNase-free conditions, we worked on sterile way during the immunohistochemistry. After deparaffinization and pressure-cooking antigen retrieval, the SMARCB1 immunostaining was done manually using the Max Polymer Detection System (Novolink, Novocastra, Newcastle, UK) to reveal antigen–antibody reaction. Immunostains were air dried for 30 min then two-step dissection was performed using PALM laser microdissector. Three thousand SMARCB1-negative tumor cells were dissected and collected into individual tubes from each case and immediately placed on ice. As positive control, SMARCB1 immunoreactive endothelial cells and lymphocytes were collected. RNA was extracted using the RNeasy-Micro Kit (Ambion), according to the protocol for laser capture microdissection provided by the manual. Residual DNA was removed by DNase treatment as an optional step.

### Quantitative Real-Time Reverse Transcriptase-PCR Analysis (qRT-PCR)

qRT-PCR was applied in order to examine the expression of *SMARCB1* in epithelioid sarcoma

**Table 2** Designed primers for the methylation-specific PCR

Primer set	Sense primer, 5' → 3' <sup>a</sup>	Antisense primer, 5' → 3' <sup>a</sup>	Size, bp	Anneal temp., °C	Genomic position <sup>b</sup>
M01	GTTGTTAAGATTTGGCGTC	TCCCGTTTCTTTACGTCTAA	80	55	+ 319
UM01	AGTGTGTTAAGATTTGGTGT	TCCCATTCTTTACATCTAACTA	80	52	+ 316
M02	GATCGGTTTCGAGGTAGTTC	CGCCCCGATAAATAAAAAAC	112	56	+ 81
UM02	ATGATTGGTTTTGAGGTAGTTT	CCACCCCAATAAATAAAAAAC	112	53	+ 79
M03	CGTTAACGTTAGCGTTTGC	ACGAAATACGAACCGAACC	111	59	– 212
UM03	TAGTGTTAATGTTAGTGTTTGT	AAAACAAAATACAAAACCAAACC	111	51	– 215
M04	CGGTTGAGGCGTTAGTATTC	CGAAACCGAAAAACGAAATA	51	57	– 140
UM04	GTTTGGTTGAGGTGTTAGTATTT	ACCAAAAACAAAAAACAAAATAC	51	53.5	– 143
M05	TCGTTTCGTTTTGTTCGTC	ATATAAACTCGCCGTCGTC	101	58	– 22
UM05	GGTTGTTTGTTTTTGTTGTT	ATATAAACTCACCATCCTCCT	101	51	– 24
M06	CGTAGTTCGGTTTCGGTC	ATATAAACTCGCCGTCGTC	117	58	– 38
UM06	TTTTGTAGTTTGGTTTTGGTT	ATATAAACTCACCATCCTCCTC	117	52	– 41
M07	GTTTTTTAAGGGTTCGC	CTCAATACGCAACCGTAAC	91	56	– 276
UM07	GTGGTTTTTTAAGGGGTTTGT	CCTCAATACACAAAACACTAACA	91	54	– 279

Abbreviations: M, methylated-specific primers; UM, unmethylated-specific primers.

<sup>a</sup>Underlined-bold cytosins in the sense and the underlined-bold guanins in the antisense primer indicated the cytosin within the CpG island of the promoter region, which could be identified with methylation-specific PCR.

<sup>b</sup>Primers were placed near the transcriptional start site. Genomic position is the location of the 5' nucleotide of the sense primer in relation to the major transcriptional start site of *SMARCB1* gene.

samples and performed using LightCycler® 480 Resolight Dye (Roche Applied Science, Indianapolis, IN, USA) with AmpliTaq Gold® 360 DNA Polymerase (Applied Biosystems) and primers for *SMARCB1* splice variants: *SMARCB1*sense (5'-TCCGTATGTTCCGAGGTTTC-3') and *SMARCB1*anti-sense (5'-CTTCCACTTCCGAGGCTTT-3'). *Glycer-aldehyde-3-phosphate dehydrogenase (GAPDH)* was used as endogenous control and for calibrator normal human liver was applied. After enzyme activation at 95 °C for 5 min, 45 PCR cycles were performed (denaturation at 95 °C for 10 s, annealing at 59 °C for 10 s and extension at 72 °C for 20 s). Gene expression levels were calculated and determined using the crossing point ( $C_p$ ) values ( $2^{-\Delta\Delta C_p}$  method). From the formalin-fixed, paraffin-embedded blocks, we got heterogeneous tissue (containing mainly tumor cells but also normal endothelial, lymphoid, etc, cells), but to obtain pure tumor RNA, that was not possible. Therefore, we performed laser capture microdissection to collect only tumor cells.

#### Primer Design for Methylation-Specific PCR

Primers were designed to discriminate between methylated and unmethylated alleles following bisulfite treatment and to discriminate between bisulfate modified and unmodified DNA. As the two strands of DNA are no longer complementary after bisulfite treatment, primers can be designed for either modified strand. For convenience, we have designed primers for the sense strand. Short amplicons, between 80 and 117 bp were designed. The primers and annealing temperatures have been described previously (Table 2).

