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High Prevalence of *CIC* Fusion with Double-Homeobox (*DUX4*) Transcription Factors in *EWSR1*-Negative Undifferentiated Small Blue Round Cell Sarcomas

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Abstract

Primitive round cell sarcomas of childhood and young adults have been problematic to diagnose and classify. Our goal was to investigate the pathologic and molecular characteristics of small blue round cell tumors (SBRCT) that remained unclassified after exhaustive immunohistochemistry and molecular screening to exclude known sarcoma-related translocations. As rare examples of *EWSR1*-negative SBRCT have been shown to carry rearrangements for *FUS* and *CIC* genes, we undertook a systematic screening for these two genes. *CIC* rearrangements by FISH were detected in 15/22 (68%), while none showed *FUS* abnormalities. RACE, RT-PCR and/or long range DNA PCR performed in two cases with frozen material showed that *CIC* was fused to copies of the *DUX4* gene on either 4q35 or 10q26.3. Subsequent FISH analysis confirmed fused signals of *CIC* with either 4q35 or 10q26.3 region in six cases each. Tumors positive for *CIC-DUX4* fusion occurred mainly in male young adult patients (median age: 29 years), with the extremities being the most frequent location. Microscopically, tumors displayed a primitive, round to oval cell morphology with prominent nucleoli, high mitotic count and areas of necrosis. O13 expression was variable, being either diffuse or patchy and tumors mostly lacked other markers of differentiation. Although *CIC-DUX4* resulting in a t(4;19) translocation has been previously described in primitive sarcomas, this is the first report implicating the related *DUX4* on 10q26 in oncogenesis. These results suggest the possibility of a newly defined subgroup of primitive round cell sarcomas characterized by *CIC* rearrangements, distinct from Ewing sarcoma family of tumors.

INTRODUCTION

Primitive round cell sarcomas of childhood and young adults have been problematic to diagnose and classify. Even with the increased use of ancillary techniques, such as immunohistochemistry and molecular/genetic methods applied to archival material, a subset of these tumors cannot be classified in specific histologic types. It remains unclear if these tumors represent distinct biologic entities or belong to the same spectrum of previously described sarcoma subtypes, being characterized by yet undescribed, novel gene fusions.

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A subset of tumors resembling microscopically Ewing sarcoma family of tumors (EFT), being composed mainly of primitive small round cells and occurring in children or young adults, remain unclassified, being negative for *EWSR1*, *SS18(SYT)*, *DDIT3(CHOP)* and *FOXO1(FKHR)* gene rearrangements by FISH/RT-PCR, which constitute most of the known SBRCT-related translocations in this age group. A small number of cases sharing the undifferentiated EFT appearance have been characterized instead by either an *FUS-ERG* or *CIC-DUX4* fusion (Shing et al., 2003; Ng et al., 2007; Berg et al., 2009). However, there have been no studies to date to systematically screen for these alternative fusions in a group of *EWSR1*-negative SBRCT. The purpose of this study is to address this issue in a group of SBRCT tumors which remained unclassified after exhaustive immunohistochemical and molecular/genetic work-up. Our hypothesis is that a more in-depth genetic characterization of these lesions may improve the present classification, which may encompass different pathologic entities under the somewhat ‘wastebasket’ terminology of undifferentiated/primitive sarcomas. This may have important clinical implications as well. Indeed, neoadjuvant and adjuvant combination chemotherapy including several drugs such as vincristine, dactinomycin, doxorubicin, ifosfamide and etoposide is the standard of care for patients with a diagnosis of Ewing sarcoma confirmed by molecular analysis. However, there is no consensus about the role of chemotherapy in “undifferentiated” sarcomas without specific molecular features, even if histology mimics Ewing sarcoma.

Material and Methods

Patients and Tumor Characteristics

We retrieved 22 cases of sarcoma, diagnosed between 1997 and 2011 and characterized by a small blue round cell morphology, which were negative for the *EWSR1* gene rearrangement by FISH and for other known pediatric sarcoma translocation by RT-PCR/FISH, from the surgical pathology files of our two institutions with available tissue for molecular analysis (IRB-protocol 02-060). The patient’s characteristics are described in Table 1. Median age was 29 years (range 14-69). Twenty cases (91%) arose from soft-tissue, one case from bone (SBRCT 18 – left ilium) and one case was visceral (SBRCT 14 – small intestine). The most frequent tumor location was the limb (10 cases, 45%). Most of the tumors were deep-seated, however, 3 cases (14%) were superficial associated with skin ulceration in two of them. Clinical follow-up information was available in 19 patients (86%). In each case, the diagnosis was confirmed by reviewing the H&E slides and the immunohistochemical stains. A subset of tumors which were initially tested only by RT-PCR studies to exclude *EWSR1-FLI1* and *EWSR1-ERG* fusion transcripts were subsequently studied for *EWSR1* gene abnormalities by FISH.

