CIC-DUX sarcomas demonstrate frequent MYC amplification and ETS-family transcription factor expression

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Recent molecular advances have identified a novel, clinically aggressive subgroup of undifferentiated round cell sarcomas defined molecularly by oncogenic fusion of the gene, *CIC,* and either *DUX4* or its paralog, *DUX4L*, herein termed CIC-DUX sarcomas. Morphologically, CIC-DUX sarcomas are round cell sarcomas with high-grade nuclear features, including vesicular chromatin and nucleoli, patchy clear cell foci, myxoid change, and necrosis. Here, we studied a cohort of 10 cases, including 6 newly identified cases, 2 with paired metastases. Given our prior observation of trisomy 8 in these tumors, we assayed for amplification and expression of *MYC* (c-Myc) and representative downstream targets. Trisomy 8 was detected in 5/7 testable cases, with further amplification of *MYC* locus in 6/7 testable cases and immunohistochemical expression of MYC in 10/10. The canonical MYC transcriptional target, p21, but not MTDH, was differentially expressed compared with Ewing sarcomas. Given prior observation of induction of ETS-family transcription factors by the fusion oncoprotein, we assayed and identified highly prevalent positivity for ERG (9/10) and FLI1 (8/8). These findings are cautionary regarding use of these immunostains in prospective case workup, whereas the prevalent *MYC* amplification may represent a therapeutically targetable oncogenic pathway in CIC-DUX sarcomas.

Recent molecular efforts have identified that a significant subset[1] of undifferentiated small round cell sarcomas harbor a recurrent translocation, involving the gene, *CIC (capicua transcriptional suppressor)*, on chromosome 19, fused with one of two poorly understood genes, either *DUX4 (double homeobox 4)*, on chromosome 4 or its paralog, *DUX4L (double homeobox 4-like)*, on chromosome 10, resulting in either *CIC-DUX4*, t(4;19)(q35;q13) or *CIC-DUX4L*, t(10;19)(q26;q13) fusions. These

sarcomas, originally characterized in the pediatric population,[2–5] have been found to occur over a wide age range, though they arise predominantly in young adults.[6–10]

We recently reported four undifferentiated round cell sarcomas harboring *CIC-DUX4* fusions,[11] which, together with reported *CIC-DUX4L*[6] cases, we term 'CIC-DUX sarcomas'. These tumors demonstrated clinical behavior at least as aggressive as Ewing sarcomas, with all four patients expiring from disease within 18 months. The tumors had reproducible histomorphology including high-grade nuclear features (vesicular chromatin, moderate nuclear pleomorphism, and prominent nucleoli), lobular growth pattern, geographic necrosis, round cell cytomorphology, patchy clear cell areas, and distinctive foci of myxoid change.

Given the limited experience with these tumors, we sought to characterize an expanded cohort to study CIC-DUX sarcoma histomorphology. As *in vitro* data suggest that the fusion oncoproteins of these sarcomas may induce expression of

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ETS-family proto-oncogenes,[8] and as we and others[12] have noted ERG or FLI1 expression in individual cases, we studied expression of these markers in light of the differential with Ewing sarcoma. Finally, proceeding from our observation of polysomy for chromosome 8 in CIC-DUX cases,[11] we tested for expression and amplification of the frequently amplified chromosome 8 proto-oncogene, *MYC* (c-Myc), as well as downstream transcriptional targets.

Materials and methods

Cohort

Under IRB-approved protocols, retrospective searches were performed using institutional pathology databases for small round cell sarcomas, predominantly arising in the soft tissue, which had been previously unclassifiable. Four of the cases identified were previously reported.[11] Six additional cases were identified at the University of Michigan, Cleveland Clinic, and Cedars-Sinai Medical Center. Demographic, pathologic, and clinical follow-up data were tabulated for each case. A previously constructed[13] tissue microarray of 70 primary and metastatic lesions of 45 cases of Ewing sarcoma (24 classic skeletal, 19 extraskeletal, 2 metastases only) was analyzed for comparison purposes.

Molecular Testing

We have previously detailed our molecular assays used to establish diagnosis in CIC-DUX sarcomas. Briefly, to assay for rearrangement at the *CIC* locus, two dual color interphase fluorescence *in situ* hybridization (FISH) strategies were used. The first used rhodamine labeled BAC clone RP11-569M1, spanning the *CIC* gene at 19q13, and fluorescein-labeled RP11-46I12, a centromeric control marker for chromosome 19. The second used conventional 'break apart' probes made from BAC clones RP11-317E13 (flanking, centromeric, Cy5 labeled) and RP11-778C1 (flanking, telomeric, fluorescein labeled) to *CIC*. For the first probe set, tumors showing >10% nuclei with three rhodamine signals (*CIC*-spanning target split) and two fluorescein signals (centromeric control) were considered positive for rearrangement. For the latter strategy, tumors with >10% of nuclei with split green/red signals were considered positive. A final three color dual fusion strategy was used to assay for fusion of either *DUX4* or *DUX4L* and *CIC*, using probes from RP11-778C1 telomeric to *CIC* (fluorescein labeled), RP11-521G19, centromeric to *DUX4* (rhodamine labeled), and RP11-108K14, centromeric to *DUX4L* (AQUA labeled). For *MYC* amplification and chromosome 8 polysomy testing, commercially available two color probes for the *MYC* locus at 8q24 and CEN-8 were purchased and used per manufacturer instructions (Poseidon Probes, Veridex, Raritan, NY, USA). Our

nested RT-PCR assay for the *CIC-DUX4* fusion transcript was used as previously reported,[11] using a primer design and control plasmid developed by Kawamura-Saito *et al*.[8] Two cases had also been tested by FISH at Memorial Sloan Kettering Cancer Center, providing additional confirmation of the diagnosis.

Immunohistochemical Analysis

Immunostain for ERG was performed as described previously using a ready-to-use, prediluted monoclonal antibody against ERG, clone EPR3864 (Ventana Medical Systems, Tucson, AZ) using a Ventana XT autostainer as detailed previously.[13–15] Immunohistochemical staining for FLI1 (Neomarkers, Fremont, CA, USA), p21/WAF1 (DAKO, Carpinteria, CA, USA) MTDH, (Millipore, Billerica, MA, USA), and CD99 (DAKO) was performed on a DAKO Autostainer (DAKO) using DAKO LSAB+ (FLI1 and MTDH) or Envision+ (p21) and diaminobenzadine as chromogen. Microwave citric acid epitope retrieval was used for FLI1, MTDH, and CD99 prior to staining. Microwave epitope retrieval in 10 mM Tris/1 mM EDTA pH9 was used for p21. MYC rabbit monoclonal (Y69, Abcam) was used at 1/50 dilution, in buffer CC1 for 36 min (pH 8.5 HIER buffer at 95 °C), with 32 min primary incubation at 37 °C. Appropriate negative (no primary antibody) and positive controls were stained in parallel with each set of antibodies studied.