#### Methylation Analysis of the CpG Islands in the Promoter Region Using Methylation-Specific PCR

The *SMARCB1* gene sequence contains many CpG islands. For the methylation analysis, CpG islands with 600 bp of DNA sequence (located in chromosome 22:24129097–24129696 based on UCSC Genome Browser) were chosen. This region is the promoter of the gene and overlap with the sequence of the first exon. To assess the methylation status of all cytosines within the region, we employed the sodium bisulfite treatment and methylation-specific PCR. After treatment with sodium bisulfite, unmethylated cytosines in the DNA were converted to uracils, whereas 5-methylcytosines remained as cytosines. After removal of the bisulfite and completion of the chemical conversion, this modified DNA was used as a template for PCR. With our primers, we could examine altogether 37 different CpG-s in the promoter region.

## Results

### Histology, Immunohistochemistry

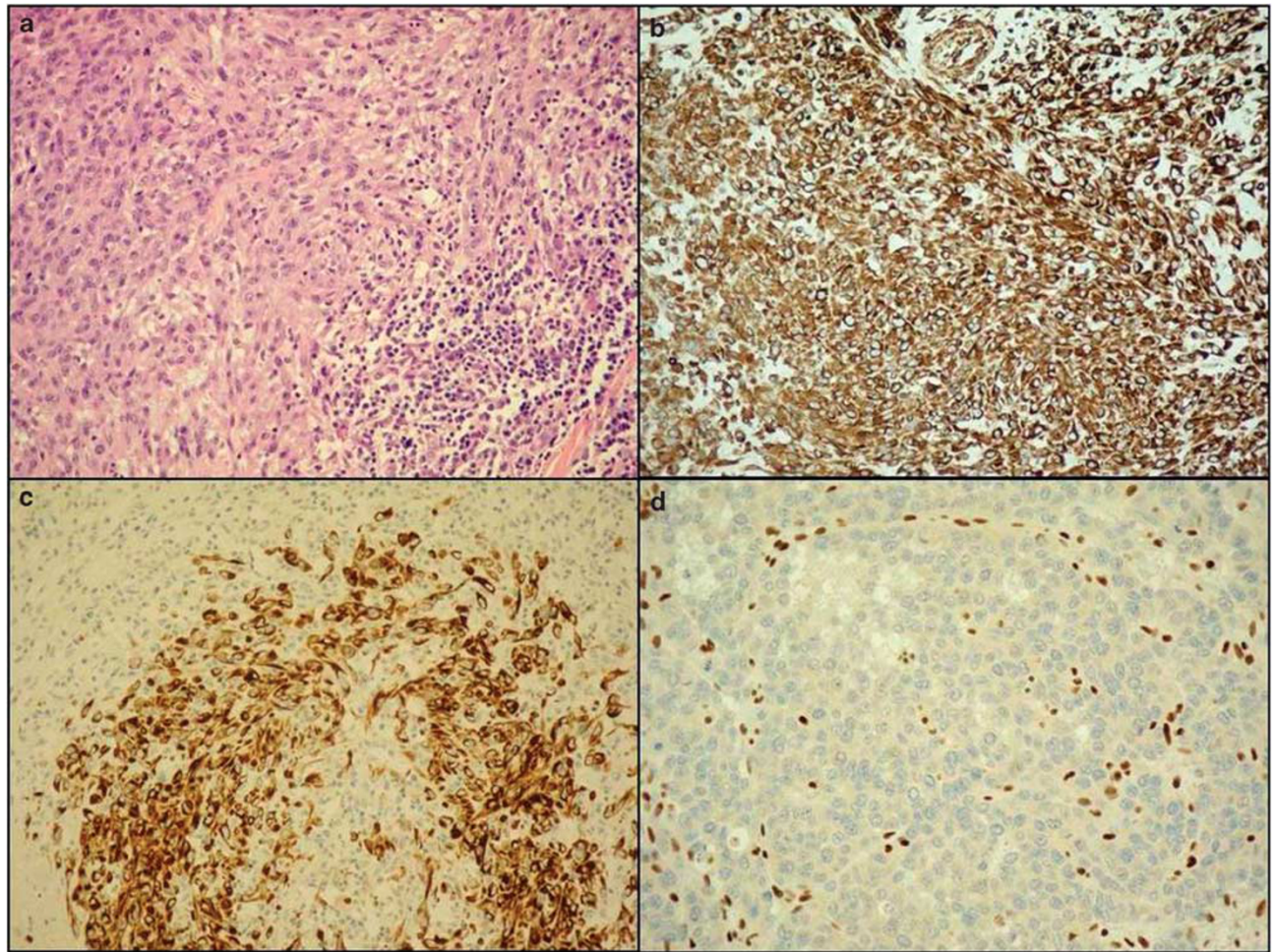
Of 36 epithelioid sarcoma cases, 20 (56%) were distal and 16 (45%) proximal type. In 31 (86%) cases, there was no detectable expression of the *SMARCB1* gene product in tumor cells with the anti-BAF47 antibody, whereas infiltrating lymphocytes and entrapped non-tumor tissue displayed immunoreactivity. Epithelioid sarcoma cases were characterized as follows: vimentin diffuse positivity, keratin (AE1/AE3) and/or EMA at least focal strong positivity, S100 negativity and *SMARCB1* negativity (31 cases; Figure 1). In 5 (14%) *SMARCB1*-positive cases, there was also CD34 positivity confirming the diagnosis of epithelioid sarcoma (Figure 2). Immunohistology data are summarized in Table 1. All 36 epithelioid sarcoma cases were evaluated and scored for EZH2 protein expression. The mean score was 2.97, which meant that EZH2 overexpression is not a general feature of epithelioid sarcoma (mean 2.97, s.d. 1.56). Twenty-five cases (69%) had a score of 3 or less (mean 2.2, s.d. 1.08) therefore they were considered to be EZH2 negative. The remaining 11 (31%) cases were regarded as positive (mean 4.73, s.d. 0.90; Figure 3) including 9 proximal type and 2 distal type. The highest score 6 was observed, however, only in three (8%) proximal-type cases, based on the age of the patients, location and lack of mutation of the *SMARCB1* gene, which is otherwise typical for malignant rhabdoid tumor.<sup>20</sup>

