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Superficial Sarcomas with *CIC* Rearrangement Are Aggressive Neoplasms: A Series of Eight Cases

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Running title: Superficial CIC-rearranged sarcoma

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ABSTRACT

CIC rearranged sarcomas have significant overlap with Ewing sarcoma, are aggressive, and typically present in deep soft tissue. They most commonly have a t(4;19)(q35;q13) with *CIC-DUX4* fusion. Superficial presentation is rare. We report eight (6F, 2M; median 45-years-old, range 14-65) superficial *CIC*-rearranged sarcomas, involving the extremities (n=4), vulva (n=2) and trunk (n=2). The tumors were composed of nodules/sheets of round cells with necrosis and hemorrhage separated by dense hyalin bands. Tumor cells had vesicular chromatin, prominent nucleoli and frequent mitoses. One showed pagetoid spread. Targeted next-generation sequencing was positive for *CIC-DUX4* fusion (6/6); FISH was positive for *CIC* rearrangement (2/3). 8/8 had evidence of *CIC-DUX4* fusion/rearrangement by molecular techniques. Immunohistochemistry was positive for CD99+ (8/8) and DUX4+ (4/4). FISH for *EWSR1* rearrangement was negative (5/5). Of 5 patients with at least 6 months follow-up, 3/5 died of disease, all within 2 years of presentation. One is alive with disease at 48 months. One is disease free at 3 months. Superficial *CIC*-rearranged sarcomas should be considered in cases exhibiting features reminiscent of Ewing

sarcoma, but with increased pleomorphism and/or geographic necrosis. In contrast to superficial Ewing sarcomas, superficial *CIC*-rearranged sarcomas are aggressive.

INTRODUCTION

Poorly differentiated round cell tumors of the skin are rare, with the main differential diagnoses including lymphoma, Merkel cell carcinoma, melanoma, rhabdomyosarcoma and undifferentiated small round cell sarcoma[1]. The last group includes Ewing sarcoma and morphologically similar *EWSR1*-gene fusion negative small round cell tumors recently discovered to harbor gene fusions in *CIC-DUX4, BCOR-CCNB3*, and *FUS-ERG* [2-8] and several other rare gene fusions[9,10].

Sarcomas with *CIC* rearrangements typically harbor a recurrent t(4;19)(q35;q13) or t(10;19)(q26;q13) translocation involving the *CIC* gene on chromosome 19, fused with either *DUX4* on chromosome 4 or, less commonly, its paralog, *DUX4L*, on chromosome 10. Less frequently, inversion within the *CIC* gene is observed. Originally described in the pediatric population, *CIC*–rearranged sarcomas have since been reported over a wide age range, with the

majority in young adults. Importantly, they nearly always arise in the deep soft tissue and behave aggressively. Common morphological features include septate growth, necrosis, vesicular chromatin, areas of clear cell change, and CD99, WT1, FLI1 and ERG immunoreactivity[11-14]. Rare case reports of tumors presenting above the fascia or in the viscera exist, but these reports lack clinical follow-up[15, 16].

We describe the clinical, histologic and molecular features of eight cases of primary superficial *CIC*-rearranged sarcoma. Rearrangements of *CIC* were detected by targeted next generation sequencing (NGS) in six cases and fluorescence *in situ* hybridization (FISH) using break apart probes in two cases. The clinical, histologic, and discriminating immunohistochemical and molecular features and differential diagnosis are discussed. Importantly, this series confirms the aggressive nature of this rare malignancy, even when it presents as a superficial tumor in the dermis or subcutis.

MATERIALS AND METHODS

Cases were received in consultation to the Dermatopathology or Soft Tissue Pathology services, or following surgical treatment, at the authors' institutions between 2015 and 2019. Clinical data related to the patient age and gender, and the tumor size and location were recorded from initial reports. Other medical information, including follow-up data, was obtained from the contributing pathologists or clinicians. The study was conducted under institutional review board approval.