Immunohistochemical scoring was performed (SCS, DGT, RMP) using two scoring systems. A conventional 0–3+ by proportion (0, negative; 1+, focal; 2+ multifocal; 3+ diffuse) was used for scoring whole-tissue sections stained utilizing antibodies used in our routine clinical practice (ERG, FLI1, MYC, CD99). For scoring the TMA format used for testing the MYC targets p21/WAF1 and MTDH, we used an Allred score (0–8, 0–5 proportion score summed with a 0–3 intensity score)[16] for comparison between expression of these targets in CIC-DUX sarcomas and Ewing sarcomas. Specifically, for the Allred score intensity and proportion, both contribute to an overall score (0, none; 1, <1%; 2, 1–10%; 3, 10–33%; 4, 33–66%; and 5, >66% plus an intensity score of 0, none; 1, weak; 2, intermediate; and 3, strong). Distributions of ordinal Allred scores for p21 and MTDH were compared between CIC-DUX sarcomas and Ewing sarcomas by the Mann–Whitney U-test, with significance assumed at an alpha of 0.05.

Results

Cohort

The final cohort included three males and seven females, with age range of 20–43 years, median 35 years. The tumors ranged in size from 3 to 14 cm

Table 1 Clinicopathologic features and molecular validation

Case	Reference	Sex	Age (years)	Size (cm)	Site	Trans-location	Positive assays	Negative assays	Metastasis	Treatment, in sequence	Outcome (Mo)
1	Choi *et al*	F	25	11	Right calf	t(4;19)	Karyotype, RT-PCR, FISH	EWSR1 and SYT FISH, EWSR1-FLI1/ ERG and SYT-SSX1/ 2RT-PCRs	Lung, brain	AI; IE; resection of primary, resection of lung and brain metastases	DOD 10
2	Choi *et al*	F	32	14	Right gluteus/ pelvis	t(4;19)	Karyotype, RT-PCR, FISH	EWSR1 and SYT FISH, EWSR1-FLI1/ ERG and SYT-SSX1/ 2RT-PCRs	Lung	CVAD; I; E; resection of primary tumor, high dose I for recurrent disease, local radiotherapy for persistent disease	DOD 14
3	Choi *et al*	F	20	6	Left shoulder	t(4;19)	RT-PCR, FISH	EWSR1 and SYT FISH, EWSR1-FLI1/ ERG and SYT-SSX1/ 2RT-PCRs	Lung	AI; high-dose I; surgical resection of lung metastases; surgical resection of primary; local radiotherapy	DOD 17
4	Choi *et al*	M	43	9.8	Left knee	t(4;19)	RT-PCR, FISH	EWSR1 and SYT FISH, EWSR1-FLI1/ ERG and SYT-SSX1/ 2RT-PCRs	Pelvis	Resection of the primary Tumor; local radiotherapy	DOD 14
5	This study	F	33	6.4	Right knee	t(4;19)	FISH	EWSR1 FISH	~	Resection of the primary tumor; local radiotherapy	NED 18
6	This study	F	36	3	Trapezius	t(10;19)	FISH	EWSR1 FISH	Lung	Local radiotherapy, combination VAC-IE, en bloc re-resection	DOD 11
7	This study	M	38	5.2	Lower arm	t(4;19)	RT-PCR, FISH	EWSR1 FISH	Lung, cubital fossa lymph node	Resection of lung metastases, local paliative radiotherapy, palliative A	AWD 17
8	This study	F	23	5.8	Left upper back	t(4;19)	RT-PCR, FISH	EWSR1 FISH	Lung	Lung metastectomy, VAC-IE, stereotactic radiosurgery to primary and lung metastases	NED 48
9	This study	M	37	4.1	Right lower leg	t(10;19)	Karyotype, FISH	EWSR1, NR4A3, FUS FISH, EWSR1-FLI 1/ERGRTPCR	Groin lymph node	Resection of primary, VAC-IE at 10 months- 1 year, resection of groin lymph node metastasis	NED 22
10	This study	F	36	6.5	Right thigh	t(4;19)	RT-PCR, FISH	EWSR1, SYT FISH	New case	New case	New case

Abbreviations: A, doxorubicin; AWD, alive with disease; C, cyclophosphamide; D, dactinomycin; DOD, dead of disease; E, etoposide; F, female; FISH, fluorescence *in situ* hybridization; I, ifosfamide; M, male; NED, no evidence of disease; RT-PCR, reverse transcriptase-polymerase chain reaction; V, vincristine.

(median 6 cm) and arose predominantly in soft tissues of the extremities, with half arising in the soft tissues of the lower extremities. The gene fusions identified were t(4;19)/*CIC-DUX4* in 8/10 cases, and t(10;19)/*CIC-DUX4L* in 2/10 cases, the latter both among the new cases. Of six new cases, follow-up was available for five, where metastasis was noted in 4/5, one demise at 11 months, one case alive with disease progressing on palliative chemotherapy, and three cases showing no evidence of disease at up to 48 months after diagnosis, two of which show no evidence of disease after metastectomy. Table 1 summarizes the clinicopathologic, molecular, and treatment/outcome parameters of these cases.

Morphology

We prospectively evaluated morphological features described previously as characteristic of CIC-DUX sarcomas,[11,12] particularly the distinctive myxoid change and patchy clear cell areas, but also including the lobular growth pattern, necrosis, high-grade nuclear features, and presence and extent of spindled cytomorphology. Compared with Cases 1–4 reported previously,[11] Cases 5–10 showed relatively more extensive myxoid stromal change in 3/6. In Case 9, this morphology resulted in resemblance to extraskeletal myxoid chondrosarcoma or myoepithelioma. Clear cell change was variable, present in 5/6 primary lesions, whereas the one case that lacked clear cell change showed this feature in its metastasis. Foci of spindled cellular morphology were identified in 5/6 cases. Metastatic deposits were available for review for Cases 7 and 9; both showed a degree of dense, sheetlike growth exceeding that of the primary tumor. Figures 1 and 2 illustrate the morphological features seen, whereas Table 2 summarizes the morphological observations by case.