### Deletion and Mutation in *SMARCB1*

As stated previously, loss of *BCR* FISH signal also indicate *SMARCB1* gene deletion. The *ABL* gene located on chromosome 9 was regarded as a positive control. Deletion of both *BCR* alleles results in loss of nuclear protein expression. Four cases showed tumor cells without detectable *BCR* by FISH in their nucleus and were regarded as biallelic deletion of *BCR/SMARCB1*. Single-allelic deletion of *BCR/SMARCB1* with one *BCR* signal in the tumor cells was found in 11 cases. In the remaining 16 cases, chromosomal abnormalities could not be detected. Tetrasomy was observed in four cases. Among those 11 cases that showed one allelic deletion, two of them revealed mutation of the retained allele (sequencing all nine exons of the *SMARCB1* gene). One of them displayed a deletion in the exon 3 resulting in a frameshift and the other showed a single basepair point mutation in the exon 7. Altogether, we found six cases with genetic aberrations (biallelic alteration) that explain the loss of *SMARCB1* function (Figure 4).

### Methylation Status

First, we tested the primers (designed for CpG islands of *SMARCB1* promoter) on negative and

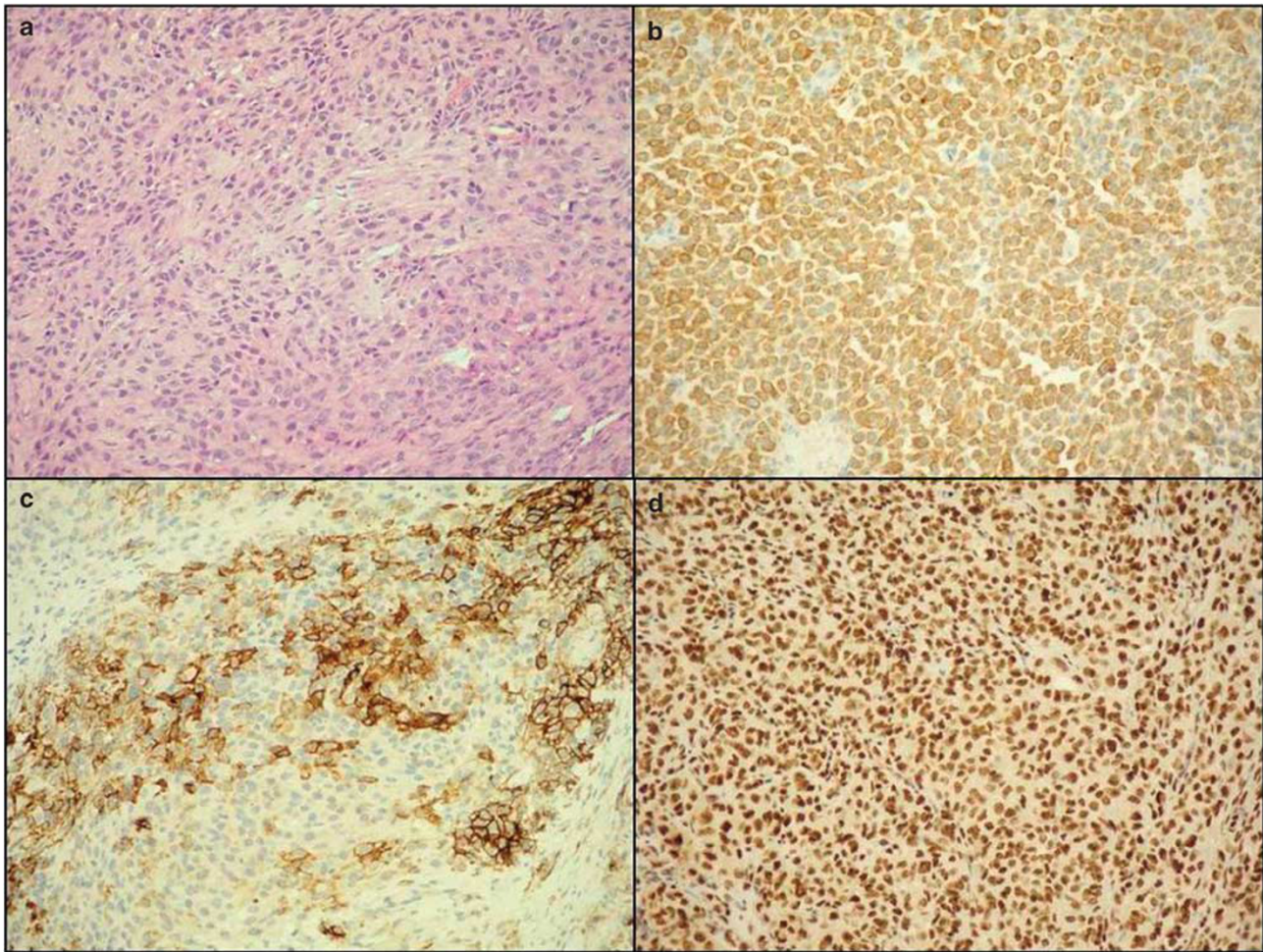


**Figure 1** Typical picture of distal-type epithelioid sarcoma: tumor cells with abundant eosinophilic cytoplasm and small nucleoli (a; H&E stain; 20 $\times$ ). Characteristic coexpression of vimentin (b; 20 $\times$ ) and cytokeratin (AE1–AE3) (c; 20 $\times$ ) is evident. SMARCB1 negativity is obvious in tumor cells while normal stromal cells and lymphocytes are positive (d; 40 $\times$ ; case number: 29).

positive control DNAs, described previously in Materials and methods section. The whole genome amplified sample, which contains only unmethylated alleles, resulted in PCR products only with unmethylated primers sets, whereas the *in vitro* (M.SssI methyltransferase) methylated DNA resulted in PCR products with both the methylated and unmethylated primer set. This methylation heterogeneity (methylated and unmethylated products) in our positive sample suggested that the *in vitro* methylation of the DNA was not totally 100% effective but still clearly indicating the reliability of this method. Twenty-five tumor DNA (bisulfite converted) samples (displaying no biallelic mutation/deletion in the *SMARCB1* gene) were analyzed. Methylation-specific PCR was performed using all the designed 14 methylated- and unmethylated-specific primers. Methylation-specific PCR analysis revealed that there were no methylated alleles in the promoter region of *SMARCB1*, so this tumor-suppressor gene displayed unmethylated status in epithelioid sarcoma (Figure 5).

#### Examination of the *SMARCB1* Gene Expression by qRT-PCR

As a result of investigation of the *SMARCB1* gene expression, low level of *SMARCB1* mRNA was found in RNA samples (16 cases) extracted from tissue blocks (9 cases could not be studied because of degradation of their RNA). We suspected that the stromal or other normal cells (eg, endothelium, lymphocyte, fibroblast, etc) might cause contamination and it results in the appearance of the *SMARCB1* mRNA in a variable expression levels among tumor samples depending on the ratio of normal and tumor tissue. Therefore, we decided to carry out laser capture microdissection to collect only tumor cells, which showed no expression of *SMARCB1* gene; correspondingly, mRNA could not be detected. For a positive control, we also collected individual stromal and/or normal cells from the tumor-free area, which revealed increased levels of *SMARCB1* transcript. *GAPDH* was amplified in all examined cases as an endogenous control,



**Figure 2** Proximal-type epithelioid sarcoma (**a**; H&E stain; 20 $\times$ ) with characteristic cytokeratin (AE1–AE3) (**b**; 20 $\times$ ) and CD34 (**c**; 20 $\times$ ) positivity but with preserved SMARCB1 (**d**; 20 $\times$ ; case number: 33). Cases with SMARCB1 positivity were omitted for further analysis.

indicating the presence of mRNA in each sample (Figure 6).

