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ORIGINAL ARTICLE – CANCER RESEARCH

DNA methylation profiling distinguishes Ewing-like sarcoma with *EWSR1–NFATc2* fusion from Ewing sarcoma

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Abstract

Purpose Recent studies revealed divergent gene expression patterns in Ewing sarcoma (EwS) with canonical *EWSR1–ETS* gene fusions and undifferentiated round cell sarcomas (URCS) with *EWSR1* rearrangements fused to the *non-ETS* gene *NFATc2*. Thus, the question arises whether the latter tumors really belong to EwS.

Methods We collected five cases matching the group of URCS with *EWSR1–NFATc2* fusion and performed DNA methylation and copy number profiling. Results were compared to methylation data of 30 EwS with various *EWSR1–ETS* fusions and one EwS with *FUS–ERG* fusion, 16 URCS with *CIC* rearrangement and 10 URCS with *BCOR* alteration and a total of 81 *EWSR1*-associated soft tissue sarcomas including 7 angiomatoid fibrous histiocytomas, 7 clear cell sarcomas of the soft tissue, 28 desmoplastic small round cell tumors, 10 extraskeletal myxoid chondrosarcomas and 29 myxoid liposarcomas.

Results Unsupervised hierarchical clustering and t-distributed stochastic neighbor embedding analysis of DNA methylation data revealed a homogeneous methylation cluster for URCS with *EWSR1–NFATc2* fusion, which clearly segregated from EwS and the other subtypes. Copy number profiles of *EWSR1–NFATc2* cases showed recurrent losses on chromosome 9q and segmental gains on 20q13 and 22q12 involving the *EWSR1* and *NFATc2* loci, respectively.

Conclusion In summary, URCS with *EWSR1–NFATc2* fusion share a distinct DNA methylation signature and carry characteristic copy number alterations, which emphasizes that these sarcomas should be considered separately from EwS.

Keywords EWSR1 · NFATc2 · Ewing · Ewing like · DNA methylation

Introduction

Ewing sarcoma (EwS) is a highly malignant tumor predominantly affecting the bones of children, adolescents and young adults. EwS belongs to the group of undifferentiated round cell sarcoma (URCS) and represents one of the most frequently diagnosed malignant mesenchymal bone tumors, accounting for approximately 5–10% of all cases (Alava et al. 2013). However, 10–15% of EwS primarily arise within soft tissue (Sankar and Lessnick 2011).

Precise separation of EwS from URCS without knowledge of the molecular phenotype is challenging for pathologists, because most tumors lack unambiguous morphological features or a specific immunophenotype (Alava et al. 2013).

EwS is genetically characterized by *EWSR1-ETS* gene fusions (Grunewald et al. 2018). *EWSR1–FLI1* represents

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the most common gene fusion in EwS (85%), followed by *EWSR1-ERG* (10%) (Delattre et al. 1992; Sorensen et al. 1994). Less frequently (< 1%), *EWSR1* is fused to other *ETS* gene members such as *ETV1, ETV4* and *FEV* (Jeon et al. 1995; Kaneko et al. 1996; Peter et al. 1997). In exceptionally rare *EWSR1* wild-type cases, a *FUS–ERG* fusion has also been described (Chen et al. 2016; Shing et al. 2003).

Some URCS show a significant morphologic overlap with EwS. However, they lack the prototypic *EWSR1–ETS* or *FUS–ERG* fusions. These tumors were termed EwS-like URCS (Antonescu 2014). Through molecular studies investigating EwS-like URCS, it was possible to subcategorize this heterogeneous group of sarcomas further. More than 90% of EwS-like URCS carry either *CIC–DUX4* fusions or *BCOR* alterations (Italiano et al. 2012; Pierron et al. 2012; Specht et al. 2016). The current concept proposes both *BCOR*- and *CIC*-positive URCS to be considered as stand-alone subtypes with distinct clinical features and differing biological behavior (Antonescu et al. 2017; Kao et al. 2018). Thus, precise subtyping of EwS-like URCS will soon become relevant in a clinical context.

