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Prognostic determinants in epithelioid sarcoma

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ABSTRACT

Background: Epithelioid sarcoma (ES) is a rare soft tissue neoplasm that usually arises in the distal extremities of young adults, presents a high rate of recurrences and metastases and frequently poses diagnostic dilemmas. In order to identify markers useful for patient stratification purposes, we investigated the prognostic impact of clinical and molecular patient characteristics, including the status of *SMARCB1* tumour suppressor gene, in a consecutive series of ES cases.

Methods: Kaplan–Meier survival curves were compared by the log-rank test. Immunophenotyping and SMARCB1 protein expression were analysed by immunohistochemistry or western blotting in 40 ES patients for which tumour material was available. Cases lacking SMARCB1 protein expression were investigated for the presence of gene mutations and gene deletions by exon sequencing, fluorescent *in situ* hybridization and quantitative PCR.

Results: FNCLCC tumour grade 3 and proximal-type histology significantly correlated with shorter overall survival (log-rank $p = 0.0046$ and $p = 0.0001$, respectively). We identified loss of SMARCB1 protein expression in the majority of ES cases (25/40, 62.5%), including 24/34 (71%) adult cases but only 1/6 (17%) paediatric/adolescent cases ($p = 0.02$, two-tailed Fisher's exact test). The absence of protein is strongly correlated with *SMARCB1* gene deletion ($p = 0.003$, two-tailed Fisher's exact test). We observed a trend towards the correlation between *SMARCB1* inactivation and both higher tumour grading and a clinical course of the disease characterised by the occurrence of multiple relapses/metastasis.

Conclusion: These data show that both tumour grading and subtype are prognostic factors in ES. Loss of SMARCB1 protein expression in ES is a frequent occurrence mediated by gene deletion events, thus pointing to a crucial role of *SMARCB1* in ES genesis. Analysis of

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SMARCB1 status in ES warrants prospective investigation as a prognostic marker and therapeutic target.

1. Introduction

Epithelioid sarcoma (ES, International Classification of Diseases for Oncology Code Number 8804/3) is a rare mesenchymal neoplasm that displays variable epithelioid morphology, presents a high rate of recurrences and metastases and frequently poses diagnostic dilemmas.[1] It usually affects young adults and arises in the distal extremities (classic-type ES) or, more rarely, in proximal sites of the trunk (proximal-type ES). Proximal-type ES is more frequently associated with epithelioid or rhabdoid morphology and higher mitotic activity,[2] but the prognostic impact of ES subtype is still unclear.

Similarly, grading schemes in ES are disputed and have been discouraged by some Authors who considered ES a high-grade soft tissue sarcoma,[1] although recently the FNCLCC grading system demonstrated prognostic value in a cohort of ES in children and adolescents.[3]

We previously reported evidence of *SMARCB1* inactivation in 6/11 ES cases.[4] The *SMARCB1* gene, located at the 22q11 chromosomal region, encodes for an invariant subunit of SWI/SNF chromatin remodelling complex and has been reported to act as a tumour suppressor gene in infantile malignant rhabdoid tumour (MRT),[5,6] a highly aggressive neoplasm affecting renal or extrarenal soft tissue and cerebral tissue in paediatric patients. Previous studies indicate that the analysis of the status of *SMARCB1* may be an informative predictor for rational targeted interventions directed against the Cdk/Cyclin pathway, such as 4-HPR[7] and flavopiridol[8] Cdk/Cyclin inhibitors.

We therefore investigated the prognostic value of relevant clinico-pathologic variables as well as the frequency and the mechanism of *SMARCB1* inactivation in a consecutive series of ES and examined potential associations with clinical parameters. Our results show that FNCLCC tumour grade 3 and proximal-type histology significantly correlated with shorter overall survival and that the loss of SMARCB1 protein expression by means of gene deletion events is a frequent occurrence, thus pointing to a crucial role of *SMARCB1* in ES genesis.