Immunophenotype and Genotype

Both the cases previously reported[11] and those newly identified were studied by immunohistochemistry (IHC) for expression of Ewing sarcoma-associated

markers CD99 and ETS transcription factor family members ERG and FLI1. CD99 was expressed in 7/7 cases, focal in 5/7, multifocal and diffuse in the two remaining cases, though the pattern of staining was cytoplasmic without the classic membranous enhancement seen in Ewing sarcoma
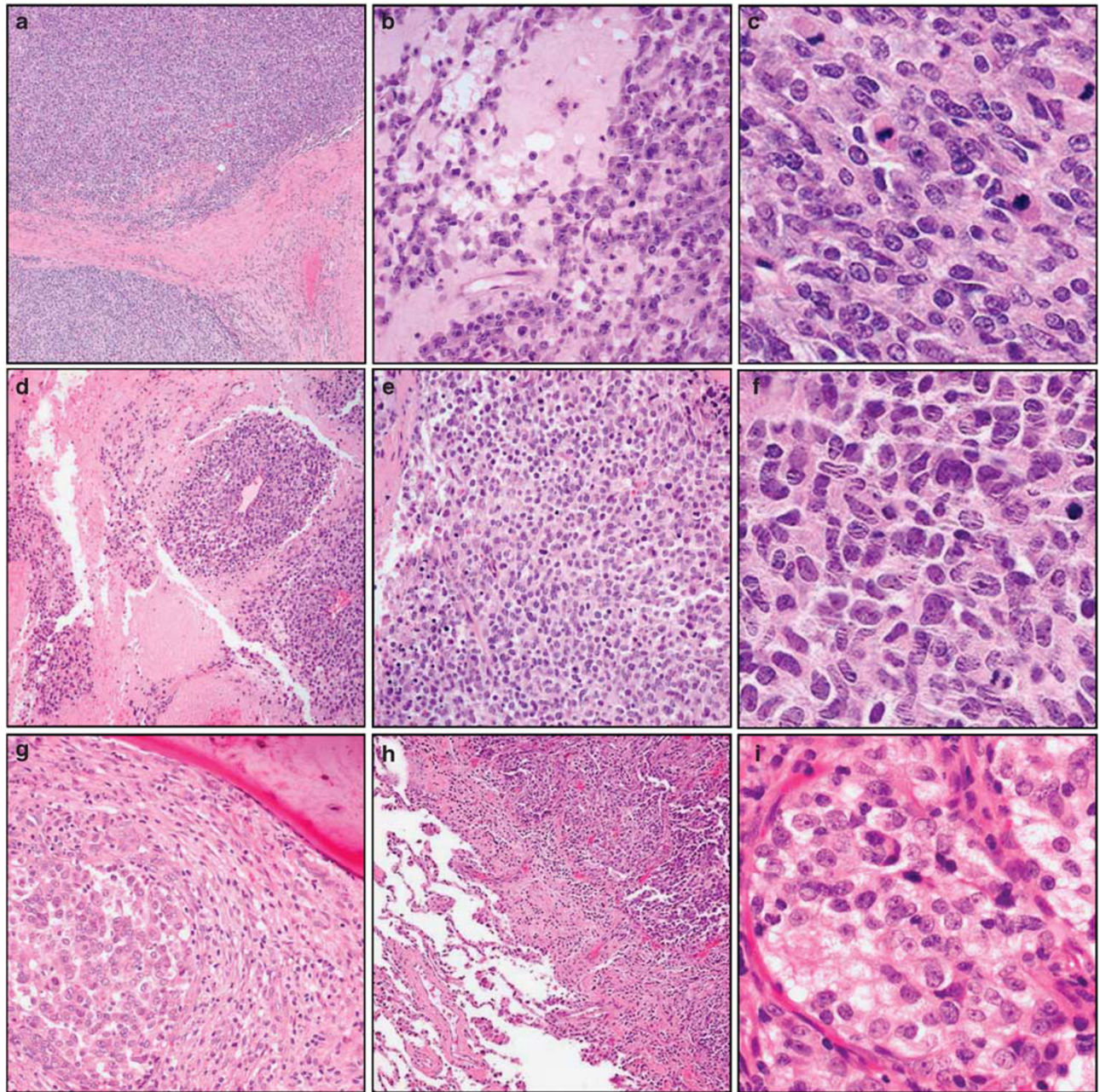
Figure 1 Morphological features of CIC-DUX Sarcomas, cases 5–7. Case 5 demonstrates prominent lobular growth (**a**, ×10) with focal stromal myxoid change (**b,** ×40). High-grade cytology was apparent with brisk mitosis, with mild to moderate nuclear variation beyond that seen in a Ewing sarcoma (**c**, ×40, zoom). Case 6, a t(10;19) case, showed predominant, geographic necrosis >50% of the untreated primary, with sparing of perivascular tumor (**d**, ×20). Areas of pale to clear cytoplasm were focal (**e**, ×40), whereas the cytology showed moderate nuclear pleomorphism and a syncytial appearance (**f**, ×40, zoom). Case 7 showed metaplastic ossification of the peritumoral fibrous septae (**g**, ×40); the case progressed to lung metastasis (**h**, ×20), where focal areas of pale to clear cytoplasm were apparent (**i**, ×40, zoom).