Immunohistochemical Staining

The cases were received with hematoxylin and eosin stained slides and various immunohistochemical stains performed by the referring institutions. Additional studies were performed at the authors' institutions. 4-µm formalin-fixed, paraffin-embedded tissue sections were stained with hematoxylin and eosin, and for immunohistochemistry using the Ventana Benchmark Ultra automated immunostainer (Ventana Medical Systems (VMS), Tucson AZ) and revealed in brown with the UltraView Universal DAB Detection kit (Ventana). Sections were treated with fully automated deparaffinization, followed by automated epitope retrieval. Localization of the antigen-antibody complex was achieved using the VMS OptiView DAB detection kit with peroxidase and diaminobenzidine tetrahydrochloride (DAB) chromogen. Immunohistochemical stains, either performed at the referring institution and provided with the case, or additionally performed at the senior author's institution included (details given for those performed by authors): S100 protein (polyclonal; dilution 1:200; Dako, Carpinteria, CA), Sox10, Melan A, HMB45 (monoclonal; predilute; Biogenex, Fremont, CA), pancytokeratin, CK7, CK20, AE1/3 (monoclonal, 1:200, Milipore, Burlington, MA), CAM5.2, EMA (monoclonal; 1:50; Dako), Ber-EP4 (monoclonal; 1:10; Dako), p63, ERG (monoclonal; 1:100; Abcam, Cambridge, UK), FLI-1, CD31 (monoclonal; 1:20; Dako), CD34 (monoclonal; predilute; Cell Marque, Rocklin, CA), SMA (monoclonal; 1:50, Dako), Desmin (monoclonal; 1:10; Dako), myogenin, caldesmon, Calretinin, INI1 (monoclonal; 1:250; BD Bioscience, San Jose, CA), WT1, DUX4 (mouse monoclonal P4H2 from ThermoFisher, cat# MA5-16147, Ventana Medical Systems (VMS) OptiView DAB detection; heat-induced epitope retrieval (HIER) with CC1 (high pH

buffer) for 16 minutes; incubate DUX4 (1:400) for 16 minutes with no heat), PAX5, PAX8 (polyclonal; 1:200; Protein Tech Group, Rosemont, IL), TTF1 (monoclonal; predilute; Ventana, Tucson, AZ), STAT6, MDM2, DOG1, androgen receptor (monoclonal; 1:10; Dako), ER, FactorXIIIa, glypican-3, PD-1L, Napsin A, vimentin, CD99 (monoclonal; predilute; Cell Marque, Rocklin, CA), CD68, CD56, CD10, CD45 (monoclonal; 1:100; Dako), CD33, CD117, CD3, CD20, CD30 (monoclonal; predilute; Ventana), CD43, and TdT.

Fluorescence *in Situ* Hybridization

Molecular evaluation for *EWSR1* (22q12) gene rearrangement was performed using FISH on interphase nuclei present on formalin fixed paraffin embedded sections. Briefly, 4µm thick paraffin sections were baked at 60°C overnight, deparaffinized and subjected to proteinase K digestion. Slides were washed ani probe applied to each slide with overnight hybridization. Slides were counterstained with 4',6-diamidino-2-phenylindole and signals visualized on an Axioskop photomicroscope (Zeiss, Oberkochen, Germany). The FISH probe to detect the presence of an *EWSR1* (22q12) rearrangement is a dual color, break apart probe consisting of a mixture of FISH DNA probes on the centromeric and the telomeric sides of the *EWSR1* gene breakpoints (Abbott Molecular, Des Plaines, IL). Signals from 200 nonoverlapping nuclei were counted in each case. Spacial separation of the two differently colored probes is indicative of a rearrangement/translocation involving *EWSR1*. Ten percent or more of the neoplastic cells must show rearrangement of the gene region to be interpreted as a positive result.

FISH to detect *CIC* rearrangement was performed similarly as previously described.[13,14] Briefly, rhodamine labeled BAC clone RP11-569M1, spanning the *CIC* gene at 19q13, and fluorescein-labeled RP11-46I12, a centromeric control marker for chromosome 19 was used, along with a second 'break apart' probe sets 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).