The tumors were assessed microscopically for growth pattern, cytomorphology (round, oval versus spindle cell), cellular pleomorphism, nucleolar size, mitotic activity, and necrosis. For each case, the location of the tumor was recorded, along with the anatomic structures involved. The immunohistochemical stains were re-reviewed and a minimum panel was required for inclusion in the study, including O13 and/or CD99, cytokeratin and/or EMA, and desmin. Most cases had in fact a much wider panel of stains performed, including neural/neuroendocrine markers, lymphoid markers (Table 2, Supplementary Table 1).

Fluorescence in Situ Hybridization (FISH) and Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

Since the immunohistochemical profile of an undifferentiated SBRCT is quite often non-specific, the differential diagnosis under consideration was typically quite wide, including in addition to Ewing sarcoma family of tumors other entities such as a poorly differentiated synovial sarcoma and less commonly a small cell variant of angiomatoid fibrous

histiocytoma, round cell/myxoid liposarcoma and an alveolar rhabdomyosarcoma. Thus, in addition to the genetic/molecular tests applied to exclude Ewing sarcoma, the molecular work-up often included RT-PCR for detecting other recurrent chromosomal translocation, such as *SS18-SSX* in 4 cases, *EWSR1-ATF1/EWSR1-CREB1* in 3 cases, *PAX-FOXO1* in 2 cases (Table 2). Additional FISH studies to exclude rearrangements in *SS18* in 5 cases and *DDIT3* in 2 cases were performed at the time of diagnosis.

FISH on interphase nuclei from paraffin embedded 4-micron sections was performed applying probes using bacterial artificial chromosomes (BAC), flanking *EWSR1* in 22q12, *FUS* in 16p11, and *CIC* in 19q13. Since copies of the *DUX4* retrogene are located at telomeric regions of both chromosomes 4 and 10 (de Greef et al., 2008), respectively, BACs centromerically flanking the *DUX4* region on 4q35 and 10q26.3 and BACs telomerically flanking *CIC* were used as fusion probes to verify the translocations of t(4;19) and t(10;19). The BAC probes spanning *DUX4* genes covered 540 kb beyond the 200kb region of high homology of subtelomeric 4q35 and 10q26.3 (which includes the 100kb region of D4Z4 tandem repeats) (Lemmers et al., 2010b). BAC clones were chosen according to UCSC genome browser (<http://genome.ucsc.edu/>) (Fig. 2). The BAC clones were obtained from BACPAC sources of Children's Hospital of Oakland Research Institute (CHORI) (Oakland, CA) (<http://bacpac.chori.org>). DNA from individual BACs was isolated according to the manufacturer's instructions, labeled with different fluorochromes in a nick translation reaction, denatured, and hybridized to pretreated slides. Slides were then incubated, washed, and mounted with DAPI in an antifade solution, as previously described (Antonescu et al., 2010). The genomic location of each BAC set was verified by hybridizing them to normal metaphase chromosomes. Two hundred successive nuclei were examined using a Zeiss fluorescence microscope (Zeiss Axioplan, Oberkochen, Germany), controlled by Isis 5 software (Metasystems). A positive score was interpreted when at least 20% of the nuclei showed a break-apart signal. Nuclei with incomplete set of signals were omitted from the score.

3' Rapid Amplification of cDNA Ends (RACE), Cloning, Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) RT-PCR and Sequence Analysis

Total RNA was extracted from two cases with available frozen tissue, using Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). One microgram of total RNA was used for cDNA preparation followed by a 3'-RACE, using the SMARTer™ RACE cDNA Amplification Kit (Clontech, Mountain View, CA). Reverse transcribed mRNA was initiated at the poly(A) tail of mRNA with appropriate 3'-RACE CDS Primer A in a 10 µl reaction volume according to the manufacturer's protocol. First round PCR was done by Clontech Advantage 2 PCR kit with the SMARTer™ RACE Universal Primer A Mix and *CIC* forward primer exon 17 (5'-TGAGTTGCCTGAGTTTCG-3'). Nested PCR was performed with the SMARTer™ RACE Nested Universal Primer A and *CIC* forward primer exon 19 (5'-GAGGACGTGCTTGGGGAGCTAGAGT-3'). Amplified PCR products were being cloned by TOPO® TA Cloning® Kit for Sequencing with One Shot® TOP10 Chemically Competent E. coli (Invitrogen, Carlsbad, CA). The constructed plasmid DNA was sequenced using Sanger's method. To confirm RACE results, RT-PCR was performed by SuperScript® First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). *CIC* exon 19 and *DUX4* exon 2 chromosome 10 (5'-CGGTGAGAGACTCCACACAGCGG-3') were used as forward and reverse primers individually.

Long Range DNA PCR (LR-PCR)

Genomic DNA was extracted from frozen tissue by Phenol/Chloroform assay and quality was confirmed by electrophoresis. One microgram of genomic DNA was used for the

LongRange PCR Kit (QIAGEN, Valencia, CA) in the presence of Q solution to improve the thermostability and long range PCR efficiency. The following primers were used: CIC exon 17 forward primer (5'-CGCAAGAAGAGGAAGAAGTCCACGG-3') and DUX4 exon 1 reverse primer (5'-CGAGGAAGAATACCGGGCTCTGC-3') to amplify the *CIC-DUX4* on chromosome 4 (SBRCT 3); and CIC intron 19 forward primer (5'-GTGAGCCTGTCTCGGAGTCTTGGG-3') and DUX4 intron 3 reverse primer (5'-CACAAAGCCCCCTGTAGACAAAGC-3') to amplify the *CIC-DUX4* on chromosome 10 (SBRCT 9). The amplified long range PCR product was sequenced using the Sanger method.