Despite the recent progress in genomic characterization and biological understanding of URCS, some cases remain ambiguous. These URCS carry fusions between *EWSR1* and members outside the *ETS* gene family, which often occur between *EWSR1* and *NFATc2* (Mastrangelo et al. 2000; Savola et al. 2009; Sumegi et al. 2011; Szuhai et al. 2009; Wang et al. 2007). The current 2013 World Health Organization (WHO) classification of soft tissue and bone tumors provisionally assigned these cases to EwS (Alava et al. 2013). However, recent gene expression-based studies observed major differences between URCS with *EWSR1–NFATc2* fusion and EwS (Baldauf et al. 2018b; Specht et al. 2014; Watson et al. 2018). Also, *EWSR1–NFATc2*-positive sarcomas show a striking predominance of affecting mainly male adults (Grunewald et al. 2018). Thus, there is an ongoing debate whether URCS with *EWSR1–NFATc2* fusion belong to EwS, or whether they represent a distinct entity (Baldauf et al. 2018a; Charville et al. 2018).

To address this issue further, we performed comparative, genome-wide DNA methylation profiling and cytogenetic analyses of five URCS with *EWSR1–NFATc2* fusion.

Materials and methods

Sample selection

We collected five cases matching the group of URCS with *EWSR1–NFATc2*. The samples were retrieved from the Gerhard-Domagk Institute of Pathology of the University Hospital Münster (Germany), the Institute of Pathology of the University Hospital in Leiden (the Netherlands), the Institute of Pathology of the University Hospital in Basel (Switzerland) and the Department of Pathology of the University Hospital Virgen del Rocío in Seville (Spain). The *EWSR1–NFATc2* fusion has been confirmed in four cases, among them two cases from the seminal paper by Szuhai and colleagues (Szuhai et al. 2009). In one previously published case, detection by next-generation sequencing and FISH analysis failed due to poor RNA quality, though the copy number profile revealed amplifications involving the *EWSR1* locus (Koelsche et al. 2018a). The five URCS with *EWSR1–NFATc2* were reviewed by H&E staining. Histological sections were scanned with the NanoZoomer 2.0-HT digital slide scanner (Hamamatsu Photonics, Hamamatsu City, Shizuoka Prefecture, Japan) and images were captured with the ImageScope software (Leica Biosystems, Buffalo Grove, IL, USA).

The control group included 31 EwS with *EWSR1–FLI1* ($n = 24$), *EWSR1–ERG* ($n = 4$), *EWSR1–ETV1* ($n = 1$), *EWSR1–FEV* ($n = 1$) and *FUS–ERG* ($n = 1$) gene fusions. Furthermore, 16 URCS with *CIC* rearrangement and 10 URCS with *BCOR* alteration were included.

In addition, the control group was expanded by including sarcomas that may comprise *EWSR1*-rearrangements, namely angiomatoid fibrous histiocytoma ($n = 7$), clear cell sarcoma of the soft tissue ($n = 7$), desmoplastic small round cell tumor ($n = 28$), extraskeletal myxoid chondrosarcoma ($n = 10$) and myxoid liposarcoma ($n = 29$). The methylation data of the control group and of two URCS with *EWSR1–NFATc2* fusions have been previously reported (Koelsche et al. 2018a, b). Basic clinical information of the investigated cases is provided in Supplementary Table 1. This investigation was performed in accordance with the Declaration of Helsinki.

DNA isolation, genome-wide DNA methylation data generation and pre-processing

Representative tumor tissue with highest available tumor content was chosen for DNA extraction. The Maxwell® 16 FFPE Plus LEV DNA Kit or the Maxwell® 16 Tissue DNA Purification Kit (for frozen tissue) was applied on the automated Maxwell device (Promega, Madison, WI, USA) according to the manufacturer's instructions. All tumors had a total amount of > 100 ng DNA and were suitable for the array-based DNA methylation analysis. All tumors were subjected to Illumina Infinium HumanMethylation450 (450 k) BeadChip or the successor EPIC/850 k BeadChip (Illumina, San Diego, USA) analysis at the Genomics and Proteomics Core Facility of the German Cancer Research Center (DKFZ) Heidelberg. DNA methylation data were normalized by performing background correction and dye bias correction (shifting of negative control probe mean intensity to zero and scaling of normalization control probe