2. Materials and methods

2.1. Patient samples

Fifty-six Bouin's- or formalin-fixed, paraffin-embedded (FFPE) tumour samples were retrieved from the Department of Pathology of the Istituto Nazionale Tumori of Milan archives. Slides were reviewed and diagnoses confirmed applying updated criteria.[1,9] The case series was formed by 40 ESs, 10 high-risk gastro-intestinal stromal tumours (GISTs) and 6 MRTs (3 renal, 1 extrarenal and 2 ATRT). ESs were subdivided into classic and proximal-type and the FNCLCC system[1,10] for ES grading was applied, in order to investigate its potential prognostic value. This system is based on a final grading which combines the scores for differentiation, mitotic index, and necrosis. Clinical features of ES patients were in part previously published.[3,11] Six cases aged 10–18 years were admitted and treated at the Paediatric Oncology Department. Thirty-two adult patients aged above 18 were admitted and treated at the Unit of Melanoma and Sarcoma Surgery. In selected cases frozen tumour tissue samples, collected following institutional review board guidelines, were available. DNA was extracted from 10 μm frozen or FFPE tissue sections using Qiagen DNA Mini kit (Qiagen, Italy, Milano I), following manufacturer's recommendations. Haematoxylin–eosin staining was performed prior to extraction to verify for the presence of more then 60% tumour cells in frozen samples. FFPE normal tissues microarray was purchased from AccuMax array (Petagen Inc., Seoul Korea).

2.2. Protein expression

Immunophenotyping with CD34, cytokeratins, epithelial membrane antigen and CD31 antibodies was performed for uniform pathologic re-examination. Protein expression of SMARCB1 was investigated by immunohistochemistry using anti-BAF47/SNF5 antibody 1:100 (BD Transduction Laboratories, Buccinasco I). Endogenous peroxidise was blocked with 0.3% hydrogen peroxide in methanol for 30 min. For antigen retrieval the slides were immersed in citrate buffer solution 5 mM pH 6 and heated in autoclave at 95 °C for 15 min. Immunohistochemistry analysis was done using Ultra vision detection system (LabVision, Fremont, CA). As controls, sarcoma samples from different subtypes were analysed concomitantly. Loss of SMARCB1 protein expression as detected by immunohistochemistry was not observed in GIST tumours, including high-risk cases (0/10) that frequently suffer of chromosome 22 losses, whilst the paediatric malignant rhabdoid tumours investigated invariably displayed SMARCB1-negative immunostaining. For Western blot analysis, protein lysates were prepared in RIPA buffer (Sigma–Aldrich S.r.l., Milano I), 40 μg of cell lysate were loaded on 4–15% gradient polyacrylamide gels (Biorad Lab, Segrate I) and electro-blotted on to polyvinylidene difluoride membranes (Amersham, GE Healthcare, Milano I). Subsequently, membranes were incubated 1 h at room temperature in a solution of phosphate-buffered saline supplemented with 5% nonfat dry milk. For immunodetection, the anti-BAF47/SNF5 (1:250, BD Transduction Laboratories) and anti-CyclinD1 (1:1000, DCS6 Santa Cruz Biotechnology, CA) antibodies were used. After overnight incubation at 4 °C with the primary antibody, membranes were washed in TBST [10 mmol/l Tris–HCl (pH 8.0), 0.15 mol/l NaCl, and 0.05% Tween 20], followed by AlexaFluor680- or IRDye800CW-conjugated antibodies (from Invitrogen Life Technologies, San Giuliano Milanese I and Li-Cor Biosciences, Lincoln US, respectively). Odyssey infrared imaging system (Li-Cor Biosciences) was used for detection. Protein loading equivalence was assessed using an anti-Gapdh antibody (Sigma–Aldrich).

2.3. Fish

Gross gene deletions were analysed by fluorescent *in situ* hybridization (FISH) with BAC (Bacterial Artificial Chromosome) probes, using BAC RP11-71g19 encompassing *SMARCB1* gene at 22q11 and control BAC RP11-262a13 located at 22q13.3 (probes kindly provided by Resources for Molecular Cytogenetics, Bari I). FISH analysis was performed on FFPE or frozen tumour samples as previously described.[4] A minimum of 100 nuclei were counted, the expected number of nuclei showing 0, 1 and 2 signals was calculated on control, normal FFPE samples or on patient's epidermis in sub-cutaneous tumours where it was part of the same tissue block. Deviation from the expected number of signals in the tumour tissue analysed was evaluated by Chi-square test. Control probe provided a technical control of the labelling and hybridization procedures.