Sequence analysis and comparison of D4Z4 sequence on chromosomes 4 and 10

The DUX4 sequences were accessed from ENST00000507734 (<http://useast.ensembl.org/index.html>) on chromosome 4 and uc001lns.2 (<http://genome.ucsc.edu/cgi-bin/hgTracks?org=human>) on chromosome 10. The starting point of the D4Z4 region was mapped by the *KpnI* restriction enzyme site (GGTACC) (Snider et al., 2009). As 4q35 D4Z4 units have a SNP combination of B-X⁺ (*BlnI* resistant and *XapI* sensitive), whereas chromosome 10q repeat arrays are usually homogeneous for B+X⁻ D4Z4 units (*XapI* resistant and *BlnI* sensitive) (Lemmers et al., 2010b), we have investigated the *BlnI* (CCTAGG) and *XapI* (RAATTY) restriction sites in the D4Z4 sequences and cross-referenced to our genomic data from SBRCT 3 and 9 (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Statistical analysis

The statistical analysis of baseline demographics and clinical outcome is based on all data available up to the cut-off date, 31 May 2011. Survival rates were estimated with the use of the Kaplan–Meier method.

RESULTS

Pathologic Findings

All cases showed a primitive population of small to medium-sized round to oval cells, packed in solid sheets with minimal or absent intervening collagen. Although some tumors showed focally a more ovoid or elongated configuration (Fig. 1A), distinct areas of spindling were seen only in two cases as a minor component. The latter was composed of short spindle cells arranged in a vague storiform or loose whorling (nodular) pattern (Fig. 1B). Areas of spindling arranged in well-defined fascicles, reminiscent of fibrosarcoma, were not present. Most tumor cells had an ill-defined cell border, with scant amount of amphophilic or lightly eosinophilic cytoplasm, except for one tumor that showed focally a plasmacytoid appearance with more abundant cytoplasm (Fig. 1C). Most tumors showed vesicular nuclei, with distinct, often enlarged nucleoli. Although the presence of larger, pleomorphic cells was not seen, there was a higher degree of heterogeneity in nuclear shape and size, compared to the rather monomorphic appearance seen in EFT. Geographic areas of necrosis were commonly seen, as well as individual cell necrosis with 'starry sky' appearance (Fig. 1D). A high mitotic rate of >10MF/10HPFs was detected in all cases. There was no difference in morphology identified between the tumors associated and those which are not with a rearrangement of the *CIC* gene.

CIC gene rearrangement by FISH detected in two-thirds of undifferentiated SBRCT negative for EWSR1 abnormalities

Of the 22 patients included in the study, 15 (68%) showed a *CIC* rearrangement by FISH (fig. 2A). There were no FUS rearrangements detected by FISH in any of the 15 tumors tested.

The *CIC*-rearranged tumors were then investigated for *DUX4* abnormalities on 4q35 by FISH. A *CIC-DUX4* fused signal was detected in 6 of the 15 tumors tested (Fig. 2B). In one of the *CIC-DUX4* positive cases by FISH (SBRCT 3), RACE and RT-PCR was attempted unsuccessfully using multiple primer combinations. The most likely explanation for this failure was the D4Z4 repetitive sequence of *DUX4*. However, by LR-PCR, the amplified 1.3 kb product confirmed the fusion of the 5' portion of *CIC* exon 20 to exon 1 of *DUX4*, located within the D4Z4 repeat and part of the coding sequence region of *DUX4* on chromosome 4 (Fig. 3 A,B). The deduced amino-acid sequence of the chimeric protein indicated that as previously described by Kawamura-Saito et al (Kawamura-Saito et al., 2006) most of the *CIC* protein was preserved while a large part of the N-terminal region of *DUX4* was lost including the two homeobox domains.

A novel t(10;19)translocation resulting in a *CIC-DUX4* fusion detected by RACE and subsequently confirmed by RT-PCR, LR-PCR and FISH

RACE-PCR on SBRCT 9 identified a fusion between the first 80 bp of *CIC* exon 20 with the *DUX4* exon 1 sequence on 10q26.3 (Fig. 4A). The same result was further obtained by RT-PCR, using the forward *CIC* exon 19 primer (5'-GAGGACGTGCTTGGGGAGCTAGAGT-3') and reverse *DUX4* exon 2 primer (5'-CGGTGAGAGACTCCACACAGCG-3'). By Sanger sequencing the 231 bp amplified product (Fig. 4B) showed the fusion of *CIC* exon 20 with *DUX4* exon 2 on 10q26.3. A FISH BAC probe was then designed to cover the *DUX4* locus on 10q26.3, which indeed showed a fused signal (Fig. 2C) and confirmed the RACE results.