RNA Sequencing

RNA sequencing studies were performed using a custom-designed Archer® FusionPlex® sequence assay, with probes for fusions of 34 known sarcoma-associated genes. Five to ten unstained slides and 1 H&E stained slide from neutral buffered formalin-fixed paraffin-embedded (FFPE) tissue were obtained from each specimen. The area of interest was circled and the percentage of tumor cells noted by a pathologist, avoiding necrosis or extensive hemorrhage. Sections were deparaffined with xylene, then underwent microdissection using a light microscope. Total nucleic acid (TNA) extraction was performed using the Maxwell® RSC RNA

FFPE Kit (Promega cat# AS1440, Madison, WI) and protocol on the Maxwell® RSC instrument. Extracted TNA was quantified using both the Qubit RNA HS Assay Kit (Invitrogen cat#Q32852, Carlsbad, CA) and Nanodrop 8000 Spectrophotometer (ThermoFisher, Carlsbad, CA). Optimally, 200 ng of extracted TNA in 20uL of ultra-pure water was used (minimal input 50 ng TNA). Extracted TNA was converted to cDNA, evaluated using the Archer PreSeq RNA QC assay, a qPCR method to assess initial RNA quality. cDNA libraries were made using anchored multiplex polymerase chain reaction (Archer® FusionPlex® standard protocol and reagents, Archer DX, Inc., Boulder, CO) and the custom designed Gene-Specific Primer (GSP) pool, targeting 34 genes involved in soft tissue neoplasms. This custom panel uses 242 unidirectional GSPs, designed by Archer, to enrich TNA for known and unknown fusion transcripts. Sequencing was performed on an Illumina® MiSeq™ (San Diego, CA) instrument with 150x2 cycle pair-end reads for a depth of >500,000 total reads. An in-house informatics pipeline and Archer analysis software (Version 6.0.3.2) was used for read alignment (genome build hg19/GRCh37), fusion gene identification and annotation. Fusions were considered reportable if the gene fusion had a minimum of five unique reads spanning the fusion junction, at least three unique start sites and at least 10% of reads surrounding the break point supporting the fusion event. All technical quality data and potential fusion reads were inspected by molecular pathology professional staff prior to clinical reporting.

RESULTS

Clinical Features

Eight cases were received in consultation which histologically resembled Ewing sarcoma. The clinical features of the patients are listed in **Table 1**. There were two men, five women, and one girl (2M:6F). Age at presentation ranged from 14 to 65 years (median 45 years). The sites involved were distal upper extremity (2), labia/vulva (2), flank (2) and distal lower leg (1) and foot (1). The clinical impression was typically non-specific ("mass") except in a single labial/vulvar case, when "Bartholin's gland cyst" was the suspected diagnosis. Each of the lesions were sampled with an incisional biopsy, and each extended to the biopsy margins. In every case, complete surgical excision was recommended and completed, along with close clinical follow-up. One patient received adjuvant radiation therapy following surgery. Of the five cases with reasonable follow-up (at least 6 months), 3/5 died of disease at between 22 and 24 months. Lungs were the most common site of metastasis, but lymph node and subcutaneous metastases were also commonly observed at the time of disease progression. 1/5 patients is alive with metastatic disease (lungs) at 48 months (received adjuvant radiation) and 1/5 is alive without disease at three months.

Pathologic Features

The tumors ranged from 0.5 to 8 cm in greatest dimension (median 2.5 cm). Representative photomicrographs from selected cases are shown in **Figures 1-3**. Histologically, tumors involved the superficial to deep dermis with extension into the subcutis. They were comprised of nodules, islands, and sheets of uniform round cells with prominent geographic necrosis in a hyalinized stroma. Hemorrhage and cystic degeneration was frequent. Ulceration of the

overlying epidermis was seen in larger tumors and one case demonstrated pagetoid spread of adjacent epidermis. Tumor cells were small to medium in size, with minimal clear to pale cytoplasm, and oval to round nuclei, with vesicular to at times coarse chromatin and prominent nucleoli. The mitotic rate was uniformly high (>10/10 HPF) and single tumor cell apoptosis and necrosis was observed in all cases. The leading histological differential diagnoses in most cases included: Ewing sarcoma, melanoma, epithelioid sarcoma, malignant PEComa, and angiosarcoma.

