HHS Public Access

Author manuscript

J Cancer Res Clin Oncol. Author manuscript; available in PMC 2020 November 03.

Published in final edited form as:

J Cancer Res Clin Oncol. 2019 May ; 145(5): 1273–1281. doi:10.1007/s00432-019-02895-2.

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

Christian Koelsche[1], **Mark Kriegsmann**[1], **Felix K.F. Kommoss**[1], **Damian Stichel**[2,3], **Katharina Kriegsmann**[4], **Christian Vokuhl**[5], **Thomas G.P. Grünewald**[6,7,8,9], **Laura Romero-Pérez**[6], **Thomas Kirchner**[7,8,9], **Enrique de Alava**[10], **Juan Diaz-Martin**[10], **Wolfgang Hartmann**[11], **Daniel Baumhoer**[12], **Cristina R. Antonescu**[13], **Karoly Szuhai**[14], **Uta Flucke**[15], **Uta Dirksen**[16], **Stefan M. Pfister**[17,18,19], **David T.W. Jones**[17,20], **Gunhild Mechtersheimer**[1,*], **Andreas von Deimling**[2,3,*]

[1.]Department of General Pathology, Institute of Pathology, University Hospital Heidelberg, Heidelberg, Germany [2.]Department of Neuropathology, Institute of Pathology, University Hospital Heidelberg, Heidelberg, Germany [3.]Clinical Cooperation Unit Neuropathology, German Cancer Consortium (DKTK), German Cancer Research Center (DKFZ), Heidelberg, Germany [4.]Department of Hematology, Oncology and Rheumatology, University Hospital Heidelberg, Heidelberg, Germany [5.]Department of Pediatric Pathology, University Hospital of Schleswig-Holstein, Kiel, Germany [6.]Max-Eder Research Group for Pediatric Sarcoma Biology, Institute of Pathology, Faculty of Medicine, LMU Munich, Munich, Germany [7.]Institute of Pathology, Faculty of Medicine, LMU Munich, Munich, Germany [8.]German Cancer Consortium (DKTK), partner site Munich, Munich, Germany [9.]German Cancer Research Center (DKFZ), Heidelberg, Germany [10.]Department of Pathology. Institute of Biomedicine of Sevilla (IBiS), Virgen del Rocio University Hospital /CSIC/University of Sevilla/CIBERONC, 41013 Seville, Spain [11.]Division of Translational Pathology, Gerhard-Domagk-Institute for Pathology, University Hospital Münster, Westfalian Wilhelms University, Münster, Germany [12.]Bone Tumour Reference Centre, Institute of Pathology, University Hospital Basel and University of Basel, Basel, Switzerland [13.]Department of Pathology, Memorial Sloan-Kettering Cancer Center, New York, USA [14.]Department of Cell and Chemical Biology, Leiden University Medical Center, Leiden, the Netherlands [15.]Department of Pathology, Radboud University Hospital, Nijmegen, the Netherlands [16.]Cooperative Ewing Sarcoma Study group, University Hospital Essen, Essen, Germany [17.]Hopp Children's Cancer Center, NCT Heidelberg (KiTZ), Heidelberg, Germany [18.]Division of Pediatric Neurooncology, German Cancer Consortium (DKTK), German Cancer Research Center (DKFZ), Heidelberg, Germany [19.]Department of Pediatric Oncology, Hematology and Immunology, Heidelberg University Hospital, Heidelberg, Germany [20.]Pediatric Glioma Research Group, German Cancer Consortium (DKTK), German Cancer Research Center (DKFZ), Heidelberg, Germany

Correspondence: PD Dr. med. Christian Koelsche, University Hospital Heidelberg, Institute of Pathology, Department of General Pathology, Im Neuenheimer Feld 224, D-69120 Heidelberg, Fon: +49 (0)6221 56 37882, Fax: +49 (0)6221 56 4566, Christian.Koelsche@med.uni-heidelberg.de or Prof. Dr. med. Andreas von Deimling, University Hospital Heidelberg, Institute of Pathology, Department of Neuropathology, Im Neuenheimer Feld 224, D-69120 Heidelberg, Fon: +49 (0)6221 56 4651, Fax: +49 (0)6221 56 4566 Andreas.vonDeimling@med.uni-heidelberg.de.