Figure 2 Morphological features of CIC-DUX Sarcomas, Cases 8–10. Case 8 demonstrates cytomorphology ranging from round to more spindled (**a**, ×20) with myxoid areas associated with spindled growth (**b**, ×20). High-grade cytology with nucleoli was apparent (**c**, ×40, zoom). Case 9, a t(10;19) case, showed superficial extension nearing the skin surface (**d**, ×10) and striking morphology evocative of a myoepithelial neoplasm (**e**, ×20). The subsequent lymph node metastasis showed dense, cellular growth more similar to other cases (**f**, ×10). Case 10 showed a necrotic lesional core (**g**, ×10). Several foci at the periphery showed stromal myxoid change (**h**, ×40, zoom), whereas spindled cellular areas were notable (**i**, ×40). In Case 9, CD99 positivity was diffuse (**j**, ×20) albeit without a membranous pattern (**k**, ×40). No more than focal staining was seen in most cases (**l**, ×40).
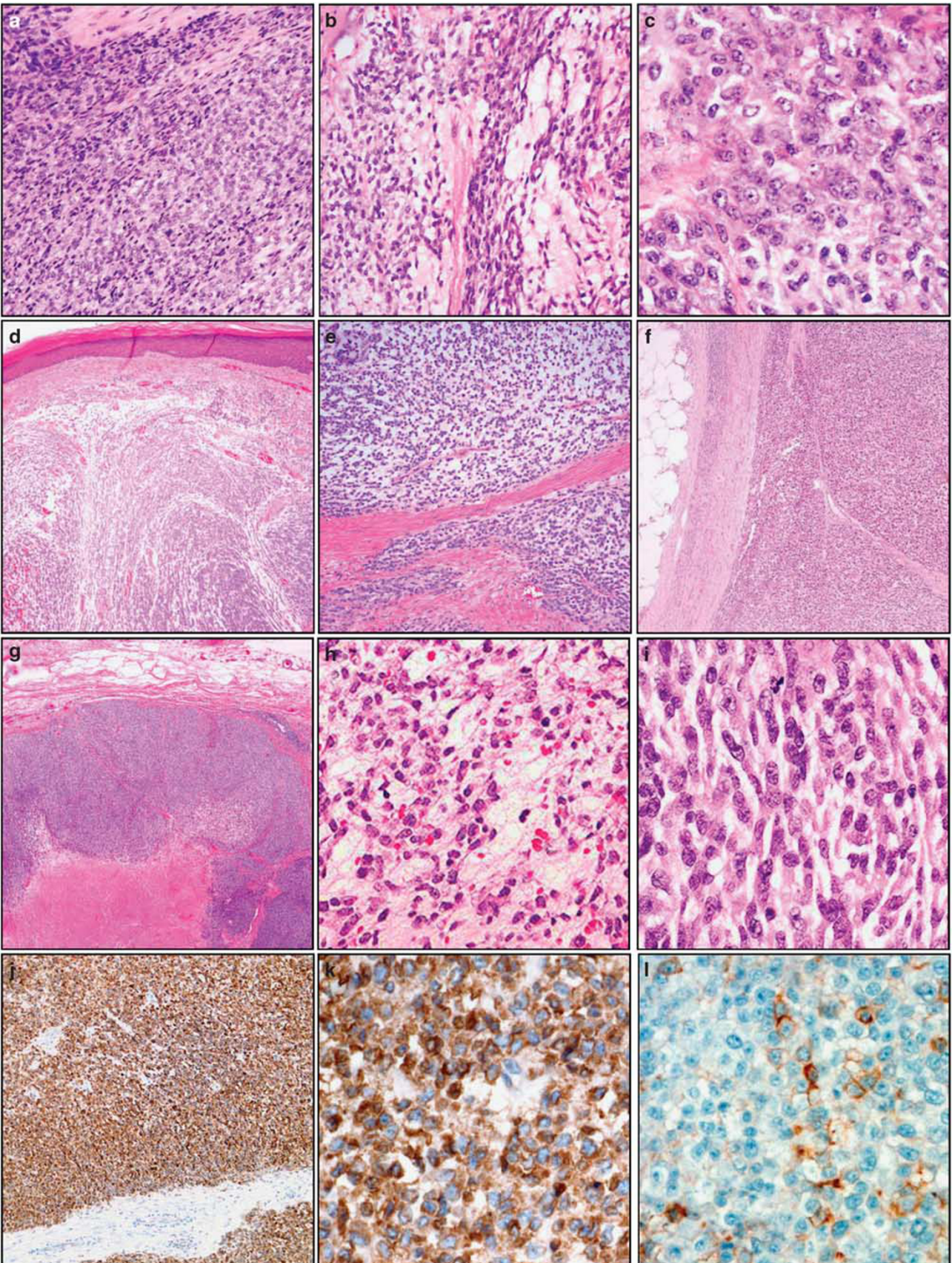
Table 2 Morphological features of new cases and metastases

Case	Lesion studied	Site	Septate growth	Necrosis	Nuclear features	Clear cell areas	Myxoid	Spindled cytology	Other notes
5	Primary	Right knee	Present	Single cell and focus of tumor cell necrosis	Vesicular chromatin, less prominent nucleoli, moderate pleomorphism	Multifocal	Focal	Focal	~
6	Primary	Right trapezius	Present	~50%, Geographic with perivascular sparing	Vesicular chromatin, less prominent nucleoli, moderate pleomorphism	Focal	Multifocal	Focal	~
7	Primary and metastasis	Lower arm, lung	Present	~5%, Focal tumor cell necrosis in primary and metastasis	Vesicular chromatin, prominent nuclei, moderate pleomorphism in primary and metastasis	Absent in primary, present in metastasis	Focal, primary	Absent	Metaplastic bone in peritumoral septae, prominent lymphohistiocytic infiltrate only in primary
8	Primary	Left upper back	Present	~5%, Multifocal tumor cell necrosis	Vesicular chromatin, prominent nuclei, moderate pleomorphism	Multifocal	Multifocal	~10%, Multifocal	~
9	Primary and Metastasis	Right lower leg, groin lymph node	Present	Focal in primary, ~30%, geographic with perivascular sparing in lymph node metastasis	Vesicular chromatin, less prominent nucleoli, moderate pleomorphism mostly in metastasis	Prominent in metastasis	Predominant in primary, focal in metastasis	Prominent in primary, patchy in metastasis	Marked increased cellularity in groin node metastasis
10	Primary	Right thigh	Present	~50%, Geographic with perivascular sparing	Vesicular chromatin, prominent nuclei, mild pleomorphism	Focal	Prominent	~30%, Multfocal	Myxoid periphery, cellular core of lesion

(Figure 2j–l). ERG was positive in 9/10 cases; FLI1 was positive in 8/8 cases. Overall, FLI1 positivity was more extensive and intense than that of ERG. The patterns of FLI1 and ERG are demonstrated in Figure 3; Table 3 details the immunophenotype by case.

We previously observed trisomy 8 in two CIC-DUX cases where karyotyping was performed in routine clinical workup.[11] Among the six newly identified cases, cytogenetic data were only available for one additional case, a t(10;19) CIC-DUX4L case, which also showed trisomy 8. Given the known locus of *MYC* (c-Myc) on chromosome 8, we hypothesized that this oncogene might be of significance in CIC-DUX sarcomas. Immunohistochemical MYC expression was positive, mostly diffuse, in 10/10 cases. On the basis of this observation, we tested for amplification of the *MYC* locus by FISH, using probes spanning the *MYC* locus to evaluate copy number, and centromeric controls for chromosome 8 as a surrogate for ploidy. Trisomy for chromosome 8 was seen in 5/7 cases successfully tested, confirming the cytogenetic observation of trisomy 8 in one of the two previous cases (the hybridization was unsuccessful in the other). Amplification of the *MYC* locus, present as multiple additional copies, was identified in six of seven cases; two cases harbored amplification without chromosome 8 polysomy. Figure 4 shows representative *MYC* FISH and IHC findings; Table 3 summarizes immunophenotypic and FISH results by case.