Immunohistochemical Results

Pertinent results obtained from immunohistochemical stains performed in each case are displayed in **Table 2**. All cases were predominantly negative for markers of epithelial origin, including various cytokeratins such as cytokeratin AE1/3, as well as EMA and P63. One case (of 6 total tested) that showed focal positivity for CK7. All markers of smooth or skeletal muscle origin (SMA, desmin, caldesmon, myogenin) were negative when tested, as were melanocytic/neural markers (Sox10 and S100). INI1 nuclear staining was retained. CD99 staining was positive in a membranous fashion in 8/8 cases, and only focally positive in 1/8 cases. WT1 and CD56 were uniformly positive when tested. ERG and CD31 staining was variable. DUX4 immunohistochemical staining was employed in cases 5-8, and showed diffuse nuclear positivity in all four cases (4/4).

Molecular Pathology Results

Targeted genomic interrogation of the tumors' genomes was performed in all eight cases. The findings are presented in **Table 3**. In most cases, FISH for *EWSR1* gene region rearrangement was performed, and it was negative in each case (5/5). In 3 of 8 cases, FISH for *CIC* gene region rearrangement was performed. Cases 3 and 4 were positive, while Case 1 yielded a false negative result. Subsequent targeted next generation sequencing, however, was positive in Case 1. All cases tested by targeted NGS (6/6) were positive for *CIC-DUX4* fusion.

DISCUSSION

Recent molecular advances have led to the identification of a novel undifferentiated round cell sarcoma with "Ewing-like" features characterized by *CIC* rearrangement - most commonly the *CIC-DUX4 or CIC-DUX4L* fusion, as defined in the 4th edition of the WHO Classification of Tumors of Soft Tissue and Bone[17]. In the largest cohort reported thus far[12], these sarcomas were shown to have a relatively uniform clinical, morphologic and immunophenotypic presentation. Noted features included presentation in young adults (mean age 32 years), slight male predominance, localization to the soft tissue (86%, equally in extremities and trunk), with less frequent presentation in the viscera or bone.

The present study describes eight new cases of cutaneous *CIC*-rearranged sarcomas. The proportion of reported tumors located above the fascia is thus far very low, and the extent of cutaneous involvement is unclear in the literature. As such, it has been unclear whether prognosis is improved by superficial presentation, as seen in certain other cancer types, including Ewing

sarcoma and clear cell sarcoma. The data herein suggests that superficial presentation in the setting of *CIC*-rearranged sarcomas does not impact clinical disease course; 3/5 patients with adequate follow-up died of their disease, and an additional patient is currently living with metastatic disease. Interestingly, the patient with a significantly delayed recurrence also received radiation therapy, suggesting radiation may have a role in adjuvant treatment.

Microscopically, the histomorphologic and immunophenotypic features in our cases were similar to those reported in deeper locations. The most important entities to include in the differential diagnosis include lymphoma, Merkel cell carcinoma, melanoma, rhabdomyosarcoma and Ewing sarcoma. While the rest can be readily excluded by immunohistochemical stains, differentiating Ewing sarcoma from *CIC*-rearranged sarcoma represents more of a challenge due to overlap in the immunohistochemical profile. Distinction is important, as Ewing sarcoma carries a significantly better prognosis, particularly in the superficial location, as evaluated by Collier *et al*.[18] In a cohort of 78 patients, only two (2.5%) presented with evidence of metastatic disease, compared to 23% of deep cases, while 62 of 78 patients (88.5%) had no evidence of disease at a mean follow-up of 4.4 years. Histologically, there are features that help distinguish *CIC*-rearranged sarcomas from Ewing sarcoma. *CIC*-rearranged sarcomas tend to have a more heterogeneous cytology than Ewing sarcoma, including spindled, rhabdoid, plasmacytoid, epithelioid and round cell forms; and, tumor cells show increased nuclear size and shape variability with myxoid stroma commonly observed at least focally[12].Geographic necrosis is also a feature common to *CIC*-rearranged sarcomas that is not typically seen in Ewing sarcomas. CD99 immunoreactivity, as shown in our