mean intensity to 20,000, respectively). Probes targeting sex chromosomes, probes containing multiple single nucleotide polymorphisms and those that could not be uniquely mapped were removed. Probes from the EPIC array were excluded if the predecessor Illumina Infinium 450 k BeadChip did not cover them, thereby making data generated by both 450 k and EPIC feasible for subsequent analyses. In total, 438,370 probes were kept for analysis.

Unsupervised clustering, t-SNE analysis and cumulative copy number plotting

For unsupervised hierarchical clustering, we selected 10,000 probes that showed the highest median absolute deviation (MAD) across the beta values. Samples were hierarchically clustered using the Euclidean distance and Ward's linkage method. Hierarchical clustering using Euclidean distance and complete linkage reordered methylation probes. The unscaled methylation levels were shown in a heat map from unmethylated state (blue color) to methylated state (red color). For unsupervised 2D representation of pairwise sample correlations, dimensionality reduction by t distributed stochastic neighbor embedding (t-SNE) was performed using the 10,000 most variable probes, a perplexity of 20 and 2500 iterations. Novel methylation groups were tested for stability by varying the number of the most variable probes. Copy number assessment was done based on methylation array data using the R-package conumee after additional baseline correction (https://github.com/dstichel/conumee).

Results

Study cohort

Five tumors from five patients matching the molecular criteria of URCS with *EWSR1–NFATc2* fusion were included, four primary tumor samples and one tumor metastatic to the lung. Patients with *EWSR1–NFATc2* positive URCS were older (median age 39 years; range 16–56 years) compared to 31 patients with EwS (median age 17 years, range 3–63 years), 10 patients with URCS with *BCOR* alteration (median age 14 years; range 0–22 years) and 16 patients with URCS with *CIC* rearrangement (median age 26 years; range 12–49 years). Clinical data of the five URCS with *EWSR1–NFATc2* fusion are summarized in Table 1.

Histologic features of undifferentiated round cell sarcomas with *EWSR1–NFATc2* fusion

The tumor cells of URCS with *EWSR1–NFATc2* fusion were round to polygonal with faint eosinophilic to clear cytoplasm, which sometimes appeared vacuolated. The tumor cell outlines were relatively ill defined, the nuclei were large with a dense chromatin pattern (Fig. 1a, b). In some areas, the tumor cells were separated by thin to coarse fibrous septae, which resulted in a nested pattern (Fig. 1c, d). Intriguingly, one case exhibited a predominant myxoid tumor matrix with tumor cells arranged in cords and groups therein (Fig. 1e). Foci of necrosis were present in two cases (Fig. 1f). Inflammatory bystander cells were composed of eosinophilic leukocytes (Fig. 1g, h).

Distinct methylation signature in undifferentiated round cell sarcomas with *EWSR1–NFATc2* fusion

URCS with *EWSR1–NFATc2* fusion formed a homogeneous methylation class by both clustering (Fig. 2a) and t-SNE analyses (Fig. 2b), which also remained stable when varying the number of CpGs used for this analysis. The methylation profiles of URCS with *EWSR1–NFATc2* fusion were distinct from the methylation class of EwS, which formed a homogeneous methylation cluster irrespective of their various *TET–ETS* gene fusion variants. URCS with *CIC* rearrangement, URCS with *BCOR* alteration and the tumor control subtypes angiomatoid fibrous histiocytoma, clear cell sarcoma of the soft tissue, desmoplastic small round cell tumor, extraskeletal myxoid chondrosarcoma and myxoid liposarcoma formed subtype-specific methylation classes, respectively.