At the DNA level, LR-PCR further confirmed the fusion of *CIC* exon 20 with *DUX4* exon 1 at the 135497789 genomic position (Fig. 4C). The *CIC* breakpoint was identical with the one identified in SBRCT 3 with t(4;19).

The comparative analysis of the D4Z4 genomic sequence showed 100% homology between the SBRCT3 *DUX4* sequence and the 4q35 D4Z4 reference sequence (ENST00000507734) and between SBRCT 9 *DUX4* and the 10q26 D4Z4 published sequence (uc001ins.2)(Fig 3C). However, few deletions in the CDS region were identified within the D4Z4 on 10q26, but not in 4q35 (Fig. 3C).

Thereafter, the *CIC*-rearranged tumors without abnormal 4q35 signals were investigated for 10q26.3 abnormalities by FISH. A *CIC-DUX4* fused signal was detected in 6 cases, in keeping with a t(10;19) translocation. There was no significant difference between tumors harboring a t(4;19) and t(10;19) in terms of histological features, clinical presentation or outcome.

Immunohistochemical Results

Among the *CIC*-rearranged tumors, 10 cases were diffusely positive for O13 and/or CD99, while 5 cases were only patchy and weakly positive (fig. 1E, Table 2). FLI1 was negative in three and weak/focal in the remaining four cases tested. Cytokeratin, EMA and S100 protein were each focally positive in two cases, while desmin was positive in one tumor. NSE was positive in all three cases tested, while none of the 11 cases tested showed reactivity for chromogranin and/or synaptophysin. Tumors were consistently negative for all lymphoid markers applied. In the *CIC*-negative rearranged group, O13 and/or CD99 were positive in all cases diffuse, except for one tumor in which staining was focal (Table S1). FLI1 staining was positive in one tumor and negative in the remaining two tumors tested.

Patient characteristics and radiological patterns—The median age for the patients with a *CIC*-rearranged tumor was 29 years (range 15-60). The M/F sex-ratio was 2. Fourteen

cases (93%) arose from soft-tissue and one case (7%) was visceral (small intestine). The most frequent tumor location was the limb (8 cases, 53%). Radiological features were non specific showing with magnetic resonance imaging intermediate signal intensity on T1-weighted images, high signal intensity on T2-weighted images, and heterogeneous contrast enhancement. Tumour necrosis was frequently observed and represented up to 50% of the tumor volume. In two cases, baseline 18 [F] Fluorodeoxyglucose positron emission tomography baseline was available for review and showed hypermetabolism of the primary tumor (SUVmax of 4 and 14, respectively).

Clinical management and impact of chemotherapy—All patients but one had surgical resection of the primary tumor. Five patients out of the 13 operated patients received 2 to 5 cycles of neoadjuvant chemotherapy. Chemotherapy consisted in administration of the VAC regimen (vincristine: 1.2mg/m² day 1, doxorubicin: 37.5 mg/m² day 1, cyclophosphamide: 2100 mg/m² day 1; 21 day cycle) in four cases and of the AI (doxorubicin: 25 mg/m² day 1-3, ifosfamide: 2000-3000 mg/m² day 1-3; 21 day cycle) regimen in one case. According to RECIST, two patients had partial response, two patients had stable disease and one patient had progressive disease. For the two patients with PR, the proportion of residual tumor cells was 0% and 30% respectively whereas no histological evidence of chemotherapy efficacy was found for the two patients with stable disease. Eight patients received chemotherapy in the metastatic setting with various chemotherapy regimens. Six patients were evaluable for response. According to RECIST, one patient had complete response (AI), one had partial response (MAID: doxorubicin: 20 mg/m² day 1-3, ifosfamide: 2500 mg/m² day 1-3, dacarbazine: 225 mg/m² day 1-3; day 1= day 21), and four had progressive disease.

Follow-up information and survival—The median follow-up was 12 months. At the time of this writing, three patients are still under treatment for the primary tumor event. Eight patients (53%) developed metastatic disease. The median metastasis-free survival was 9.8 months (95% CI: 0-22). Metastases were synchronous in one case and metachronous in 7 cases. Lung was the most frequent metastatic site (six patients), but other metastatic sites were also observed in four cases including unusual ones such as brain. The median overall survival has not been reached. The one-year overall survival was 81%.

DISCUSSION

The diagnostic challenge and subsequent uncertain clinical management related to the undifferentiated/primitive sarcoma of children and adolescents remains unresolved. The confusion is also enhanced by the grouping of SBRCT together with sarcomas displaying a spindle-fascicular growth, resembling ‘infantile fibrosarcoma’, under the meaningless term of undifferentiated sarcoma. Some of the larger series investigating this issue comes from the Intergroup Rhabdomyosarcoma Study (IRS)(Pawel et al., 1997). Among the 1,527 patients entered on IRS-III and IRS pilot-IV, 34 (5%) remained unclassified after retrospective re-review and application of immunohistochemical ancillary techniques (but not molecular diagnostic techniques)(Pawel et al., 1997). The incidence of similar histology among soft tissue sarcoma in adults remains undefined.