*equal contribution

Conflict of Interest

The authors state no conflict of interest.

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 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 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.

Material 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 Rocio 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; Koelsche et al. 2018b). 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 (450k) BeadChip or the successor EPIC/850k 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 20000, 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 450k BeadChip did not cover them, thereby making data generated by both 450k and EPIC feasible for subsequent analyses. In total, 438370 probes were kept for analysis.

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

For unsupervised hierarchical clustering, we selected 10000 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 10000 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 (Figure 1 a, b). In some areas, the tumor cells were separated by thin to coarse fibrous septae, which resulted in a nested pattern (Figure 1 c, d). Intriguingly, one case exhibited a predominant myxoid tumor matrix with tumor cells arranged in cords and groups therein (Figure 1 e). Foci of necrosis were present in two cases (Figure 1 f). Inflammatory bystander cells were composed of eosinophilic leukocytes (Figure 1 g, 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 (Figure 2 a) and t-SNE analyses (Figure 2 b), which also kept 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.

Copy number analysis

We next analyzed the copy number profiles of URCS with *EWSR1-NFATc2* (Figure 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.

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).

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 was 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 was 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; Antonescu et al. 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank the Microarray Unit of the Genomics and Proteomics Core Facility, German Cancer Research Center (DKFZ), for providing excellent methylation services. We also thank the HUVR-IBiS Biobank (Andalusian Public Health System Biobank and ISCIII-Red de Biobancos PT17/0015/0041) for providing tissue.

Funding

The work was supported by the German Cancer Aid (grant 70112499). The laboratory of T.G.P.G. was supported for this project by grants from the Dr. Leopold und Carmen Ellinger Stiftung and the German Cancer Aid (grant 70112257).

References

de Alava E, Lessnick SL, Sorensen PH (2013) Ewing Sarcoma In: Fletcher CDM, Bridge JA, Hogendoorn PCW, Mertens F (eds) WHO Classification of Tumours of Soft Tissue and Bone. International Agency for Research on Cancer (IARC), Lyon, pp 305–310

Antonescu C (2014) Round cell sarcomas beyond Ewing: emerging entities Histopathology 64:26–37 doi:10.1111/his.12281 [PubMed: 24215322]

Antonescu CR et al. (2011) EWSR1-ATF1 fusion is a novel and consistent finding in hyalinizing clear-cell carcinoma of salivary gland Genes Chromosomes Cancer 50:559–570 doi:10.1002/gcc.20881 [PubMed: 21484932]

Antonescu CR et al. (2017) Sarcomas With CIC-rearrangements Are a Distinct Pathologic Entity With Aggressive Outcome: A Clinicopathologic and Molecular Study of 115 Cases Am J Surg Pathol 41:941–949 doi:10.1097/PAS.0000000000000846 [PubMed: 28346326]

Antonescu CR et al. (2010) EWSR1-POU5F1 fusion in soft tissue myoepithelial tumors. A molecular analysis of sixty-six cases, including soft tissue, bone, and visceral lesions, showing common involvement of the EWSR1 gene Genes Chromosomes Cancer 49:1114–1124 doi:10.1002/gcc.20819 [PubMed: 20815032]

Baldauf MC et al. (2018a) Are EWSR1-NFATc2-positive sarcomas really Ewing sarcomas? Mod Pathol 31:997–999 doi:10.1038/s41379-018-0009-7 [PubMed: 29895896]

Baldauf MC et al. (2018b) Robust diagnosis of Ewing sarcoma by immunohistochemical detection of super-enhancer-driven EWSR1-ETS targets Oncotarget 9:1587–1601 doi:10.18632/oncotarget.20098 [PubMed: 29416716]

Charville GW et al. (2018) PAX7 expression in sarcomas bearing the EWSR1-NFATC2 translocation Mod Pathol doi:10.1038/s41379-018-0095-6