Two established transcriptional targets of MYC signaling include Metadherin (*AEG-1/Astrocyte Elevated Gene 1/Lyric*) encoded at *MTDH*[17] and p21 (WAF1/CIP1) encoded at *CDKN1A*.[18] To study how the pattern of expression of these transcriptional targets in CIC-DUX sarcomas compares with Ewing sarcoma, we used a tissue microarray, constructed of Ewing sarcoma cases described previously.[13] Comparing semiquantitative Allred IHC scores[16] between CIC-DUX and Ewing sarcoma cases, we found that MTDH was expressed similarly ($P = 0.20$), whereas p21 expression was significantly greater in the CIC-DUX cases ($P < 0.001$) (see Figure 5).

Discussion

Recent molecular advances, especially next generation sequencing, have allowed identification of a number of subtypes of undifferentiated round cell and spindle cell sarcomas as defined by the 2013 WHO classification,[19] several of which lack EWSR1 gene rearrangement[12] and harbor characteristic alternative genomic alterations. Among recently recognized molecular subtypes of these undifferentiated sarcomas are tumors harboring *CIC-DUX4* and *CIC-DUX4L* fusions,[6,11] which may constitute a substantial subset of such cases.[6] The distinctive features described by others,[2,3,5–8] in our prior cohort,[11] and replicated herein, lead us to suggest that these tumors be regarded as a distinct entity, defined not only by their distinctive molecular but also by reproducible histopathologic characteristics.

We would argue, as have others,[12] that diagnostic recognition of this entity as a high-risk malignancy
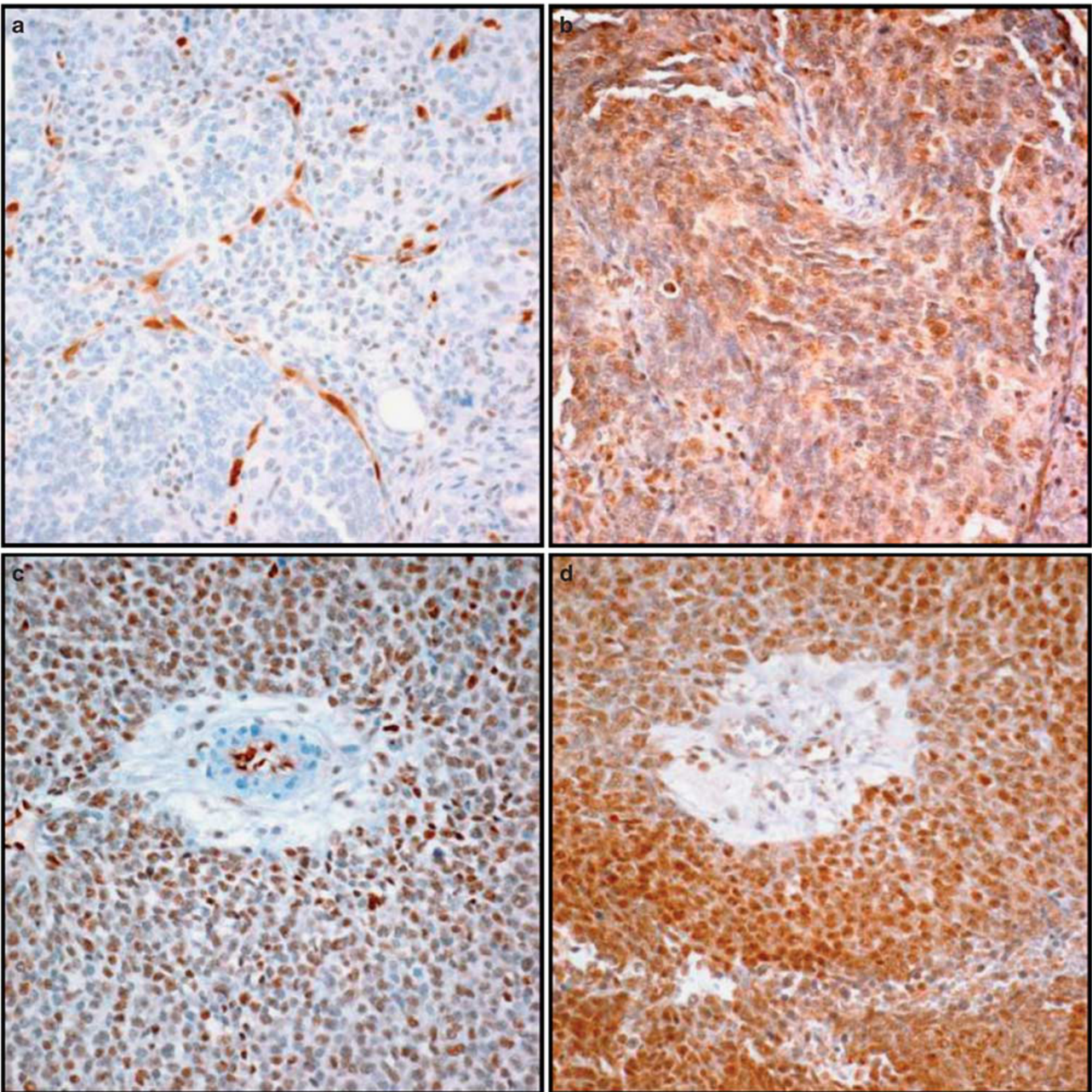
Figure 3 ERG and FLI1 positivity of CIC-DUX Sarcomas. Representative micrographs from Case 7 (**a**, **b**), Case 9 (**c**, **d**), and Case 10 (**e**, **f**), for ERG (**a**, **c**, **e**) and FLI1 (**b**, **d**, **f**) illustrate positivity of these Ewing sarcoma-associated markers. ERG ranged from focal (Case 7, panel **a**, scored as 1 +) to 3 + , diffuse in Cases 9 and 10. FLI1 showed overall greater proportion and intensity; each of these was graded as 3 + positivity.