Allaggio et al. studied a group of seven undifferentiated pediatric and adolescent sarcoma lacking the known translocations associated with Ewing sarcoma, synovial sarcoma, etc (Allaggio et al., 2009). They observed two histologic patterns: one composed of primitive round cells and the other with elongated, spindle cells arranged in poorly formed fascicles. Although the two subgroups had overlapping immunoprofile and ultrastructural features, the tumors showing a primitive round cell morphology were associated with a more aggressive clinical course, compared to the ones with an undifferentiated spindle cell appearance.

Specifically, the primitive small blue cell subgroup occurred more centrally on the trunk or proximal extremity and presented with advanced stage at diagnosis. They postulated that the differences in clinical presentation, outcome and morphology may suggest distinct biologic subgroups. In fact one of their cases of undifferentiated primitive sarcoma, arising in the chest wall of 5 year-old boy was associated with a 4q35 rearrangement, a der(4)t(4;?8)(q35;q22)(Alaggio et al., 2009). Corroborated with the tumors of similar morphology reported by both Pawel (Pawel et al., 1997) and Sebire (Sebire et al., 2002), it emerges that undifferentiated sarcomas with a spindle cell phenotype and resembling infantile-fibrosarcoma are associated with a relatively favorable outcome. Of note, six undifferentiated sarcomas composed of a mixed round and spindle cell 'fibrosarcoma-like' appearance studied here by FISH did not show the presence of *CIC* rearrangements (data not shown).

As EWSR1 and FUS belong to the same FET family of RNA-binding proteins and appear to be functionally interchangeable, it is maybe not surprising that *FUS* was found to be fused to one member of ETS transcription factor family in a group of EFT (Shing et al., 2003; Ng et al., 2007; Berg et al., 2009). Thus 5 tumors with *FUS-ERG* fusion (Shing et al., 2003) (Berg et al., 2009) and one case with *FUS-FEV* have been reported to date (Ng et al., 2007). Despite these results, none of our 15 SBRCT tested showed *FUS* rearrangements by FISH, suggesting an infrequent involvement of *FUS* in this specific group of tumors.

CIC, a human homologue of *Drosophila capicua*, encodes for a high mobility group (HMG) box transcription factor. *Drosophila Cic* has been shown to mediate c-erbB (Egfr) signaling via transcriptional repression (Roch et al., 2002). Like several of the Sox genes, Cic is also predominantly overexpressed in the cerebellum (Lee et al., 2002), as well in subsets of medulloblastoma (Lee et al., 2005).

Human 4q and 10q subtelomeric regions contain the polymorphic macrosatellite repeat D4Z4, which share high sequence homology over a region of >200 kb (Lemmers et al., 2010b). Each D4Z4 repeat unit contains a conserved open reading frame for the *DUX4* retrogene, encoding for the double-homeobox transcription factor (van Geel et al., 2002). *DUX4* is normally expressed in human testis and germline cells, and is epigenetically suppressed in differentiated cells. Residual *DUX4* transcripts are spliced to remove the carboxyterminal domain that has been associated with cell toxicity (Snider et al., 2010). The contraction of the D4Z4 macrosatellite repeat array in the subtelomeric region of 4q35, but not 10q26, causes fascioscapular muscular dystrophy (FSMD). However, this contraction is pathogenic only in certain permissive configurations, such as the formation of a canonical polyadenylation signal for transcripts derived from *DUX4*, by single nucleotide polymorphisms distal to the last D4Z4 repeat (Lemmers et al., 2010a). These findings suggest that FSMD arises through a toxic gain of function attributable to the stabilized distal *DUX4* transcript.

CIC-DUX4 was previously shown to transform NIH 3T3 fibroblasts (Kawamura-Saito et al., 2006). As a consequence of fusion with the C-terminal of *DUX4*, the transcriptional activity of *CIC* is enhanced, suggesting a deregulation of downstream targets. *CIC-DUX4* binds to the ERM/ETV5 promoter by recognizing a novel target sequence, up-regulating its expression. From the fusion transcript, the deduced chimeric protein would include the HMG box of *CIC*, the putative binding site of TLE proteins, and highly conserved regions between human *CIC* and *Drosophila capicua*. In contrast, a large part of N-terminal *DUX4* region was lost, including the DNA-binding double homeodomain. Tetracycline-inducible *CIC-DUX4* expression in U2OS cells was associated with overexpression of ERM/ETV5, ETV1/ER81, RaLP, and CCL2. The same pattern of up-regulation was also present in the t(4;19) positive tumors. ChIP assays showed that PEA3 family genes are targets for the *CIC-*

DUX4 by a t(4;19) translocation. PEA3 represents one major subgroup of the superfamily of Ets-related transcription factors and is composed of three members, PEA3 (E1AF or ETV4) (Xin et al., 1992), ER81/ETV1 (Brown and McKnight, 1992) and ERM/ETV5 (Monte et al., 1994). These three factors regulate the expression of several target genes involved in tumorigenesis (de Launoit et al., 1997). Interestingly, both *PEA3* and *ETV1* have been described as fusion partner genes of *EWSR1* in EFT (Jeon et al., 1995). Therefore, it has been suggested that up-regulation of *PEA3* family genes by CIC-DUX4 may represent a molecular change equivalent to the EWSR1-ETS fusion (Kawamura-Saito et al., 2006).