Table 1 Clinical characteristics of undifferentiated round cell sarcomas with *EWSR1–NFATc2* fusion

Case ID	*EWSR1–NFATc2*	Age at diagnosis	Gender	Location	Manifestation	References
103662	Non-determinable	51 years	Male	Humerus	Primary	Koelsche et al. (2018a, b)
128854	Exon 8–Exon 3	39 years	Male	Lung (primary humerus)	Metastasis	Szuhai et al. (2009)
110244	Exon 8–Exon 3	56 years	Female	Femur	Primary	–
128856	Exon 8–Exon 3	16 years	Male	Femur	Primary	Szuhai et al. (2009)
97480	Exon 8–Exon 3	17 years	Female	Humerus	Primary	Koelsche et al. (2018a, b)

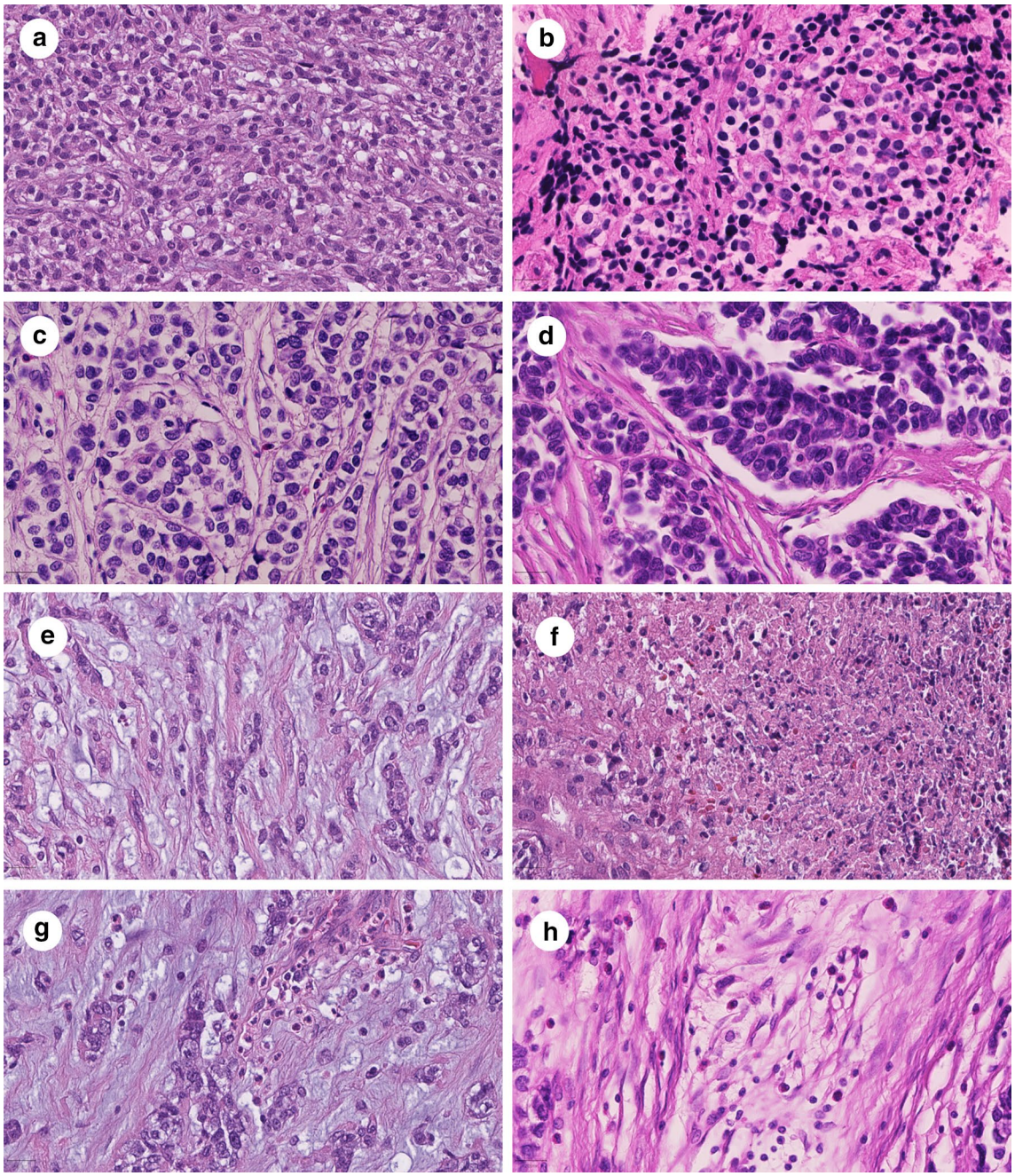

Fig. 1 Histologic features of undifferentiated round cell sarcomas with *EWSR1–NFATc2* fusion. Undifferentiated round cell sarcomas with *EWSR1–NFATc2* fusion were composed of relatively monotonous cells with a faint eosinophilic to clear cytoplasm and large, chromatin dense nuclei (**a, b**). The tumor matrix variably contained thin to coarse collagen bundles, which separated tumor cells and appeared as a nested growth pattern (**c, d**). One case presented with a myxoid tumor matrix (**e**). Focal necrosis was observed in two cases (**f**). Inflammatory cells were variably present and were composed of eosinophilic leukocytes (**g, h**). Magnification: 400-fold. Scale bars: 20 µm

Copy number analysis

We next analyzed the copy number profiles of URCS with *EWSR1–NFATc2* (Fig. 3). A segmental gain on chromosome 22q12 involving the *EWSR1* locus and losses on chromosome 9q were observed in all five cases. A segmental gain on chromosome 20q13 covering the *NFATc2* locus was observed in four cases. None of these copy number alterations were present in the 31 EwS, 16 URCS with *CIC* rearrangement and 10 URCS with *BCOR* alteration.