(at least analogous to Ewing sarcoma) is important given the different treatment considerations, as compared with conventional adult soft tissue sarcomas. Though interdisciplinary consultation is recommended, national comprehensive cancer network general guidelines for adult soft tissue sarcomas, depending on stage, may recommend surgery with observation, with or without adjuvant or neoadjuvant radiotherapy[20] rather than a Ewing sarcoma-type multidrug chemotherapeutic protocol. Data on treatment and response to therapy in CIC-DUX sarcomas remain limited; recognition of CIC-DUX sarcomas will be requisite to make progress in this regard going forward. We report herein two cases showing metastatic progression with prolonged survival after combination chemotherapy (including the Ewing sarcoma regimen, VAC-IE) and radiotherapy, now without evidence of disease at 22 and 48 months. These outcomes suggest that a multidrug Ewing sarcoma-type regimen may be advantageous in these patients and call for larger studies of treatments and outcomes going forward.
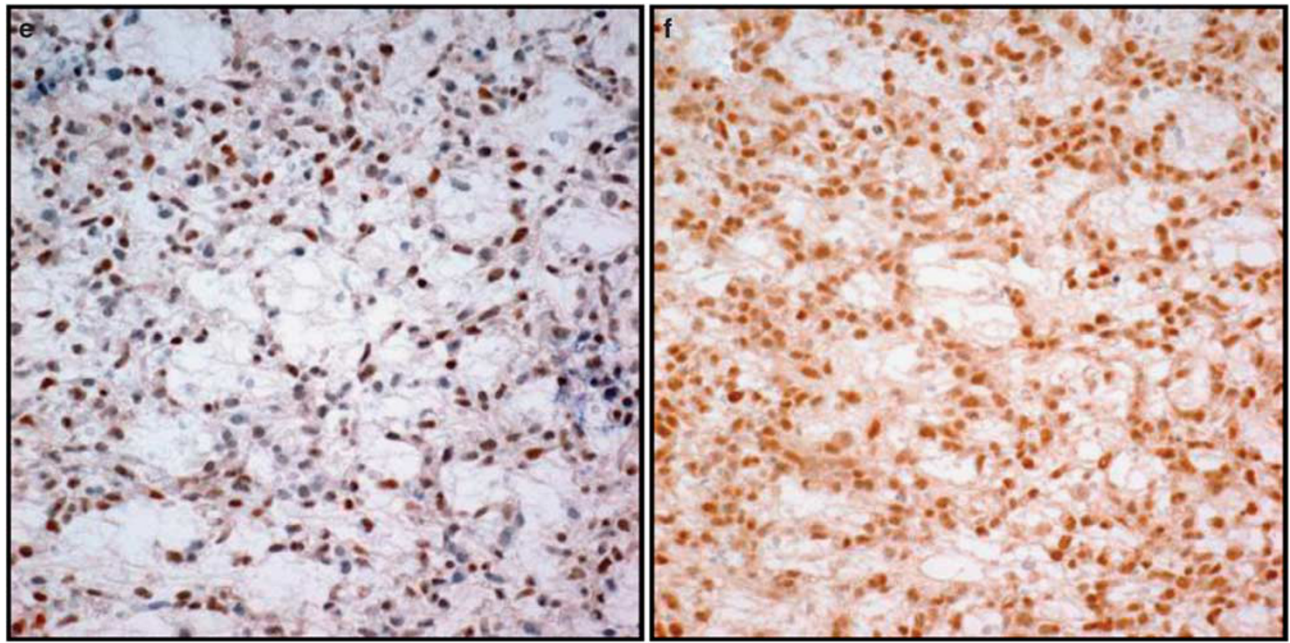
Figure 3 (Continued).

Table 3 Molecular and immunophenotypic features

Case	Lesion studied	Site	Chr.8, cytogenetics	Chr.8, FISH	MYC amplification	c-Myc IHC	ERG IHC	FLI1 IHC	CD99 IHC
1	Primary, Choi *et al*	Right calf	+ 8, Trisomy	NC	NC	2 +	2 +	2 +	1 +
2	Primary, Choi *et al*	Right gluteus/pelvis	+ 8, Trisomy	+ 8, Trisomy	+	3 +	2 +	3 +	1 +
3	Primary, Choi *et al*	Left shoulder	ND	−	+	3 +	3 +	3 +	2 +
4	Primary, Choi *et al*	Left knee	ND	−	+	3 +	3 +	3 +	1 +
5	Primary	Right knee	ND	+ 8, Trisomy	+	3 +	0	ND	ND
6	Primary	Right trapezius	ND	+ 8, Trisomy	−	3 +	2 +	ND	ND
7	Metastasis	Lung	ND	NC	NC	1 +	1 +	3 +	2 +
8	Primary	Left upper back	ND	+ 8, Trisomy	+	3 +	2 +	2 +	ND
9	Metastasis	Groin lymph node	+ 8, Trisomy	NC	NC	3 +	2 +	3 +	3 +
10	Primary	Right thigh	ND	+ 8, Trisomy	+	3 +	2 +	3 +	1 +

Abbreviations: Chr., chromosome; FISH, fluorescence *in situ* hybridization; IHC, immunohistochemistry; NC, non-contributory technical failure; ND, tissue insufficient—not done.