The identification of a subgroup of undifferentiated sarcomas characterized by a fusion between genes which are not related to EWSR1 or the ETS family may have significant clinical implications. A recurrent t(4;19)(q35;q13.1) translocation, resulting in a *CIC-DUX4* fusion, has been reported in seven other cases (Table 3) (Richkind et al., 1996; Kawamura-Saito et al., 2006; Rakheja et al., 2008; Yoshimoto et al., 2009; Graham et al., 2011). The combined analysis of the clinical data from these case reports and from our series indicates a male predominance (sex ratio M/F=1.2), a median age at diagnosis of 28 years (range 6-69), a frequent tumor location in the limb (10 cases out of 22, 45%) and a high rate of metastatic relapse (11 cases out of 18 with available follow-up, 61%). Therefore, besides similar histological patterns, these tumors share with EFT a very aggressive clinical course. This raises the question of the optimal management of patients with *EWSR1*-negative SBRCT and more particularly of the role of adjuvant (or neoadjuvant) chemotherapy. Indeed, the role of adjuvant chemotherapy in the management of young adult or adult patients with soft-tissue sarcoma remains a controversial issue due to the lack of clearly proven survival benefit (Blay and Le Cesne, 2009). However, adjuvant (or neoadjuvant) chemotherapy is the standard of care for adults with a diagnosis of EFT or rhabdomyosarcoma as well as for children (Balamuth and Womer, 2010; Huh and Skapek, 2010). Many clinicians managing patients with localized soft-tissue sarcoma would not propose chemotherapy besides the loco-regional treatment except in the case of a diagnosis of EFT or rhabdomyosarcoma. Therefore, we believe that our results showing a high prevalence of rearrangement of *CIC* in *EWSR1*-negative SBRCT support the routine testing of this gene in this group of tumor. In our study, four patients presenting with localized disease at diagnosis were treated according to the institution's guidelines for EFT (neoadjuvant VAC regimen). Two patients had radiologically stable disease, one partial response and one had progressive disease. The low number of cases does not allow us to compare the chemosensitivity of *CIC*-rearranged tumors to that of *EWSR1*-rearranged EFT. Therefore, further studies are needed to clarify the role of chemotherapy in *CIC*-rearranged SBRCT. We propose that these patients should not be denied inclusion in EFT clinical trials, but rather be analyzed as a separate stratum.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Morphologic appearance of *CIC*-rearranged lesions. A. Tumor composed of mixed round and oval cells, displaying less monomorphic cytomorphology compared to EFT, with a higher degree of variability in nuclear size and shape (SBRCT 9, 200x); B. rare tumors showed areas of short spindle cells, arranged in a vague storiform pattern without a well-defined fascicular growth (SBRCT 7, 100x); C. although most tumors cells had scant cytoplasm, this lesion showed focally sheets of round cells with moderate amount of amphophilic cytoplasm, with eccentric nuclei, with open chromatin and prominent nucleoli (SBRCT 3, 200x); D. high magnification showing uniform round cells with open, vesicular chromatin and eosinophilic, macronucleoli (SBRCT 14, 400x); E. although most tumors showed geographic areas of necrosis, few tumors showed a distinctive ‘starry-sky’ appearance due to individual tumor cells necrosis (SBRCT 7, 200x); F. O13 immunostaining showing patchy areas of reactivity (SBRCT 9, 200x).

Figure 2. *CIC* gene rearrangements and fusion with candidate genes on 4q35 and 10q26.3 by FISH. A. Split-apart signal in a *CIC*-rearranged tumor; upper-right showing location and designation of BACs flanking *CIC*, three on each side (labeled with red, centromeric and with green, telomeric); B. fused yellow signal, resulting from the fusion of telomeric *CIC* in green with centromeric portion of *DUX4* in red, on chromosome 4q35; C. fused signal of telomeric *CIC* (green) to centromeric *DUX4* in red on chromosome 10q26.3.

Figure 3. Long range genomic PCR of *CIC-DUX4* in SBRCT 3. An 1,296 bp was amplified by LR-PCR and shown by gel electrophoresis (A), which by Sanger sequencing confirmed the fusion of 5' portion of *CIC* exon 20 with *DUX4* exon 1 on 4q35; * represents a short fragment between *CIC* and *DUX4* breakpoints, which matches the anti-parallel 4760-4782 region of *CIC* exon 20 (B). Comparative analysis of the D4Z4 genomic sequence between 4q35 (including the sequence obtained from SBRCT 3; position 1 represents the *DUX4* breakpoint) and 10q35 (including the sequence from SBRCT 9; position 374 represents the distance from the *DUX4* breakpoint) shows deletions in the CDS region of D4Z4 of 10q26 but not in 4q35(C).