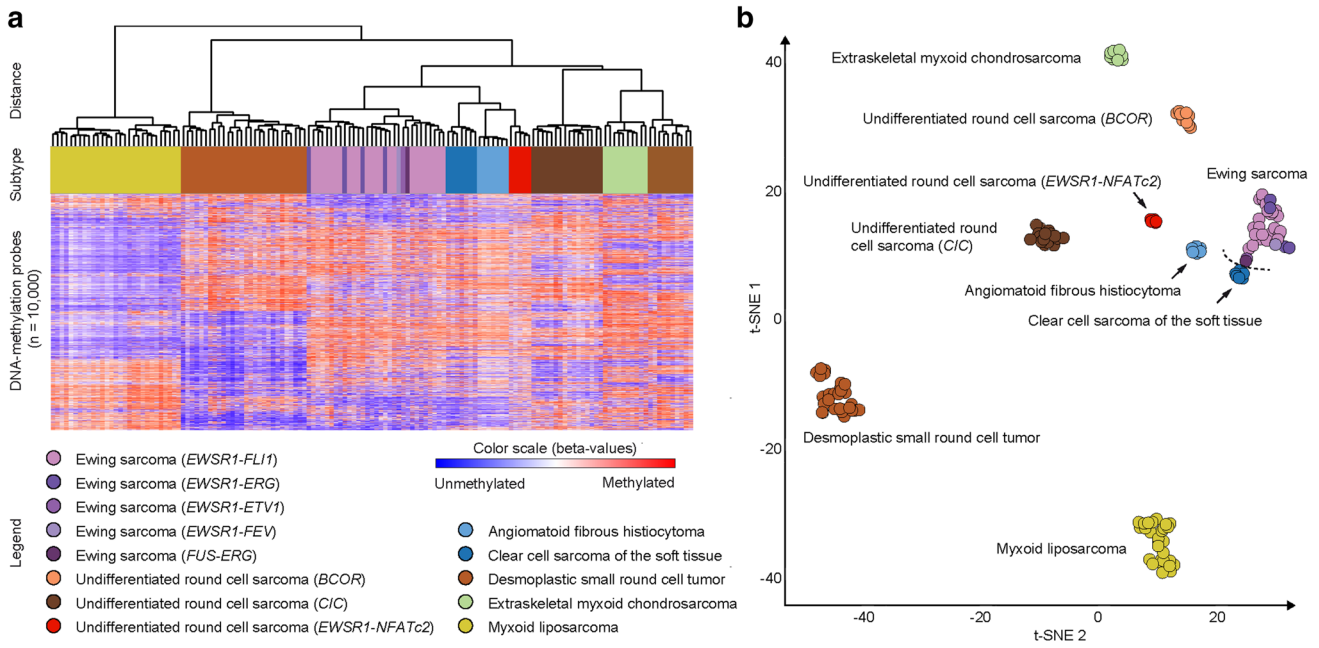

Fig. 2 Distinct DNA methylation patterns in undifferentiated round cell sarcomas with *EWSR1–NFATc2* fusion. Unsupervised hierarchical clustering (**a**) and t-Distributed Stochastic Neighbor Embedding (t-SNE) analysis (**b**) of DNA methylation data from undifferentiated round cell sarcoma variants and a reference set of prototypical soft tissue and bone sarcomas with frequent rearrangements involving gene members of the TET transcription factor family. The arrow points out the novel methylation group

Discussion

Genome-wide tumor DNA methylation patterns likely most often relate to the epigenetic state of their originating tissue and, therefore, can be used to distinguish molecular subgroups within morphologically homogeneous tumor groups. We here describe a distinct DNA methylation signature for URCS with *EWSR1–NFATc2* fusion, which is clearly distinguishable from the DNA methylation signature of EwS. Accordingly, our results strongly support the concept of considering URCS with *EWSR1–NFATc2* fusion separate from EwS. This concept has recently been fueled by gene expression studies, which revealed major differences between URCS with *EWSR1–NFATc2* fusion and EwS with canonical *TET–ETS* gene fusions (Baldauf et al. 2018b; Watson et al. 2018).

Less than 20 URCS with *EWSR1–NFATc2* fusion have been reported since their first description in 2009 (Antonescu 2014; Kinkor et al. 2014; Koelsche et al. 2018a; Machado et al. 2018; Romeo et al. 2012; Sadri et al. 2014; Szuhai et al. 2009; Watson et al. 2018). Overall, these cases share a significant pathological overlap with EwS, which retrospectively justifies their listing as variant EwS in the 2013 WHO classification of soft tissue and bone tumors (Alava et al. 2013).