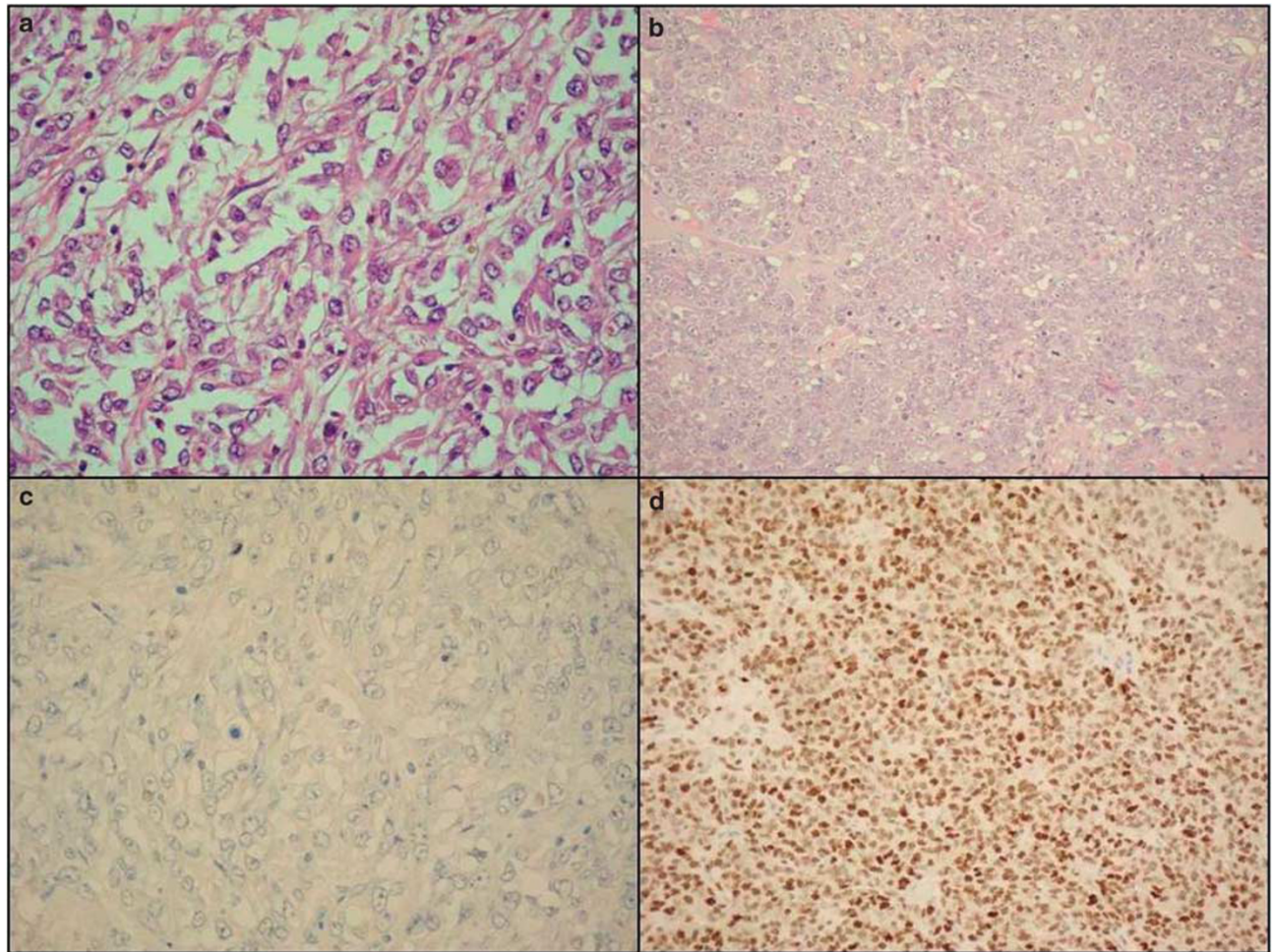
## Discussion

Epigenetic changes may explain hallmark features of cancers and may produce the same effect as genetic damages. A good example is the malignant rhabdoid tumor—epithelioid sarcoma carcinogenesis with the same SMARCB1 protein loss of both tumors but with genetic changes in the malignant rhabdoid tumor and epigenetic changes in epithelioid sarcoma. Epigenetic changes can occur on three levels in general; DNA, RNA and protein, resulting in the same effect as mutation. Hypermethylation of DNA promoter region and histone methylation via EZH2 overexpression of suppressor genes are well known and extensively investigated causes of carcinogenesis and sarcomagenesis. CpG islands are quite numerous in the promoter region of the *SMARCB1* gene, suggesting a possible hypermethylation mechanism for gene silencing. In our study, we wanted

to determine whether DNA or histone methylation of the promoter region of *SMARCB1* could result in nuclear SMARCB1 protein loss in epithelioid sarcoma using methylation-specific PCR and EZH2 immunohistochemistry.

Biallelic damage of the *SMARCB1* gene on chromosome 22q11.2 and the consequent absence of nuclear staining of SMARCB1 were first recognized back in 1990 in malignant rhabdoid tumors and were thought as being a specific and characteristic mark for this tumor.<sup>21</sup> However, very recently it was shown that loss of SMARCB1 nuclear protein is characteristic for a variety of soft tissue tumors, especially for epithelioid sarcoma. In fact, approximately 90% of epithelioid sarcoma cases have negative nuclear staining. At the same time, it was also revealed that the mechanism of SMARCB1 protein loss is different because the vast majority of epithelioid sarcoma cases did not harbor biallelic mutation of the *SMARCB1* gene. This result suggests that epigenetic events may be responsible.