Chen S, Deniz K, Sung YS, Zhang L, Dry S, Antonescu CR (2016) Ewing sarcoma with ERG gene rearrangements: A molecular study focusing on the prevalence of FUS-ERG and common pitfalls in detecting EWSR1-ERG fusions by FISH Genes Chromosomes Cancer 55:340–349 doi:10.1002/gcc.22336 [PubMed: 26690869]

Cohen JN, Sabnis AJ, Krings G, Cho SJ, Horvai AE, Davis JL (2018) EWSR1-NFATC2 Gene Fusion in a Soft Tissue Tumor with Epithelioid Round Cell Morphology and Abundant Stroma: A Case Report and Review of the Literature Hum Pathol doi:10.1016/j.humpath.2018.03.020

Delattre O et al. (1992) Gene fusion with an ETS DNA-binding domain caused by chromosome translocation in human tumours Nature 359:162–165 doi:10.1038/359162a0 [PubMed: 1522903]

Flucke U, Palmedo G, Blankenhorn N, Slootweg PJ, Kutzner H, Mentzel T (2011) EWSR1 gene rearrangement occurs in a subset of cutaneous myoepithelial tumors: a study of 18 cases Mod Pathol 24:1444–1450 doi:10.1038/modpathol.2011.108 [PubMed: 21725291]

Folpe AL, Goldblum JR, Rubin BP, Shehata BM, Liu W, Dei Tos AP, Weiss SW (2005) Morphologic and immunophenotypic diversity in Ewing family tumors: a study of 66 genetically confirmed cases Am J Surg Pathol 29:1025–1033 [PubMed: 16006796]

Grunewald TGP et al. (2018) Ewing sarcoma Nat Rev Dis Primers 4:5 doi:10.1038/s41572-018-0003-x [PubMed: 29977059]

Hornick JL, Fletcher CD (2003) Myoepithelial tumors of soft tissue: a clinicopathologic and immunohistochemical study of 101 cases with evaluation of prognostic parameters Am J Surg Pathol 27:1183–1196 [PubMed: 12960802]

Italiano A, Sung YS, Zhang L, Singer S, Maki RG, Coindre JM, Antonescu CR (2012) High prevalence of CIC fusion with double-homeobox (DUX4) transcription factors in EWSR1-negative undifferentiated small blue round cell sarcomas Genes Chromosomes Cancer 51:207–218 doi:10.1002/gcc.20945 [PubMed: 22072439]

Jeon IS, Davis JN, Braun BS, Sublett JE, Roussel MF, Denny CT, Shapiro DN (1995) A variant Ewing's sarcoma translocation (7;22) fuses the EWS gene to the ETS gene ETV1 Oncogene 10:1229–1234 [PubMed: 7700648]

Jo VY, Fletcher CD (2015) Myoepithelial neoplasms of soft tissue: an updated review of the clinicopathologic, immunophenotypic, and genetic features Head Neck Pathol 9:32–38 doi:10.1007/s12105-015-0618-0 [PubMed: 25804378]

Kaneko Y et al. (1996) Fusion of an ETS-family gene, EIAF, to EWS by t(17;22)(q12;q12) chromosome translocation in an undifferentiated sarcoma of infancy Genes Chromosomes Cancer 15:115–121 doi:10.1002/(SICI)1098-2264(199602)15:2<115::AID-GCC6>3.0.CO;2-6 [PubMed: 8834175]

Kao YC et al. (2018) BCOR-CCNB3 Fusion Positive Sarcomas: A Clinicopathologic and Molecular Analysis of 36 Cases With Comparison to Morphologic Spectrum and Clinical Behavior of Other Round Cell Sarcomas Am J Surg Pathol 42:604–615 doi:10.1097/PAS.0000000000000965 [PubMed: 29300189]

Kinkor Z, Vanecek T, Svajdler M Jr., Mukensnabl P, Vesely K, Baxa J, Kokavec M (2014) [Where does Ewing sarcoma end and begin - two cases of unusual bone tumors with t(20;22)(EWSR1-NFATc2) alteration] Cesk Patol 50:87–91 [PubMed: 24758504]

Koelsche C et al. (2018a) Array-based DNA-methylation profiling in sarcomas with small blue round cell histology provides valuable diagnostic information Mod Pathol 31:1246–1256 doi:10.1038/s41379-018-0045-3 [PubMed: 29572501]