From the standpoint of histomorphology, our observations on newly collected cases confirm observations from our previously reported cohort[11] and further establish characteristic, if variable, histomorphological features. A distinctive and prevalent feature is the observation of myxoid change, which in some of the newly identified cases was extensive, resulting in some resemblance to soft tissue myoepithelial carcinoma or extraskeletal myxoid chondrosarcoma. Characteristic also is the patchy clear cell change, which ranged from focal to diffuse in the newly identified cases, apparent only in the metastasis of one case. We also observed areas showing spindled to fusiform cytomorphology, often associated with areas of myxoid stromal change, similar to that seen in myxoid synovial sarcoma,[21] which were not observed in our prior series. The nodular growth of sheets of high-grade cells with attenuated cytoplasm separated by fibrous septae is a consistent architectural feature. Nuclear morphology, including a vesicular nuclear appearance, often with readily identified nucleoli and mild-to-moderate pleomorphism, is distinctive as compared with Ewing sarcoma. For that matter, rosette formation was not identified in any case evaluated. Prominent
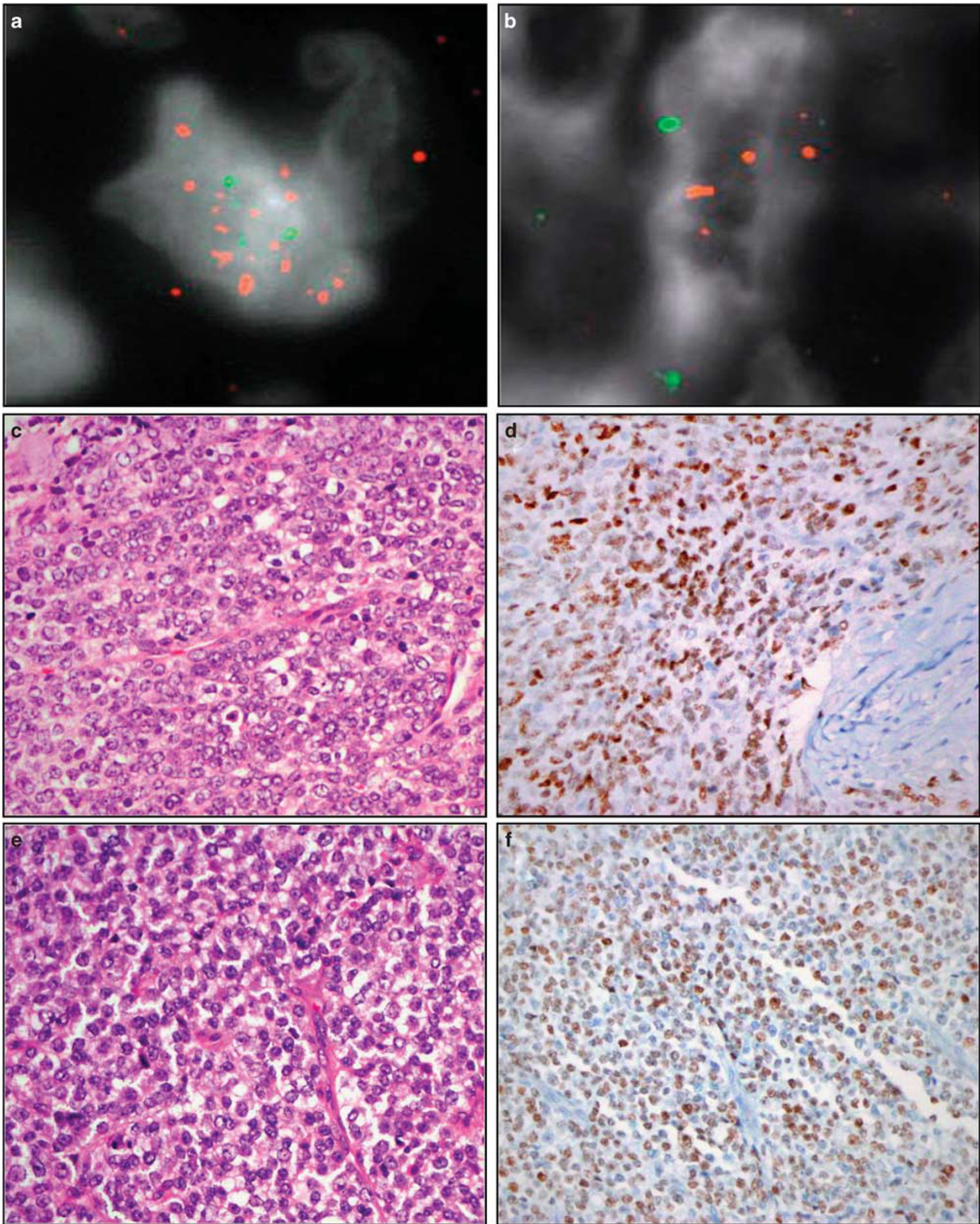
Figure 4 MYC amplification and overexpression. (**a**) Representative epifluorescence micrograph of Case 2 with chromosome 8 trisomy, indicated by three (green) centromeric probes, confirming the cytogenetic observation. The *MYC* locus is amplified (red). (**b**) Similar micrograph for case 8, which demonstrated disomy for chromosome 8 (green), still with *MYC* locus amplification (red). Both cases 2 (**c**, **d**) and 3 (**e**, **f**), demonstrated high-grade cytology and diffuse expression of MYC protein by IHC in >50% of lesional cells.
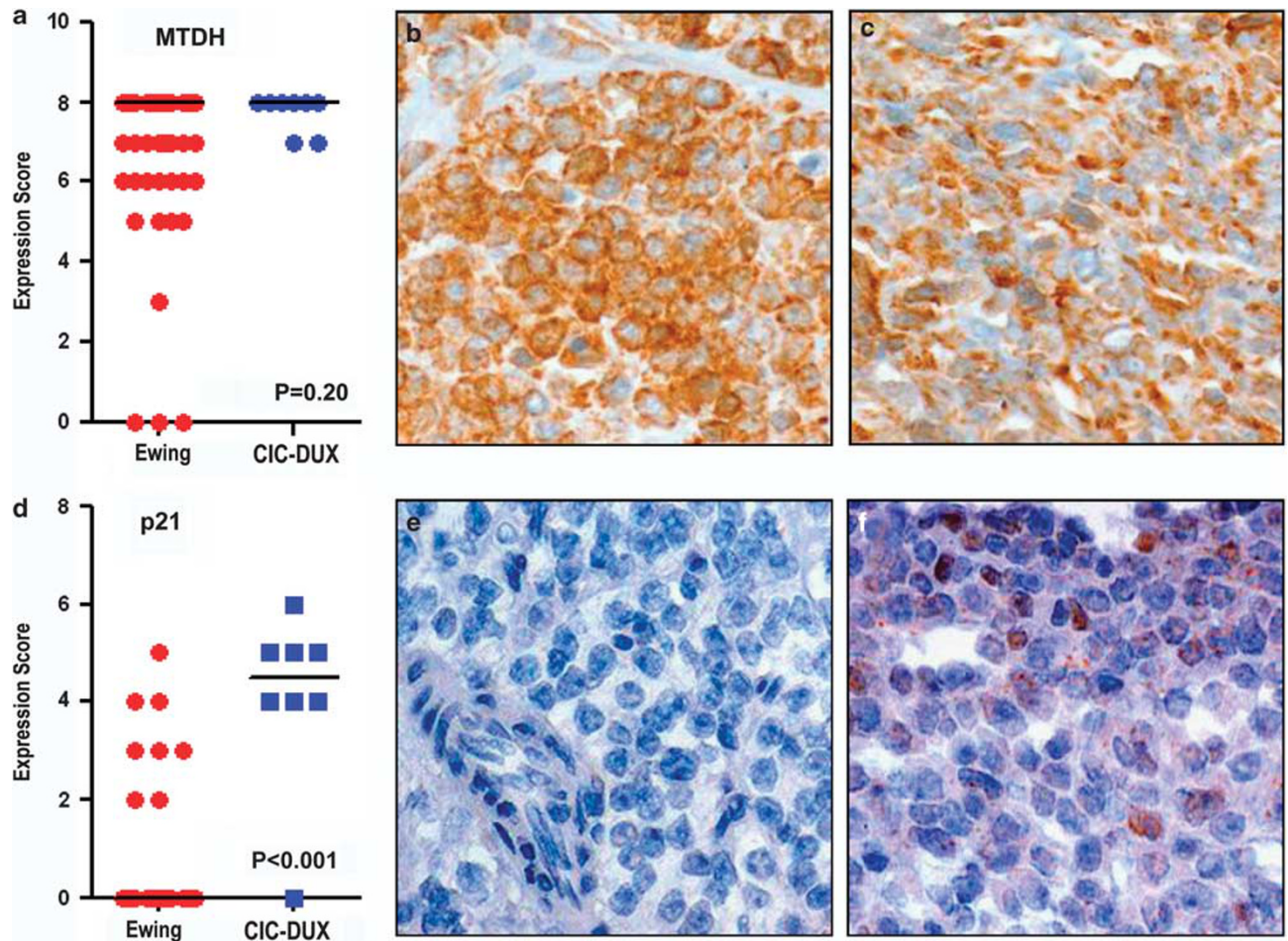
Figure 5 Differential expression of MYC targets MTDH and p21 in CIC-DUX sarcomas and Ewing sarcomas. (**a**) A dot plot comparing MTDH IHC scores in CIC-DUX sarcomas to Ewing sarcomas (median and Mann–Whitney *P*-value). Both entities show a similar pattern of membranocytoplasmic expression of this marker (**b**, representative Ewing; **c**, CIC-DUX). (**d**) Similar plot for p21, showing differential expression between these tumors, with representative micrographs showing lack of nuclear expression in Ewing (**e**) and patchy/subset expression in CIC-DUX (**f**).