In our study, the initial step was to select the SMARCB1-negative cases and from the negative



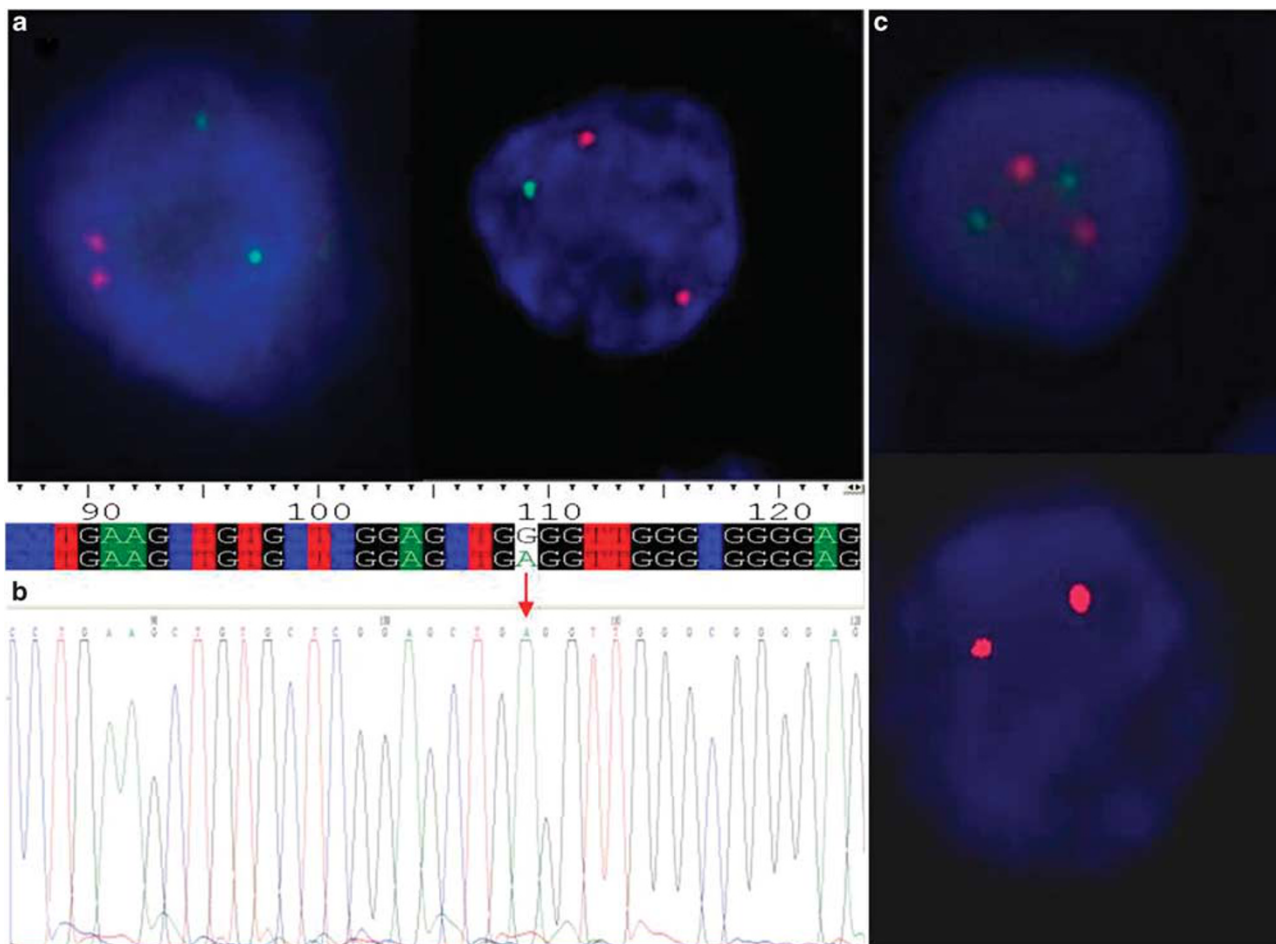
**Figure 3** Distal (a; H&E stain; 40 ×) and proximal-type (b; H&E stain; 20 ×) epithelioid sarcoma with EZH2 complete negativity (c; 40 ×) and with strong positivity (d; 20 ×), (c, d: EZH2 immunohistochemistry), (case number 21: a, c; case number 5: b, d). EZH2 positivity (4 or more score) was not a characteristic feature of epithelioid sarcoma.

cases to exclude those cases, which displayed biallelic alterations. We found 31 (86%) SMARCB1-negative epithelioid sarcoma and from these cases, 6 (19%) contained biallelic alterations, thus 25 cases for methylation analysis remained. Our results are in good harmony with others, except the ratio of biallelic mutant cases, which is slightly higher than Kohashi *et al.*<sup>10</sup> This can be explained by the fact that our analysis is the third one in this regard and therefore there are not much data to compare.

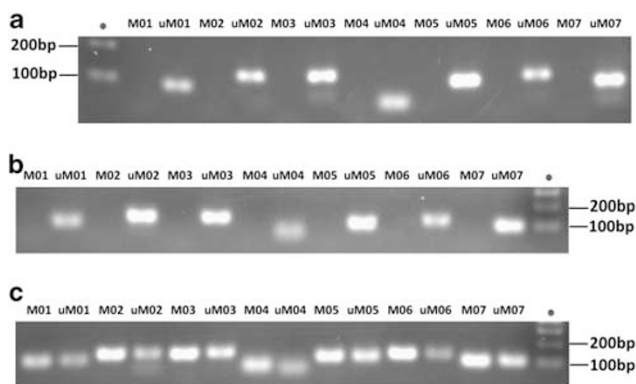
The published data regarding the promoter methylation status of *SMARCB1* gene are limited. To our best knowledge, only one article investigated the promoter methylation status of *SMARCB1* gene in malignant rhabdoid tumor is available which showed no hypermethylation.<sup>22</sup> Therefore, we used chemically induced methylation as positive control for the quality assurance of our designed primer sets. To our surprise, we found no hypermethylation in any of our 25 cases using positive and negative controls. We designed 14 methylated and unmethylated-specific primers for this purpose,