Koelsche C et al. (2018b) Primary intracranial spindle cell sarcoma with rhabdomyosarcoma-like features share a highly distinct methylation profile and DICER1 mutations Acta Neuropathol 136:327–337 doi:10.1007/s00401-018-1871-6 [PubMed: 29881993]

Machado I et al. (2018) Review with novel markers facilitates precise categorization of 41 cases of diagnostically challenging, "undifferentiated small round cell tumors". A clinicopathologic, immunophenotypic and molecular analysis Ann Diagn Pathol 34:1–12 doi:10.1016/j.anndiagpath.2017.11.011 [PubMed: 29661713]

Mastrangelo T et al. (2000) A novel zinc finger gene is fused to EWS in small round cell tumor Oncogene 19:3799–3804 doi:10.1038/sj.onc.1203762 [PubMed: 10949935]

Peter M, Couturier J, Pacquement H, Michon J, Thomas G, Magdelenat H, Delattre O (1997) A new member of the ETS family fused to EWS in Ewing tumors Oncogene 14:1159–1164 doi:10.1038/sj.onc.1200933 [PubMed: 9121764]

Pierron G et al. (2012) A new subtype of bone sarcoma defined by BCOR-CCNB3 gene fusion Nat Genet 44:461–466 doi:10.1038/ng.1107 [PubMed: 22387997]

Romeo S et al. (2012) Malignant fibrous histiocytoma and fibrosarcoma of bone: a reassessment in the light of currently employed morphological, immunohistochemical and molecular approaches Virchows Arch 461:561–570 doi:10.1007/s00428-012-1306-z [PubMed: 23001328]

Sadri N et al. (2014) Malignant round cell tumor of bone with EWSR1-NFATC2 gene fusion Virchows Arch 465:233–239 doi:10.1007/s00428-014-1613-7 [PubMed: 24993903]

Sankar S, Lessnick SL (2011) Promiscuous partnerships in Ewing's sarcoma Cancer Genet 204:351–365 doi:10.1016/j.cancergen.2011.07.008 [PubMed: 21872822]

Savola S et al. (2009) Combined use of expression and CGH arrays pinpoints novel candidate genes in Ewing sarcoma family of tumors BMC Cancer 9:17 doi:10.1186/1471-2407-9-17 [PubMed: 19144156]

Shing DC et al. (2003) FUS/ERG gene fusions in Ewing's tumors Cancer Res 63:4568–4576 [PubMed: 12907633]

Song W, Flucke U, Suurmeijer AJH (2017) Myoepithelial Tumors of Bone Surg Pathol Clin 10:657–674 doi:10.1016/j.path.2017.04.010 [PubMed: 28797507]

Sorensen PH, Lessnick SL, Lopez-Terrada D, Liu XF, Triche TJ, Denny CT (1994) A second Ewing's sarcoma translocation, t(21;22), fuses the EWS gene to another ETS-family transcription factor, ERG Nat Genet 6:146–151 doi:10.1038/ng0294-146 [PubMed: 8162068]

Specht K, Sung YS, Zhang L, Richter GH, Fletcher CD, Antonescu CR (2014) Distinct transcriptional signature and immunoprofile of CIC-DUX4 fusion-positive round cell tumors compared to EWSR1-rearranged Ewing sarcomas: further evidence toward distinct pathologic entities Genes Chromosomes Cancer 53:622–633 doi:10.1002/gcc.22172 [PubMed: 24723486]

Specht K et al. (2016) Novel BCOR-MAML3 and ZC3H7B-BCOR Gene Fusions in Undifferentiated Small Blue Round Cell Sarcomas Am J Surg Pathol 40:433–442 doi:10.1097/PAS.0000000000000591 [PubMed: 26752546]

Sumegi J, Nishio J, Nelson M, Frayer RW, Perry D, Bridge JA (2011) A novel t(4;22)(q31;q12) produces an EWSR1-SMARCA5 fusion in extraskeletal Ewing sarcoma/primitive neuroectodermal tumor Mod Pathol 24:333–342 doi:10.1038/modpathol.2010.201 [PubMed: 21113140]