Figure 4. Demonstration of novel t(10;19) translocation resulting in *CIC-DUX4* fusion in SBRCT 9. (A) RACE results identified the 5' portion of *CIC* exon 20 fused to the first nucleotide of *DUX4* exon 2 on 10q26.3; (B) RT-PCR confirmed an identical breakpoint and sequence, here illustrated by a 231 bp amplified product; (C) Diagrammatic representation of the LR-PCR results, confirming the fusion of 301 bp of *CIC* exon 20 to 662 bp of *DUX4* exon 1. The break point in *DUX4* was located at the distal end of the last D4Z4 repeat. The BlnI+ (CCTAGG) restriction site identified in exon 2 is shown in bolded black font; BlnI+ D4Z4 unit is typically present in chromosome10, compared to the D4Z4 BlnI-pattern in chr. 4 (Lemmers et al., 2010b). The underlined sequence in the pLAM region (exon 3) highlights the proposed polyadenylation site (Lemmers et al., 2010a). (D) Schematic representation of genomic sequence of *DUX4* on chromosome 10, adapted from (Snider et al., 2010) showing the break within exon 1 of *DUX4* (lightning bolt symbol). The sites of polyadenylation signals: ATCAAA, ATTTAA, CTAGCG, and TTTAAA, depicted by arrows, as described by (Lemmers et al., 2010a), and <http://www.imtech.res.in/raghava/polyapred/index.html>). Lower part illustrates the *DUX4* transcript variants identified by RACE among the *CIC-DUX4* fusion products of SBRCT 9; the predominant transcript (*DUX4* exon 2-exon 6) being depicted in the ABI sequence in (A).

Table 1

Clinical and Pathologic Findings of Small Blue Round Cell Tumors

Case	Age/ Gender	Location/Depth	Tumor Size (cm)	Metastatic disease	Sites of metastasis	Last status	Follow- up (months)
1	19/M	foot soft tissue with bone extension/D	NA	NA	NA	LOF	NA
2	30/F	peritonsillar/man- dible	5	Metachronous	Lung	LOF	13.4
3	33/F	paraspinal soft tissue/D	9.4	No	-	NED	7.1
4	35/F	Groin/D	NA	Metachronous	Lung	AWD	28.9
5	16/M	Thigh/D	13.7	Synchronous	Lung	AWD	1
6	28/M	Thigh/D	8.4	No	-	NED	6.0
7	15/M	Back/S	3	Metachronous	Lung	DOD	14.2
8	45/M	Back/S	4.2	Metachronous	Lung, bone, brain	DOD	10.4
9	49/M	Arm/D	3	Metachronous	Lung	DOD	14.6
10	26/M	chest wall soft tissue/D	8	No	-	NED	9.6
11	26/M	upper arm with skin ulceration/S	4	Metachronous	Peritoneum	AWD	22.6
12	29/F	Arm/D	1.6	No	-	NED	46.7
13	60/M	Thigh/D	9.5	No	-	AWD	3
14	27/F	Small intestine	8	Synchronous	Peritoneum Liver	DOD	2
15	38/M	upper arm/D	11	No	-	NED	55.2
16	16/F	Chest wall soft tissue/D	NA	No	-	NED	43.7
17	29/F	Paraspinal soft tissue/D	11	Metachronous	Lung, soft- tissue	DOD	10.3
18	14/F	Iliac bone	5.8	No	-	NED	35.2
19	24/F	Thigh/S	4.5	No	-	LOF	1
20	69/M	Pelvis soft tissue/D	NA	Synchronous	Lung	DOD	7.4

Case	Age / Gender	Location/Depth	Tumor Size (cm)	Metastatic disease	Sites of metastasis	Last status	Follow-up (months)
21	22/M	Finger soft tissue/S	3	No	-	NED	7.1
22	40/F	Retropertitoneum/D	23	Synchronous	Lung	DOD	8.7

SBRCT¹⁻⁶: t(4;19)/CIC-DUX4 fusion-positive; SBRCT⁷⁻¹²: t(10;19)/CIC-DUX4 fusion-positive; SBRCT¹³⁻¹⁵: CIC-rearranged; SBRCT¹⁵⁻²² negative for CIC rearrangement by FISH; i-musc, intramuscular, LOF, lost to follow-up; DOD, dead of disease, NED, no evidence of disease, AWD, alive with disease, NA, not available; D, DEEP; S, superficial.

Table 2

Immunohistochemical, Molecular and FISH findings in *CIC*-positive small blue round cell tumors