2.4. Mutational and deletion analysis

Mutational analysis was performed by exon amplification and sequencing as previously described.[4] Intragenic deletions were detected by quantitative genomic PCR using primer pairs spanning *SMARCB1* exon 4. As control loci, 2 loci located in a separate chromosome (chr11:98891904–98891994 and chr11:100863268–100863358) that we previously validated for proper amplification efficiency and ability to reveal copy-number changes (Fig. S1) were also analysed by genomic q-PCR. Primers (see Fig. S1) were designed uniformly in relation to Tm, GC content, primer and amplicon length using Primer-Express software (Applied Biosystems Foster City, CA, USA). Quantitative PCR assays were performed in 20 μl volume of 1× SybrGreen PCR master mix (Applied Biosystems), using 100 nM of primers and 10 ng of genomic DNA, with the thermal profile 52° 2 min, then 40 cycles of 92° 15 s, 60° 1 min. Data were analysed with the ddCt method for relative quantification, using the 11q22 locus amplicons as endogenous controls and the normal, donor-derived genomic DNA as calibrator. We preliminarily validated the relative quantification method by calculating the amplification efficiency of each primer pair at different DNA concentrations ranging from 1 to 100 ng. We subsequently verified the sensitivity of the assay using G401 rhabdoid sarcoma cell line, which carries a homozygous deletion of *SMARCB1* gene. Dilution of G401 genomic DNA with donor-derived normal DNA titrated at different ratios (0%, 25%, 50%, 75%, 90% and 100%) showed that under the conditions applied a 50% mixture (corresponding to a uniform hemizygous deletion or a homozygous deletion in 50% of cells analysed) was consistently measurable (Fig. S1). Four paediatric malignant rhabdoid tumours could be analysed concomitantly at the molecular level (Fig. S3) and revealed *SMARCB1* gene deletion in two cases (1 extrarenal soft tissue and 1 cerebral atypical theratoid–rhabdoid tumour) and point mutations in two other cases (1 renal and 1 cerebral atypical theratoid–rhabdoid tumour).

2.5. Cell cultures

G401 malignant rhabdoid tumour cancer cell line (American Type Culture Collection, Manassas, VA) was maintained in RMPI + 10% foetal bovine serum medium, in conventional 5% CO_2 atmosphere and 100% humidity incubators. Short-term cell cultures were established from fresh tumour material by mechanical and collagenase II enzymatic dissociation, followed by culturing in Amniomax-C100 medium (Gibco Life Technologies, San Giuliano Milanese I). Conventional and Spectral Karyotyping (Applied Spectral Imaging, Vista, CA) was performed as previously described.[12]

2.6. Statistics

All statistical analyses were carried out using GraphPad software (GraphPad Inc., La Jolla, CA). Comparisons between two classes have been performed by two-tailed Fisher's exact test. Kaplan–Meier survival curves were plotted and compared by the log-rank test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Clinical variables analyses

Fifty-two ES cases were treated at the Istituto Nazionale Tumori of Milan in the period 1986–2006. Pathologic re-examination was preformed in 40 cases for which suitable tumour material was available (Table 1). Median age was 31 years (range 10–66), histology was classic-type in 27 (67.5%) and proximal-type in 13 (32.5%), FNCLCC grading was 2 in 15 (37.5%) and 3 in 25 (62.5%). Six were paediatric/adolescent cases aged 10–18 years and 34 were adult patients above 18 years. In 14 patients, a neoadjuvant chemo- or radiotherapy regimen was administered prior to surgery. Ten cases presented multiple nodules or metastasis at the time of first surgery. Twenty patients experienced pulmonary or extrapulmonary metastasis during the disease course and 18 of them died of disease. In total, 26 patients relapsed, 19 patients died of their disease and two patients presented relapsing disease at last follow-up. Average and median follow-up time for patients alive were 51 and 50 months, respectively. Event-free survival and overall survival at 5 years were 21% and 60%, respectively.

As shown by Kaplan–Meier survival curves in Fig. 1, both tumour grade 3 and proximal-type histology were significantly correlated with shorter overall survival (log-rank test $p = 0.0046$ and $p = 0.0001$, respectively). The risk ratio (HR) conferred by grade 3 was 8.6 (95% confidence interval (CI) 1.9–38) and by proximal-type histology was 5.6 (95% CI 2.4–13.2). There was no significant correlation between occurrence of patient relapse and FNCLCC grading ($p = 0.50$) or ES histological subtype ($p = 0.15$).

3.2. SMARCB1 protein expression analysis

Immunohistochemistry revealed the absence of SMARCB1 protein expression (Fig. 2) in 25/40 (62.5%). SMARCB1 loss was predominant in the adult cases (24/34, 71%) compared to the paediatric/adolescent ones (1/6, 17%) ($p = 0.02$). The difference was still significant when the cut-off was set at 25 years of age ($p = 0.04$, Table 2), suggesting that SMARCB1 inactivation is likely to play a minor role in the juvenile form of ES compared to the adult form. No correlation was found between SMARCB1 expression and staging, location, subtype

Table 1 – Characteristics of epithelioid sarcoma patients.