The question of histiogenesis, however, has remained controversial in *EWSR1–NFATC2* fused sarcomas ever since. Morphologically, URCS with *EWSR1–NFATc2* fusion exhibit atypical features, e.g., presence of a hyaline stroma, a nested growth, enlarged cells with a clear cell appearance and nuclei with prominent nucleoli, characteristics that are not commonly seen in EwS (Folpe et al. 2005). Epithelial marker expression has been consistently described in URCS with *EWSR1–NFATc2* fusion, which is rarely observed in EwS (Antonescu 2014; Folpe et al. 2005).

URCS with *EWSR1–NFATc2* fusion carry a t(20;22)(q13;q12) chromosomal translocation (Szuhai et al. 2009). It is assumed that NFATc2, which acts as a transcription factor, recognizes similar core motifs compared to FLI1 and ERG (Sankar and Lessnick 2011). However, it has not been proven yet whether similar functional consequences may emerge from the *EWSR1–NFATc2* fusion compared to *TET–ETS* gene fusions. Unique for the *EWSR1–NFATc2* fusion is the amplification of the fusion partner *NFATc2* on chromosome 20q13 and *EWSR1* on chromosome 22q11 (Szuhai et al. 2009). This focal amplification leads to an increased number of *EWSR1* probe signals, which may hamper fluorescence in situ hybridization evaluation or may be misjudged as an artifact (Machado et al. 2018). We observed the *EWSR1* amplification in all five cases and the *NFATc2* amplification in all but one case, which might have been missed due to relatively noisy signals in the copy number profile. It should be noted that besides the *EWSR1* and *NFATc2* loci, also the remaining CNV profiles of the *EWSR1–NFATc2* sarcomas reported here are not typical for EwS (Grunewald et al. 2018).