Case	O13/ CD99	CK/ EMA	S100	Des	Chr/ Syn	Lymphoid Markers	FISH ^Δ	RT-PCR ^Δ
1	CD99 focal	Neg /Neg	Neg	Neg	Neg	ND	EWSR1, FUS	ND
2*	++	Neg	Neg	++	Neg	Neg CD3, CD20	EWSR1, FUS	PAX-FOXO1; EWSR1-Fli1; EWSR1-ERG
3 [†]	O13+ focal	Neg /Neg	Neg	Neg	Neg	Neg CD3, CD20,CD45	EWSR1, FUS	ND
4	O13+ focal	Neg /Neg	Fpos	Neg	Neg	Neg CD45	EWSR1, DDIT3, FUS	ND
5	O13++	Neg /Neg	Neg	Neg	ND	Neg TdT, CD3, CD20	EWSR1, SS18	EWSR1-Fli1; EWSR1-ERG
6	CD99++	Neg /Neg	Fpos	Neg	Neg	Neg CD45	EWSR1, SS18	ND
7	O13++	Neg/ Fpos	Neg	Neg	Neg	Neg CD45	EWSR1, FUS	EWSR1-Fli1, EWSR1-ERG, EWSR1-CREB1, EWSR1- ATF1
8	O13+ focal	Neg	Neg	Neg	Neg	Neg TdT, CD3, CD20	EWSR1, FUS	EWSR1-Fli1, EWSR1-ERG
9 [€]	++	Neg	Neg	Neg	Neg	Neg TdT, CD3, CD20	EWSR1, FUS	EWSR1-Fli1, EWSR1-ERG
10	+++	Neg	Neg	Neg	Neg	Neg TdT	EWSR1, FUS	ND
11	CD99++	Neg	Neg	Neg	ND	Neg CD20	EWSR1	ND
12	Neg/CD 99 focal	Neg /Neg	Neg	Neg	Neg	Neg TdT, CD3, CD20	EWSR1	EWSR1-Fli1, EWSR1- ATF1, EWSR1-CREB1, FUS-ATF1, SS18-SSX
13	CD99++	Neg /Neg	Neg	Neg	ND	ND	EWSR1, SS18	ND
14	CD99++	Fpos /N	Neg	Neg	Neg	Neg CD20, CD3, CD45	EWSR1	ND
15	CD99++	Fpos / Fpos	+	Neg	ND	ND	EWSR1, SS18	ND

SBRCT¹⁻⁶; t(4;19)/*CIC-DUX4* fusion-positive; SBRCT⁷⁻¹²; t(10;19)/*CIC-DUX4* fusion-positive; SBRCT¹³⁻¹⁵; *CIC*-rearranged

⁷LR-PCR confirmed a *CIC-DUX4* fusion
⁸RACE, RT-PCR and LR-PCR confirmed a *CIC-DUX4* fusion.
^{*}EM was done (pools of glycogen, uniform NS-granules); N, negative; Fpos, focally positive, ND, not-done; CK, cytokeratin; Des, desmin; Chr, chromogranin; Syn, synaptophysin
^Δ negative results.

Table 3

Previously Reported Undifferentiated Sarcomas with t(4;19) or Rearrangements Involving 4q35 or 19q13

Age/Sex/Site (reference)	Abnormality	Diagnosis	Immuno (+)	Clinical FU
12/M /foot (Richkind et al., 1996)	t(4;19)(q35;q13.1)	Primitive mesenchymal sarcoma	NSE, vimentin	DOD, 10mo
62/F/buttock/pelvis (Kawamura-Saito et al., 2006)	t(4;19)(q35;q13.1) CIC-DUX4	Ewing sarcoma- like tumor	O13 weak	DOD, 10mo
31/M/shoulder (Kawamura-Saito et al., 2006)	t(4;19)(q35;q13.1) CIC-DUX4	Ewing sarcoma- like tumor	O13 weak	NED, 30mo
16/F/trunk (Yoshimoto et al., 2009)	t(4;19)(q35;q13.1) CIC-DUX4	Undifferentiated SBRCT	CD99m strong	DOD
14/M/ head &neck (Yoshimoto et al., 2009)	t(4;19)(q35;q13.1) CIC-DUX4	Undifferentiated SBRCT	CD99 weak, focal	DOD
6/M/hip (Rakheja et al., 2008)	t(4;19)(q35;q13.1)	Undifferentiated SBRCT	CD99 weak, focal; FLI1	NA
11/F/ flank (Graham et al., 2011)	CIC-DUX4 *	Undifferentiated SBRCT	CD99m, S100 focal	NA
9/F/ paraspinal (Graham et al., 2011)	CIC-DUX4 *	Undifferentiated SBRCT	S100 focal	NA
11/F/inguinal (Graham et al., 2011)	CIC-DUX4 *	Undifferentiated SBRCT		NA
9/F/thigh (Roberts et al., 1992)	t(4;12;19)(q35;q13;q13.1)	ERMS	ND	NED, 6 mo
5/M/chest wall (Alaggio et al., 2009)	der4t(4;?8)(q35;q22)	Undifferentiated SBRCT	NR	DOD 11mo
19/F/thigh (Sirvent et al., 2009)	t(4;22)(q35;q12) <i>EW/SRI</i> and 4q35 <i>DUX4</i> region rearrangements by FISH	ERMS	Desmin myogenin	NED, 6 yrs
15/M/abdominal wall (Riccardi et al., 2010)	t(18;19)(q23;q13.2)	Undifferentiated SBRCT	CD99 focal; FLI1	NA

M, male, F, female; DOD, dead of disease, mo, months, yrs, years; FU, follow-up, NED, no evidence of disease, ERMS, embryonal rhabdomyosarcoma; ND, not done; SBRCT, small blue round cell tumor; NR, not-reported, NA, not available

*
by RT-PCR; CD99m, CD99 membranous positivity