ES no.	Age	Gender	Site	Size (cm)	ES subtype	FNCLCC grading	SMARCB1 IHC	*SMARCB1* status	Relapses	Metastasis	Follow-up	Status
1	29	M	Forearm	10	C	2	neg	HD	4	No	46	NED
2	36	M	Forearm	4	C	2	neg	na	No	No	4	NED
3	29	M	Hand	6	C	3	neg	na	No	Y	14	DOD
4	33	M	Hand	5	C	3	neg	HD	4	No	42	AWD
5	25	M	Hand	1	C	2	neg	na	1	No	4	NED
6	66	F	Trunk	6	P	3	neg	HD	4	No	9	DOD
7	31	M	Hand	2	C	3	pos	wt	1	Y	41	DOD
8	26	F	Hand	1	C	2	neg	HD	3	No	50	NED
9	36	M	Hand	4	C	3	neg	HD	1	Y	82	DOD
10	31	F	Thigh	9	P	3	neg	HD	No	Y	43	DOD
11	47	F	Thigh	4	P	3	pos	wt	No	No	50	NED
12	25	M	Hand	3	C	3	neg	wt	2	Y	77	DOD
13	55	F	Foot	3	C	2	pos	wt	1	Y	11	DOD
14	40	M	Forearm	2	C	3	neg	HD	3	No	56	AWD
15	30	M	Hand	1	C	3	neg	wt	3	No	55	NED
16	25	M	Leg	5	C	2	neg	wt	2	No	121	NED
17	34	M	Hand	1	C	2	neg	wt	1	Y	74	NED
18	25	M	Proximal arm	8	C	3	pos	wt	1	Y	10	DOD
19	34	F	Trunk	12	P	3	neg	na	No	Y	7	DOD
20	21	M	Hand	2.5	C	2	pos	wt	4	Y	58	DOD
21	25	F	Thigh	1	C	3	pos	wt	No	No	53	NED
22	32	F	Trunk	4	P	3	neg	HD	No	No	13	NED
23	66	M	Thigh	19	P	3	pos	na	No	Y	2	DOD
24	65	M	Trunk	6	P	3	neg	na	1	No	11	NED
25	27	M	Leg	1	C	3	neg	wt	No	Y	24	DOD
26	34	M	Forearm	0.5	C	3	neg	HD	2	Y	96	DOD
27	71	M	Trunk	5	P	3	pos	wt	1	Y	9	DOD
28	47	F	Trunk	3	P	3	neg	HD	2	Y	13	DOD
29	48	F	Trunk	3	P	3	neg	na	1	Y	2	NED
30	22	M	Hand	1	C	3	neg	HD	5	Y	24	DOD
31	36	F	Trunk	4	P	3	neg	wt	No	Y	9	DOD
32	27	M	Proximal arm	5	C	2	neg	wt	No	No	88	NED
33	64	M	Trunk	8	P	3	pos	na	No	Y	2	DOD
34	45	M	Thigh	2	C	2	pos	na	No	No	12	NED
35	16	F	Leg	3	C	2	pos	na	1	No	124	NED
36	17	M	Proximal arm	8	C	3	neg	wt	1	Y	54	DOD
37	10	M	Forearm	6	C	2	pos	wt	2	No	126	NED
38	16	F	Hand	3	C	2	pos	na	1	No	66	NED
39	13	M	Neck	4	P	2	pos	na	1	No	20	NED
40	18	F	Proximal arm	3	C	2	pos	na	No	No	20	NED

Abbreviations: ES = epithelioid sarcoma; age in years; C = classic-type; P = proximal-type; HD = homozygous deletion; na = not available; wt = normal; Y = yes; NED = no evidence of disease; DOD = dead of disease; and AWD = alive with disease.