geographic necrosis, often striking at low power, is a feature of the majority of cases; a rim of preserved tumor cells is often present around tumoral vasculature. Single-cell necrosis, imparting a 'starry sky' appearance,[12] was observed in these cases, as well.

The diagnosis of Ewing sarcoma of bone or extraskeletal soft tissues is clinical and morphological, assisted by adjunctive molecular studies. CD99 IHC, when positive with a diffuse membranous pattern, is supportive of a diagnosis of Ewing sarcoma. Although most cases of CIC-DUX sarcoma demonstrated some CD99 immunoreactivity, in our experience, the CD99 pattern in CIC-DUX sarcomas is different from that seen in Ewing sarcoma cases. We,[11] and others[2,5–8] have observed mostly focal to patchy staining, though our single CIC-DUX4L case with diffuse CD99 (although not membranous) is cautionary and correlates a with a few other reported CIC-DUX cases that showed diffuse CD99[2,3,6] immunoreactivity.

More specific to the molecular abnormalities seen in Ewing sarcomas is IHC for two members of the ETS family of proto-oncogenic transcription factors, ERG, and FLI1. FLI1 IHC has been used as supportive evidence of Ewing sarcoma.[22] More recently, a monoclonal antibody against the closely related ETS family member, ERG, in widespread clinical use as a specific marker of prostatic adenocarcinoma[23,24] and benign and neoplastic endothelium,[25] has shown cross reactivity with FLI1, and hence promise in diagnosis of Ewing sarcomas harboring FLI1[13] or ERG rearrangement,[26] depending on the dilution used. Although nuclear pleomorphism, nucleoli, geographic necrosis, spindling, clear cell change, and, especially, myxoid change are not typically seen in Ewing sarcoma, the two tumors may be difficult to distinguish in small samples. We observed positivity for FLI1 and/or ERG in essentially all of the CIC-DUX sarcomas studied, a finding similar to that expected in Ewing sarcoma, and therefore, a relevant and significant pitfall in diagnosis.

The observations of FLI1 and ERG immunoreactivity in our CIC-DUX4 sarcomas corroborate those

noted in previous cases,[4,6] as well as a very recent report that these sarcomas very frequently coexpress nuclear WT1 and ETS-family factors,[27] the former overlapping with the immunophenotype of desmoplastic small round cell tumor. Although such immunostains may have a role for triage of limited biopsy samples, we regard confirmation of a morphological diagnosis of Ewing sarcoma of bone or extraskeletal soft tissue (or exclusion of Ewing in a possible CIC-DUX case) to be best accomplished through molecular testing for *EWSR1* rearrangement. Also notable are recently reported findings of strong induction of expression of other ETS-family transcription factors, including ETV1, ETV4 (PEA3), and ETV5, in gene expression profiling studies.[8,27] This observation implies testable hypotheses regarding the oncogenic mechanism of the CIC-DUX fusion transcripts. We are exploring the potential for use of these additional ETS members as diagnostic adjuncts by RNA *in situ* hybridization.[28]

On the basis of observation of additional copies of chromosome 8 in the karyotypes in three of our cases, we hypothesized that *MYC* amplification might be important in the development of CIC-DUX sarcomas. We observed polysomy for chromosome 8 in the majority of our cases, with amplification of the *MYC* locus in 6/7 and MYC protein overexpression in all cases tested. Although this is not entirely surprising given the high prevalence of MYC amplification or overexpression in high-grade carcinomas, lymphomas, and sarcomas,[29–33] this observation may have potential therapeutic implications, in light of a number of emerging strategies to target this pathway.[29]

The differential expression of two well-studied MYC targets, p21[18] and MTDH[17] in CIC-DUX sarcomas as compared with Ewing sarcomas is also worth noting. Expression of MTDH (*Metadherin/AEG-1/Astrocyte Elevated Gene 1/Lyric*), which we found to be a shared and prevalent feature of both CIC-DUX sarcomas and Ewing sarcoma, may be important mechanistically and as a therapeutic target. A growing body of literature implicates MTDH as a specific mediator of lung metastasis in breast adenocarcinoma,[34] a provocative finding in light of the frequent lung metastasis among the CIC-DUX sarcomas (and, for that matter, among Ewing sarcomas). Expression of MTDH has been recently noted as a poor prognostic factor in Ewing sarcoma and other sarcomas,[35,36] and proposed as a targetable cell surface marker.[37] In contrast, we found that expression of p21 was significantly greater in CIC-DUX sarcomas. Though only a subset of nuclei in these tumors was positive for p21, this observation identifies another difference in cell signaling between CIC-DUX and Ewing sarcomas despite their shared expression of, and implicitly, shared dependency on, oncogenic ETS transcription factor signaling.

In summary, based on the findings of an expanded set of CIC-DUX sarcoma cases, we have validated prior observations of characteristic demographic features, aggressive clinical behavior, and histomorphology. Recognition of this entity is clinically relevant, and we highlight prevalent ERG and FLI1 positivity as an important consideration during diagnostic workup. Finally, prevalent *MYC* amplification with differential expression of downstream targets may represent a therapeutically targetable oncogenic pathway in CIC-DUX sarcomas, but additional studies are needed.

Acknowledgments

Disclosure/conflict of interest

The authors declare no conflict of interest.

Note added to the proof

Following acceptance of this manuscript, Specht *et al*. reported similar findings regarding FLI1, ERG, and CD99 expression in their cohort of 21 CIC-DUX sarcomas (Specht K, Sung YS, Zhang L, *et al*. 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 2014;53:622–633). Though ERG expression (assayed with more dilute antibody) was less prevalent than we observed, they also further report prevalent expression of WT1. Together, these reports amplify the distinctive features of CIC-DUX sarcomas and argue for their prospective recognition as a new class of undifferentiated small round-cell sarcomas.

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