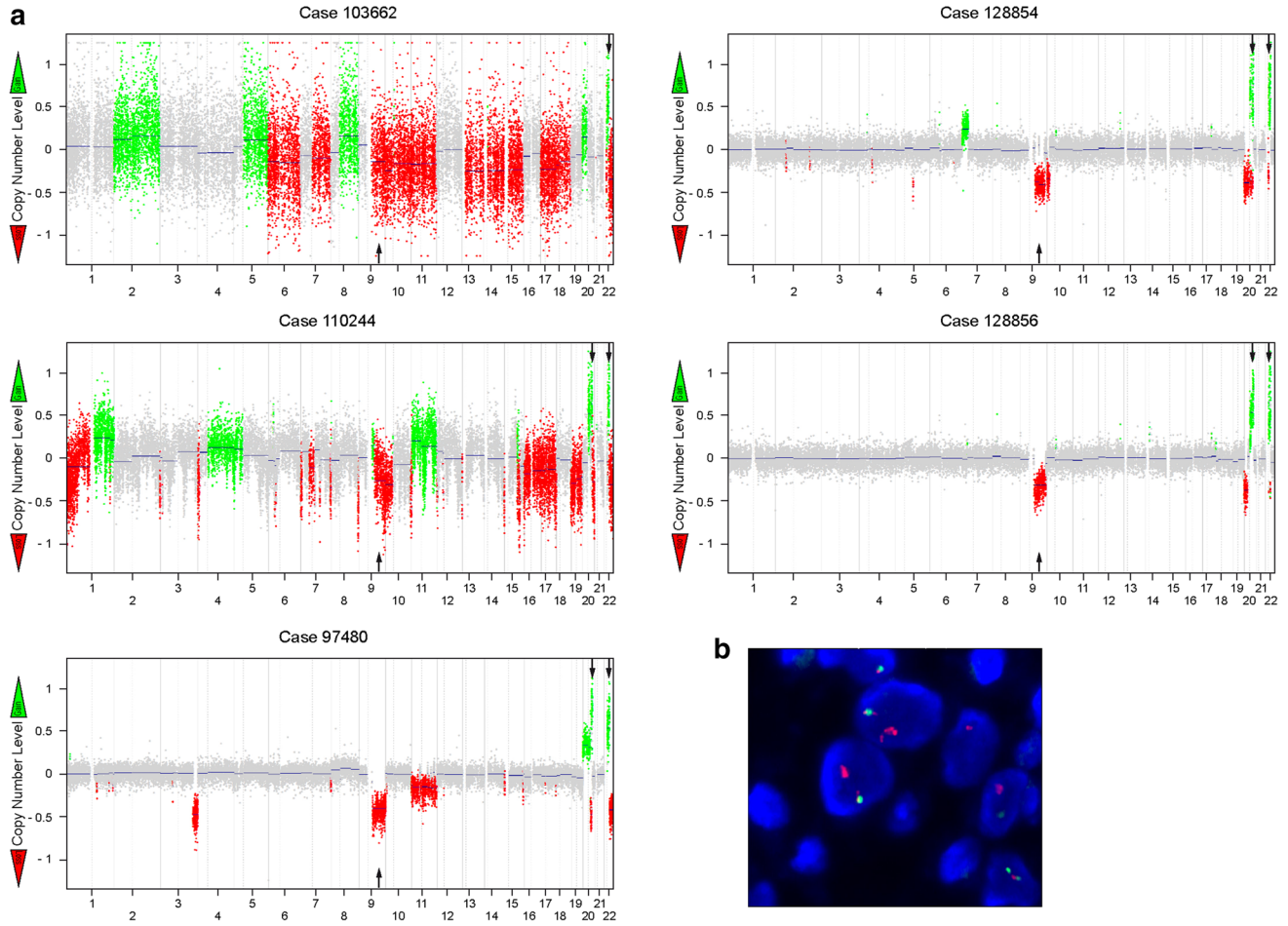

Fig. 3 Copy number alterations in undifferentiated round cell sarcomas with *EWSR1–NFATc2* fusion. Copy number profiles were generated using DNA methylation data. The arrows point out recurrent copy number alterations (**a**). Fluorescence in situ hybridization studies indicate a break and a copy number gain of *EWSR1* (**b**)