Szuhai K, Ijszenga M, de Jong D, Karseladze A, Tanke HJ, Hogendoorn PC (2009) The NFATc2 gene is involved in a novel cloned translocation in a Ewing sarcoma variant that couples its function in immunology to oncology Clin Cancer Res 15:2259–2268 doi:10.1158/1078-0432.CCR-08-2184 [PubMed: 19318479]

Wang L, Bhargava R, Zheng T, Wexler L, Collins MH, Roulston D, Ladanyi M (2007) Undifferentiated small round cell sarcomas with rare EWS gene fusions: identification of a novel EWS-SP3 fusion and of additional cases with the EWS-ETV1 and EWS-FEV fusions J Mol Diagn 9:498–509 doi:10.2353/jmoldx.2007.070053 [PubMed: 17690209]

Watson S et al. (2018) Transcriptomic definition of molecular subgroups of small round cell sarcomas J Pathol 245:29–40 doi:10.1002/path.5053 [PubMed: 29431183]

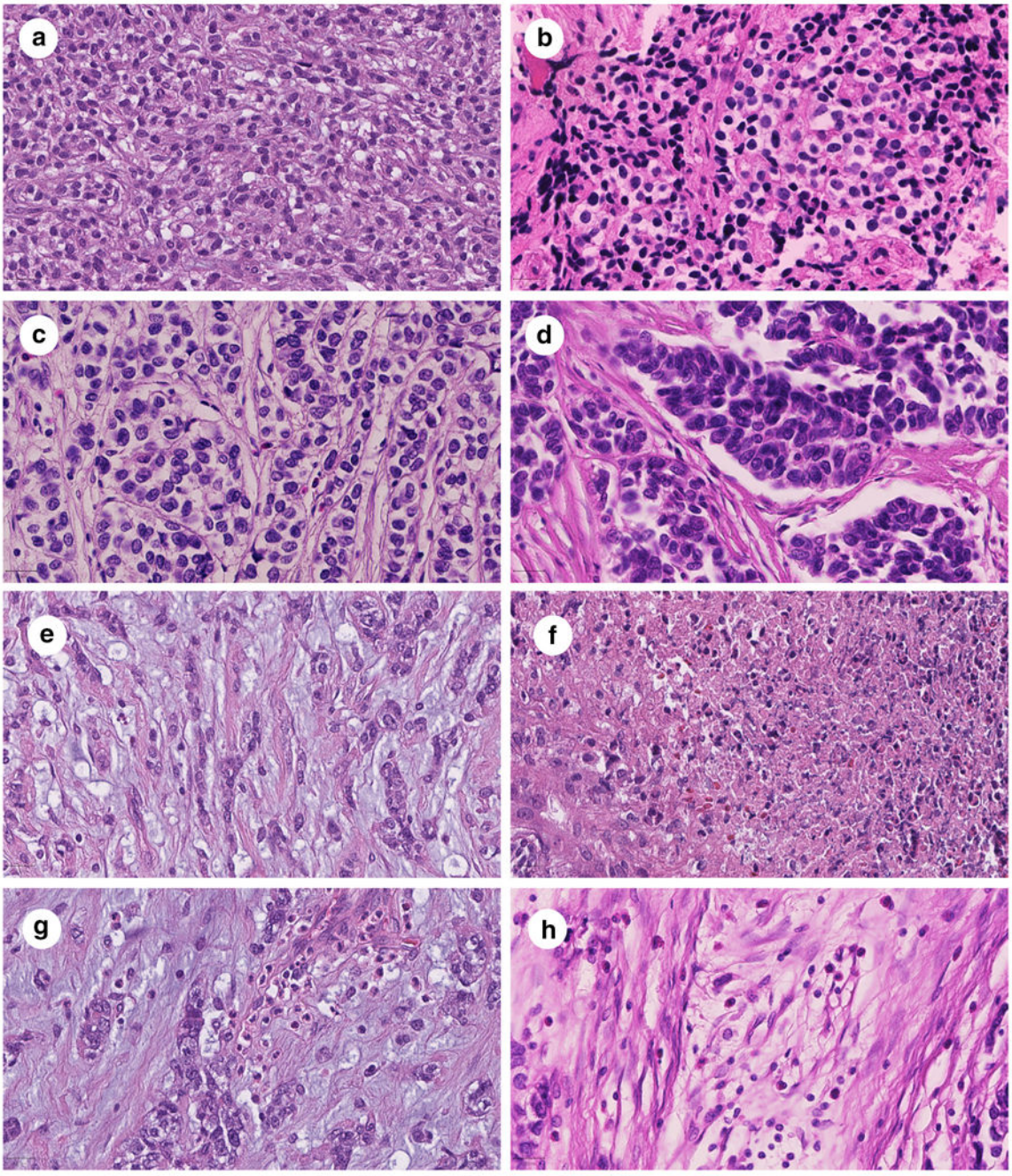

Figure 1: Histologic features of undifferentiated round cell sarcomas with *EWSR1-NFATc2* fusion.

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.

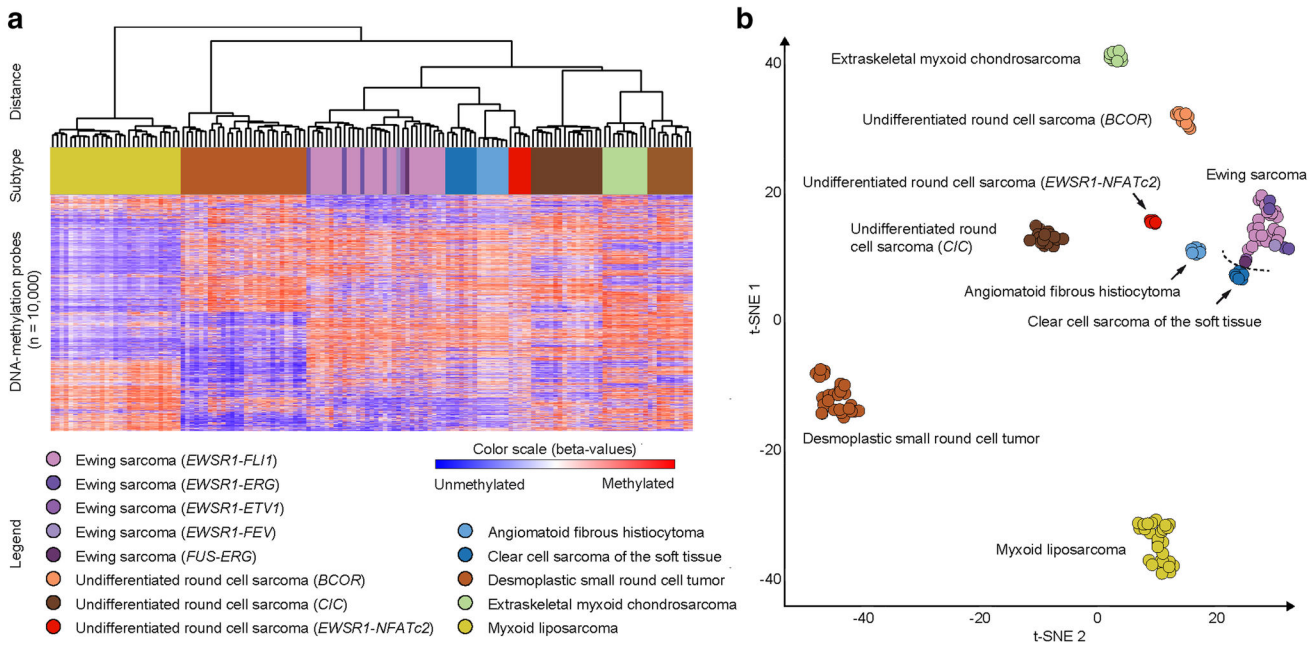

Figure 2: Distinct DNA methylation patterns in undifferentiated round cell sarcomas with *EWSR1-NFATc2* fusion.

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.