covering about 40% of the CpG islands of the promoter region. In general, a single methylated and unmethylated primer is sufficient to detect promoter methylation, as it means that the other CpG islands are also methylated.<sup>23,24</sup> The unmethylated DNA status of the promoter region suggests an epigenetic event not on the DNA level, such as histone methylation. EZH2 is the enzymatically active component of the PRC2, which catalyzes the repressive histone methylation H3K27m3 mark thus resulting in gene silencing. We tested the possible overexpression of EZH2 in our 36 epithelioid sarcoma cases and found that EZH2 overexpression is not a general feature of epithelioid sarcoma. The mean score was 2.97, which means the majority of epithelioid sarcoma cases are EZH2 negative. In fact, we found only three cases with the highest score of 6 (each proximal type) indicating that strong positivity is a very rare event among epithelioid sarcomas. In contrast, EZH2 is usually strongly positive in most 'blastic' tumors such as neuroblastoma, Ewing sarcoma, alveolar rhabdomyosarcoma, malignant rhabdoid tumor,<sup>25,26</sup>





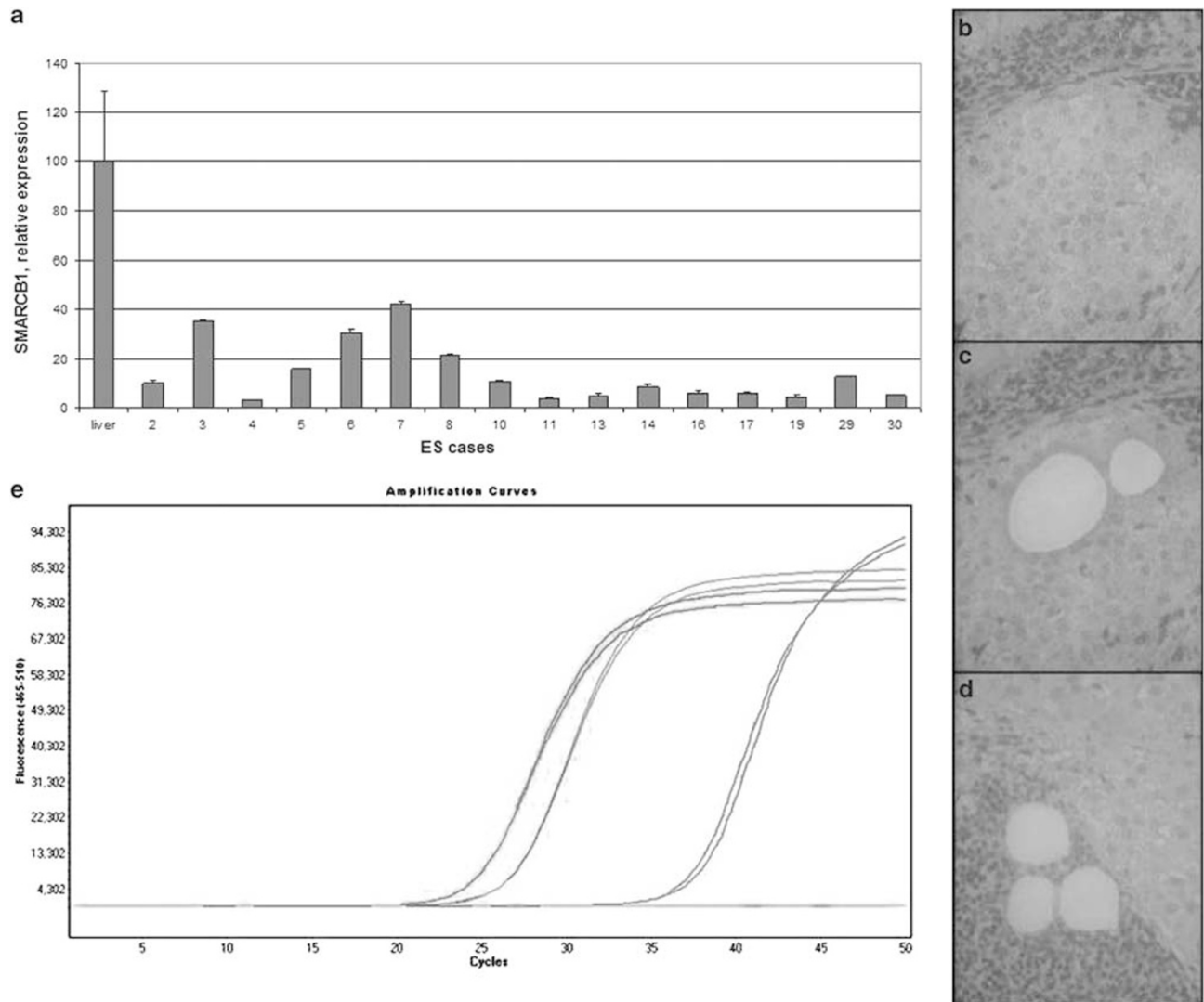
**Figure 4** Genetic examination of the *SMARCB1* gene using FISH (red signals correspond to *ABL* and green signals to *BCR/SMARCB1*) and direct sequencing. Left cell showing normal 2–2 signals and right cell displays deletion of one allele of *BCR/SMARCB1* gene (a); in the same case sequence profile for a point mutation (GGG/AGG) of exon 7 was observed (b), indicating biallelic damage of *SMARCB1* gene (case number 15). Upper normal cell with 2–2 signals and lower cell showing biallelic deletion of *BCR/SMARCB1* gene (c; case number: 8).