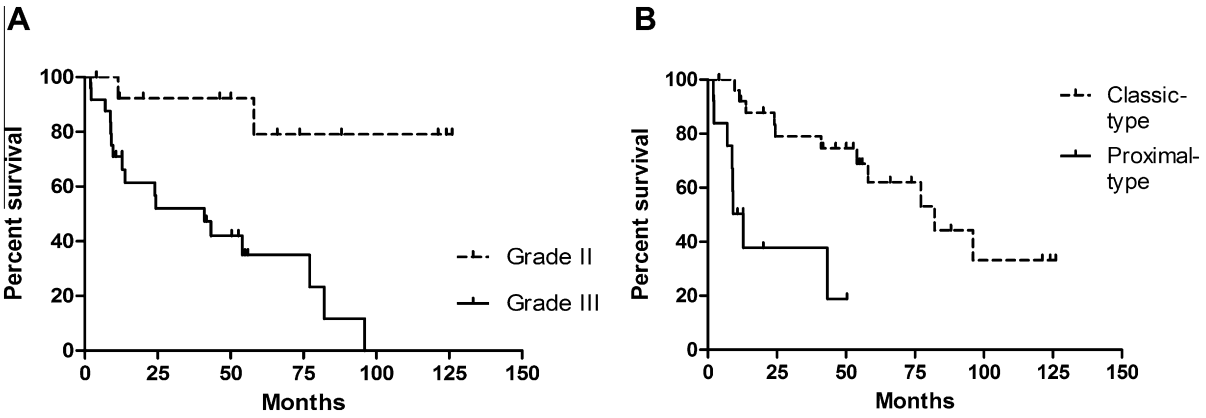

Fig. 1 – Kaplan–Meier survival curves of epithelioid sarcoma patients according to (A) grading II versus III ($p = 0.0046$ by log-rank test) and (B) classic versus proximal-type histology ($p = 0.0001$ by log-rank test).

or other clinical characteristics investigated (Table 2), including overall survival (HR = 2.1, 95% CI 0.7–6.1, log-rank $p = 0.16$ for adult cases only; HR = 1.2 95% CI 0.4–3.4, log-rank $p = 0.69$ for all patients). Interestingly, loss of SMARCB1 expression was prevalent in tumours that tended to evolve to metastatic/highly recurrent disease (more then two consecutive relapses): 19/25 IHC-negative cases (76%), in contrast to only 7/15 IHC-positive cases (47%), developed metastatic disease or more then two relapse events ($p = 0.09$). Neoadjuvant chemo- or radiotherapy regimen administered prior to surgery had no effect on SMARCB1 expression: the fraction of SMARCB1-positive and -negative tumours was the same in pre-treated versus naive cases (9/14 pre-treated cases were SMARCB1-negative compared to 16/26 of non pre-treated cases, $p = 1.00$).

The CD34 marker, which is used in routine diagnostics and is reported consistently negative in malignant rhabdoid tumours, was expressed in 14/34 (41%) cases analysed, including 10/24 (42%) SMARCB1-negative cases and 4/10 (40%) SMARCB1-positive cases, indicating that negative staining for SMARCB1 in ES did not correlate with lack of CD34 expression (Table 2).

3.3. *SMARCB1 gene status*

FISH analysis was performed successfully in 27/40 cases (Fig. 2), the main cause for FISH failure being fixation in Bouin's reagent. A strong correlation between SMARCB1 expression and gene status was observed: the SMARCB1 locus was retained in all 9/9 IHC-positive cases tested and lost in 9/18 IHC-negative cases analysed ($p = 0.01$). Similar to IHC analyses, no association between *SMARCB1* gene status and ES subtype, staging or location was detected. Instead, there was a significant correlation between tumour grading and SMARCB1 gene deletion as detected by FISH: all the 9 cases showing *SMARCB1* deletion by FISH were grade 3 compared to only 10/18 cases without deletion ($p = 0.03$).

Mutational analysis by exon amplification and sequencing in 18 cases, for which frozen or adequate FFPE tissue was available, did not reveal mutations. These cases included 7/9 IHC-negative cases that displayed normal gene copy-number as detected by FISH.

Since the BAC probe used for FISH analysis spans 148,000 bp, encompassing 9 genes (7 completely and 2 partially contained within the BAC probe), to investigate the presence of subtle *SMARCB1* gene deletions that may have escaped the FISH analysis, we applied a gene dosage assay by quantitative PCR in 13 IHC-negative cases for which frozen or adequate FFPE tissue was available (Figs. 3 and S1). In addition to confirming the presence of gene deletion previously detected by FISH, quantitative PCR uncovered significant loss of *SMARCB1* signal in two additional cases for which FISH signal was normal. These data suggest the presence of more subtle deletion events in a fraction of ES cases. Therefore, combining FISH and q-PCR results, we detected retention of *SMARCB1* in all 9/9 IHC-positive cases tested and loss of the entire *SMARCB1* gene in 11/18 IHC-negative cases tested ($p = 0.003$). Correlation between tumour grading and the occurrence of gene deletion was no more statistically significant, whilst a trend for an association with older age, already observed with IHC analysis, was present ($p = 0.09$, Table 2). There was no significant association between administration of neoadjuvant chemo- or radiotherapy regimen prior to surgery and *SMARCB1* gene status, suggesting that the deletion events identified are not caused by the treatment received in the short time preceding surgery ($p = 0.67$).

3.4. *Additional tissues and sarcoma samples analysed*

In addition to the previously reported cases,[4] we analysed a further short-term cell culture from one of the ES samples, which displayed the absence of SMARCB1 protein expression and gene deletion. Interestingly, this tumour sample showed weak SMARCB1 expression by western blotting and the absence of nuclear staining by immunocytochemistry, whilst presenting high CCND1 protein expression (Fig. S2), a phenomenon linked to *SMARCB1* ablation in paediatric MRTs.