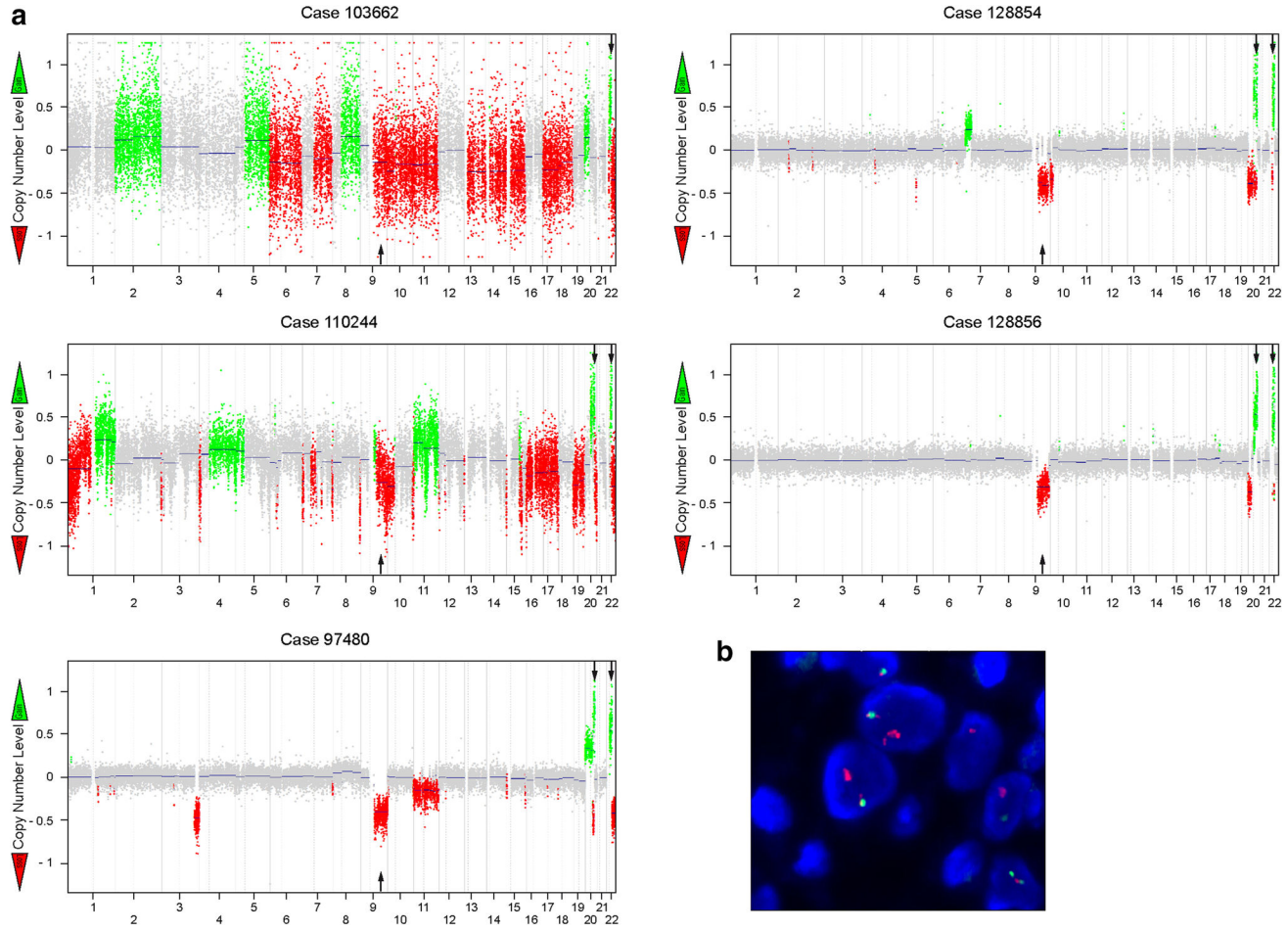

Figure 3: Copy number alterations in undifferentiated round cell sarcomas with *EWSR1-NFATc2* fusion.

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**).

Table 1:

Clinical characteristics of undifferentiated round cell sarcomas with *EWSR1-NFATc2* fusion.

Case ID	*EWSR1-NFATc2*	Age at diagnosis	Gender	Location	Manifestation	Reference
103662	Non-determinable	51 years	Male	Humerus	Primary	Koelsche et al. 2018
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. 2018