**Figure 5** Methylation-specific PCR of the *SMARCB1* gene promoter. Amplification of bisulfite-treated epithelioid sarcoma DNA: note, there are no bands with the methylated primers (a). Pre-amplified/unmethylated DNA (negative control) (b) and *in vitro* methylated DNA (positive control) (c) with *SMARCB1* methylated and unmethylated-specific primer sets. Primer sets used for amplification are designated as methylated (M) and unmethylated (uM). \*Molecular weight marker 1 kb Plus DNA Ladder (Life Technologies, Carlsbad, CA, USA). The accurate size of methylation-specific PCR products are shown in Table 2.

but in these tumors the *SMARCB1* nuclear protein is intact except in the malignant rhabdoid tumor where genetic damage occurs. It is well known that repression of E-cadherin by *EZH2* is associated with the aggressiveness of many carcinomas,<sup>27</sup> but it is still not clear what kind of other genes are silenced by *EZH2* in different tumors, although it seems that *SMARCB1* is not affected. To our best knowledge, this is the first report of larger series of *EZH2* expression in epithelioid sarcoma although five cases with a 16% labeling index were reported by Yamaga K *et al.*<sup>28</sup> It is possible, although unlikely, that the H3K27me3 mark is present without overexpression of *EZH2*, which could be assessed by chromatin immunoprecipitation followed by PCR.

If there is no epigenetic change on a DNA level, the next step is to examine the mRNA level. We could get proper RNA from 16 out of 25 cases using tissue blocks. In each case, a low but variable level of *SMARCB1* mRNA was found depending on the



**Figure 6** Results of *SMARCB1* gene expression from paraffin slides of epithelioid sarcomas. Quantification of mRNA levels for *SMARCB1* as determined by qRT-PCR analysis. The data represent mean  $\pm$  s.d. Note the different, usually low levels of individual cases compared with calibrator normal tissue (liver) (a). *SMARCB1* immunohistochemistry, performed on membrane mounted slide specific for laser capture microdissection (case number: 5). Note the *SMARCB1*-negative tumor cells and *SMARCB1*-positive lymphocytes (b;  $40\times$ ). In the course of laser capture microdissection, *SMARCB1*-negative tumor cells (c;  $40\times$ ) and lymphocytes (d;  $40\times$ ) were separately dissected for qRT-PCR. Amplification curves of *GAPDH* (from left to right curve 1 represents lymphocytes, curve 2 tumor cells), *SMARCB1* (curve 3 shows lymphocytes); the bottom straight line indicates tumor cells with no amplification of *SMARCB1* mRNA (e; the same fifth case).

ratio of normal to tumor tissue. It was unknown whether this meant a total lack of expression or low levels of expression. To clarify this issue, we chose seven cases for laser capture microdissection, including cases with very low and also with higher levels of mRNA. Separating tumor cells and normal control cells, tumor cells contained no detectable mRNA, whereas normal cells and the internal control of *GAPDH* displayed equivalent expression. A lack of mRNA of *SMARCB1* gene means that neither normal nor altered *SMARCB1* protein exists, ruling out the possibility of epigenetic changes on the protein level, such as protein degradation. These results suggest that the *SMARCB1* mRNA is produced then silenced by expression of microRNA.

Gene silencing by microRNA resulting in a decreased level or lack of mRNA is a well-known epigenetic mechanism in different tumors.<sup>29</sup>

In our study, we confirm that the majority of epithelioid sarcomas do not harbor biallelic genetic alterations of the *SMARCB1* gene but could not prove the putative role of hypermethylation of DNA and/or histone methylation to silence gene activity. The absence of *SMARCB1* mRNA clarifies that altered protein is not responsible and calls attention to the possible role of microRNA. It seems that although the malignant rhabdoid tumor and epithelioid sarcoma (especially proximal type) share morphologic features as well as loss of expression of the *SMARCB1* protein, they differ in the

molecular mechanisms leading to loss of SMARCB1 protein expression. Indeed, malignant rhabdoid tumors frequently show biallelic loss of the *SMARCB1* locus, whereas loss of SMARCB1 in epithelioid sarcomas appears to depend on epigenetic mechanisms.

## Disclosure/conflict of interest

The authors declare no conflict of interest.

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