Loss of SMARCB1 protein expression as detected by immunohistochemistry was not observed in GIST tumours, including high-risk cases (0/10) that frequently suffer of chromosome 22 losses, whilst the paediatric malignant rhabdoid tumours investigated displayed SMARCB1-negative immunostaining associated with the presence of gene mutations (Fig. S3).

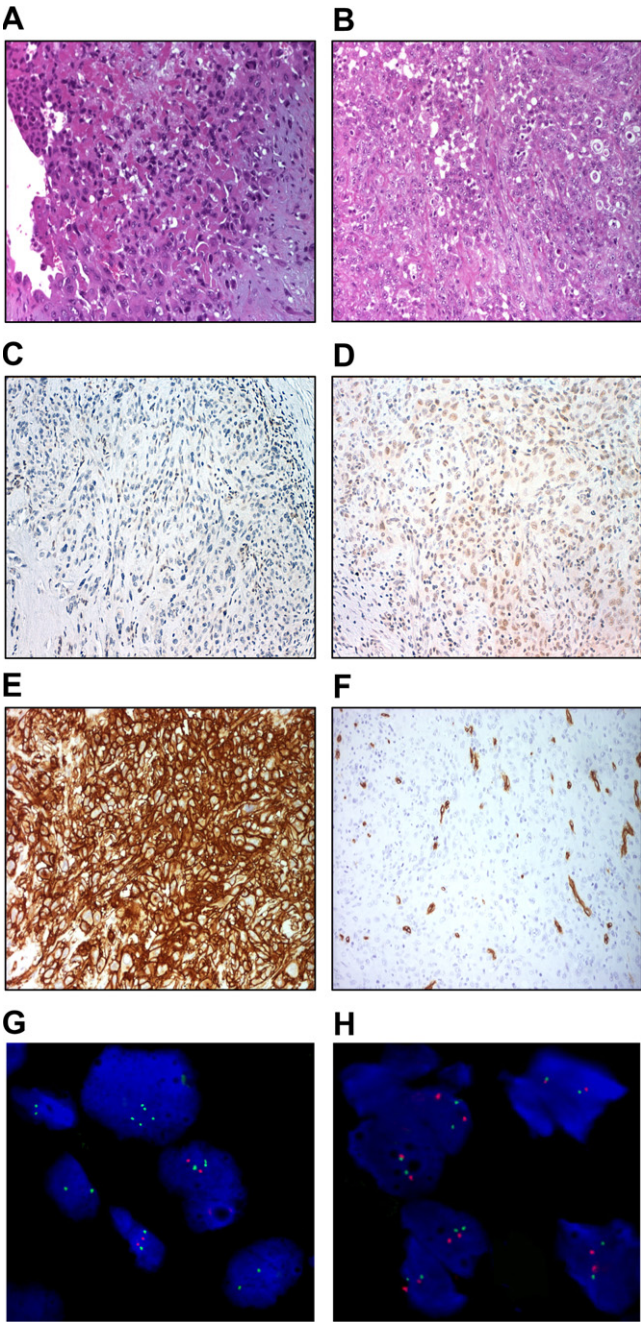

Fig. 2 – Immunohistochemistry and FISH analysis of epithelioid sarcoma samples. Representative IHC and FISH results from a SMARCB1-negative, FISH-deleted case (A–D) and a SMARCB1-positive, FISH wild-type case (E–H). Panels represent: haematoxylin–eosin staining (A and E); immunohistochemistry analysis for SMARCB1 (B and F) and CD34 (C and G) markers; FISH analysis (D and H) of *SMARCB1* (red signal) and control locus (green signal).

SMARCB1 is considered an invariant subunit of SWI-SNF chromatin remodelling complex and is predicted to be expressed ubiquitously. Nevertheless, scanty information are actually available on the actual pattern of expression of SMARCB1 in non-cancerous tissues. Therefore, we investigated SMARCB1 expression by IHC analysis in different normal tissues and established that the expression at the protein level is not ubiquitous (Fig. S2). The highest expression levels were detected in lymphoid tissue, followed by epithelial tissues. Kidney, the site of classic malignant rhabdoid tumour, showed strong staining at tubular structures. In addition, epithelial linings of colon and ovary displayed intense staining, whilst brain compartments showed only moderate to weak protein expression. Staining was invariably nuclear.