From a clinical perspective, URCS with *EWSR1–NFATc2* fusion predominantly occurred as intraosseous lesions, had a striking male predominance and occurred in patients with a slightly higher age at diagnosis compared to EwS (Antonescu 2014; Grunewald et al. 2018; Kinkor et al. 2014; Koelsche et al. 2018a; Machado et al. 2018; Romeo et al. 2012; Sadri et al. 2014; Szuhai et al. 2009; Watson et al. 2018). Surprisingly, two cases of our series were diagnosed in female patients, contradicting published data suggesting that these mainly intraosseous tumors affect male patients only. Our findings are in line with a recent summary reported in the literature, which indicated that *EWSR1–NFATc2*-positive sarcomas might also arise outside of bone and affect female patients (Grunewald et al. 2018).

Due to the exceptional rarity of URCS with *EWSR1–NFATc2* fusion, clinical data regarding the biological behavior is very limited. In studies where follow-up data were available, these cases seemed to follow a more favorable clinical course when compared to classical EwS (Machado et al. 2018; Romeo et al. 2012). In our series, follow-up data were available for three patients, which responded to neoadjuvant chemotherapy and were subsequently treated with tumor resection. In line with previously published data, one of these patients had complete remission, the second patient, which had been primarily diagnosed with metastatic stage disease, is still alive 6 years after diagnosis and the third case had a local recurrence 2 years after diagnosis.

Despite the growing evidence that URCS with *EWSR1–NFATc2* fusion may be distinct from EwS, their origin has not yet been resolved. Some *EWSR1–NFATc2* fused sarcomas exhibited morphological features matching those of myoepithelial tumors (Cohen et al. 2018; Romeo et al. 2012). Myoepithelial tumors typically arise in the soft parts, but skeletal counterparts have been described (Hornick and Fletcher 2003; Song et al. 2017). Histologically, most of these tumors show lobulated growth and are composed of cords or nests of epithelioid, ovoid, or spindle cells with a

chondromyxoid or collagenous/hyalinized stroma (Hornick and Fletcher 2003). They strongly express epithelial markers and S-100 protein, often myogenic markers and in roughly half of the cases glial fibrillary acid protein (Jo and Fletcher 2015). This immunophenotype has not been described in URCS with *EWSR1–NFATc2* fusion (Antonescu 2014; Kinkor et al. 2014; Koelsche et al. 2018a; Machado et al. 2018; Romeo et al. 2012; Sadri et al. 2014; Szuhai et al. 2009; Watson et al. 2018). However, myoepithelial tumors often carry *EWSR1* gene fusions with a growing number of non-ETS fusion partner genes, e.g., *POU5F1* (6p21), *PBX1* (1q23), *ZNF444* (19q23), *ATF1* (12q13) and *PBX3* (9q33) (Antonescu et al. 2011, 2010; Flucke et al. 2011). Most recently, a subcutaneous lesion with a phenotype matching with myoepithelial tumors has been described in a young female patient, which carried a *EWSR1–NFATc2* fusion and additionally had amplifications in the *EWSR1* and *NFATc2* regions. This tumor was positive for epithelial markers, but interestingly lacked any myoepithelial marker expression like S-100, desmin or calponin, among others (Cohen et al. 2018).

Unfortunately, myoepithelial tumors were not part of this study and, therefore, could not be used for comparative purposes. However, it seems to become apparent that URCS with *EWSR1–NFATc2* fusion is a multifaceted tumor, which is neither restricted to bone, nor exclusively occurs in male patients.

In conclusion, DNA methylation profiling segregates URCS with *EWSR1–NFATc2* fusion from EwS with canonical *TET–ETS* fusions. It is, therefore, unlikely that URCS with *EWSR1–NFATc2* and EwS share a common origin.

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Compliance with ethical standards

Conflict of interest The authors state no conflict of interest.

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