4. Discussion

In an attempt to define prognostic factors that may provide useful information for the management of ES, we analysed a consecutive series of 40 ES surgically treated at the INT National Cancer Institute in Milan. In our case series, histological subtype and FNCLCC grading represent strong prognostic indicators of poor prognosis. We also found suggestive evidence of correlation at the univariate analysis between tumour grading or aggressive evolution (metastasis/multiple relapse events) and *SMARCB1* gene and protein loss, respectively. Intrinsic limitations related to disease rarity and heterogeneity in treatment approach over long periods of time, even within a single Institution, hamper further statistical analyses. Therefore, only prospective investigation in the frame of multicentric clinical trials will have the potential to ascertain the prognostic value of *SMARCB1* in ES.

We previously reported evidence of *SMARCB1* alteration in 6/11 ES cases.[4] In the present report, we confirm in a larger patient series the frequent inactivation of *SMARCB1* tumour suppressor in ES, a tumour entity distinct from MRTs previously known to be caused by *SMARCB1* mutation. Very recently, additional studies concordantly reported SMARCB1 protein silencing in ES,[13,14] but the gene status was rarely investigated at the molecular level.[15,16] Herein we provide evidence that SMARCB1 protein loss in ES is associated with homozygous gene deletion in over 60% of the cases. We failed to detect gene point mutations in our series and the causative genetic or epigenetic event leading to *SMARCB1* inactivation remains elusive in 39% of the IHC-negative cases, which retain an apparently normal gene copy-number. However, by a genomic q-PCR approach we were able to unveil two additional cases of gene deletion that were missed by FISH. It should be emphasised that our q-PCR approach was performed by focusing on a small region spanning exon 4. Thus, it is likely that other small deletions, involving genomic regions other then exon 4, may account for IHC negativity in ES. More refined deletion–detection techniques, such as multiplex ligation-dependent probe amplification (MLPA)[18] may further improve the sensitivity of the assessment of *SMARCB1* intragenic deletions. Unfortunately, also for these approaches the use of old FFPE tissue samples may represent a limitation. Moreover, other mechanisms of gene silencing operating at the transcriptional or post-transcriptional level cannot be ruled out. Notably, consistent with our results, the other reports of ES cases investigated for *SMARCB1* gene status reported the occurrence of deletions[4,15,16] and so far, only one case of ES carrying a 1-bp substitution has been reported,[17] showing

Table 2 – Association between SMARCB1 status and clinical characteristics.

	SMARCB1 IHC		*p*-Value	SMARCB1gene status		*p*-Value
	neg	pos		HD	wt	
ES subtype						
C	17	10	1.00	7	13	0.39
P	8	5		4	3	
FNCLCC grading						
2	7	8	0.18	2	6	0.40[b]
3	18	7		9	10	
Tumour site						
extremity	15	6	0.33	7	9	1.00
other	10	9		4	7	
TNM staging						
T1N0M0	12	10	0.33	5	10	0.45
higher	13	5		6	6	
Metastasis or multiple relapses[a]						
yes	19	6	0.09	10	10	0.18
no	7	8		1	6	
Age						
>25	20	7	0.04[c]	10	9	0.09
⩽25	5	8		1	7	
CD34 stain						
neg	14	6	1.00	5	10	0.44
pos	10	4		5	5	
SMARCB1 status						
HD	11	0	0.003			
wt	7	9				

Abbreviations: ES = epithelioid sarcoma; C = classic-type; P = proximal-type; and HD = homozygous deletion as assessed by FISH and/or q-PCR.
[a] Patients were distinct based on the occurrence of metastatic disease or more than two consecutive recurrence events.
[b] The *p*-value = 0.03 if considering SMARCB1 gene status by FISH only.
[c] The *p*-value = 0.02 if considering the 18 years cut-off age.

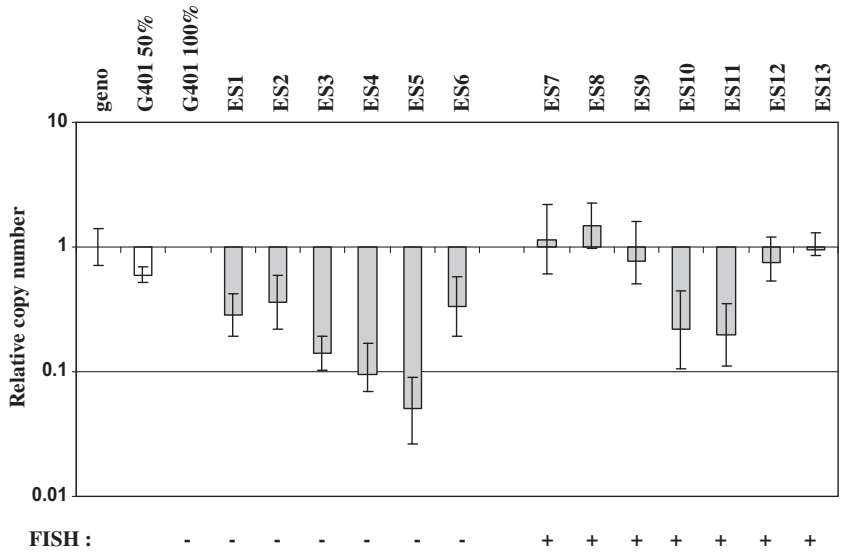

Fig. 3 – Quantitative PCR analysis of *SMARCB1* exon 4 copy-number in immunohistochemistry-negative epithelioid sarcoma cases. Normal genomic DNA (geno) was used as calibrator, genomic DNA from G401 cell line (carrying a homozygous deletion of *SMARCB1* gene) and a 1:1 mixture of the two DNAs were used as controls. Amplification of an unrelated genomic locus was used as endogenous control. Data show evidence of gene deletion in FISH-deleted epithelioid sarcoma cases, and also in two additional FISH-positive cases, suggesting the presence of an intragenic deletion.

that, although rarely, even point mutations can contribute to the malignant transformation of epithelioid sarcoma.

Soft tissue sarcomas encompass the paediatric and adult age groups and so far it is not established whether the distinct sarcoma subtypes have the same clinical behaviour when arising in adults or in children. In addition, to our knowledge no published data clearly showed the presence of alternative molecular features in the different age groups affected by the same sarcoma subtype. Notably, in our case series *SMARCB1* inactivation was more frequent in patients older than 25 years, suggesting that juvenile ES are only rarely associated with *SMARCB1* inactivation. Investigation of a larger cohort of ES will be necessary to establish whether different molecular alterations affect paediatric versus adult patients.

Several issues raise the possibility that ES and MRT may actually represent distinct manifestations of a unique tumour entity, and that the age-window in which *SMARCB1* inactivation occurs does influence the different clinical course observed between ES and MRT. Nevertheless, a number of facts are still difficult to reconcile with this hypothesis and rather support an independent origin of the two entities. In fact, to the best of our knowledge, no ES occurrences have been reported so far in the spectrum of the rhabdoid predisposition syndrome, despite the existence of adult mutation-carriers; a different mutational spectrum has been observed in ES and MRT; morphologic and immunophenotyping analyses are reported to distinguish between ES and MRT tumours.[19,20] Thus far, the issue remains unresolved.

A number of different neoplasms are characterised by the loss of genetic material at the chromosome region 22q11. Indeed, in addition to *SMARCB1*, several other known or candidate tumour suppressor genes lie on this chromosome arm including CHEK2, EP300 and NF2, which may explain the importance of 22q loss in tumours retaining *SMARCB1* expression, such as the high-risk GISTs samples we analysed. Interestingly, SAGE data show a significant reduction of *SMARCB1* expression in lymphomas compared to non-cancerous lymph nodes (http://cgap.nci.nih.gov/SAGE), which is the tissue holding the highest expression of SMARCB1 protein, as we determined by immunohistochemistry. In addition, very recently SMARCB1 protein loss of expression has been reported as a prognostic marker in melanoma, but no genetic investigations have been performed so far.[21] Due to the subtle nature of *SMARCB1* gene alterations, particularly small homozygous deletions, and to the difficulty in detecting downregulation at the RNA level, which is greatly masked by infiltration of normal cells expressing high level of SMARCB1 transcript, we propose that a broader involvement of *SMARCB1* inactivation in cancer is worth to be investigated by combined analyses at the genomic DNA level by FISH and/or q-PCR and at the protein level by immunohistochemistry.

Previously published studies indicate that *SMARCB1/INI1* gene is involved in the control of genomic stability and in the regulation of cell cycle progression.[22] SMARCB1 stimulates the p16/Rb tumour suppressor pathway by activation of CDKN2A and inhibition of Cdk/CyclinD.[23] As a result, in MRT cell lines it has been demonstrated that *SMARCB1* loss is associated with responsiveness to Cdk/Cyclin inhibitors, such as 4-HPR[7] and flavopiridol.[8] In addition, the *in vivo* spontaneous tumourigenesis in *SMARCB1* knock-out mice is prevented by CCND1 ablation.[24] These results suggest that the analysis of the status of *SMARCB1* may be an informative predictor for rational targeted interventions directed against the Cdk/Cyclin pathway. Our results provide a rationale for the investigation of the therapeutic potential of such molecules in any type of *SMARCB1*-negative neoplasm. In this regard, the availability of *in vitro* models to test this hypothesis remains mandatory.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2010.09.003.

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