cases, overlaps with Ewing sarcoma, even though some studies highlight a weaker CD99 staining pattern in *CIC* rearranged sarcoma.[19] Strong nuclear WT-1 staining, as demonstrated in case 3, is also finding in contrast with Ewing sarcoma.[19] Worth noting is the patchy strong nuclear ERG positivity in two of our cases. These findings are consistent with those noted in previous cases, yet they are not specific[20]. Patchy expression of ERG, with areas of hemorrhage, may prompt consideration of angiosarcoma, and this pitfall can be avoided by using a battery of endothelial markers. Finally, nuclear staining with DUX4 antibody, was previously shown to be highly sensitive (5/5, 100%) and specific (0/76, 100%) for diagnosing *CIC*-rearranged sarcoma.[21] Our results are consistent with this finding, though this should be confirmed with a larger cohort before relying solely on immunohistochemical stains for diagnostic confirmation.

Interestingly, *CIC* break apart FISH has recently been shown to miss a significant subset of CIC-DUX4 sarcomas[22], for reasons which are not well characterized. This may explain the negative result in case 1, and gives additional weight to using other testing modalities in cases with unexpected negative results. In our experience targeted NGS shows good sensitivity, but a subset of *CIC*-rearranged sarcomas may also be negative by this technique[23]. CIC-rearranged sarcomas demonstrate up regulation of *ETV1/4/5* and immunohistochemistry for ETV4 may also be an effective diagnostic tool, especially if there are negative molecular results in a tumor suspected to be a *CIC*-rearranged sarcoma based on morphologic features [24,25].

In conclusion, primary superficial presentation of *CIC*-rearranged sarcoma should be considered in any dermal-based poorly differentiated round cell tumor, especially those with features of

Ewing sarcoma, but additionally harboring more pleomorphism, necrosis, and/or lack of *EWSR1* rearrangement. Less robust staining for CD99 may also be a clue in some cases. The morphologic features and clinical biologic course of primary superficial *CIC*-rearranged sarcoma appears to parallel that seen in deeply seated tumors, which contrasts with many other sarcomas arising in the skin. In contrast to most superficial Ewing sarcomas, most superficial *CIC*-rearranged sarcomas appear to behave in an aggressive fashion. Detailed histologic assessment and targeted immunohistochemical and molecular studies are important in confirming the diagnosis. For molecular confirmation, next generation sequencing appears superior to FISH. Patients require treatment with a wide excision; and, adjuvant treatment such as chemotherapy with radiation may be of some benefit.

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FIGURE LEGENDS

Table 1. Clinical features of patients in series.

Figure 1. Case 1. A. Gross tumor shows a purpuric nodule with superficial erosion and serous crust. **B.** Tumor cross section demonstrates a hemorrhagic tumor surface and the superficial dermal origin with pushing into the subcutis. **C.** At low magnification, there are multiple tumor nodules, sheets and geographic shapes in a hyalinized stroma with attenuation and ulceration of the overlying epidermis and hemorrhage (H&E, 20X). **D.** Geographic and single tumor cell necrosis is prominent. Neoplastic cells are small to medium sized with pale eosinophilic to clear cytoplasm, and round to oval nuclei with vesicular chromatin and variably

prominent nucleoli (H&E, 200X). **E.** Focal intraepidermal tumor spread was noted (H&E, 200X). **F.** CD99 staining was membranous and diffuse to patchy (20X).

Figure 2. Case 5. A-B. As in the other cases, the tumor was composed of small, undifferentiated round cells in nodules and sheets with necrosis (H&E, 20X and 200X). **C-D.** This case also exhibited a spindle cell component with areas of fascicular and reticular growth (H&E, 400X).

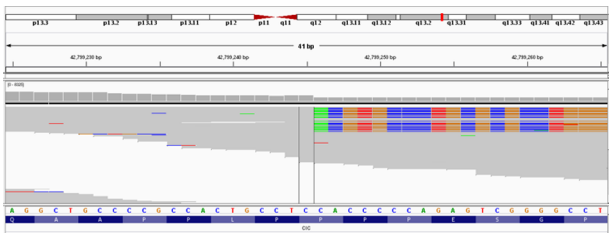
Figure 3. A. Case 6. Occasional microcystic and corded architecture (H&E, 200X) noted. **B-C. Case 3.** Foci of rhabdoid and plasmacytoid features (H&E, 40X and 200X). **D.** A representative image of DUX4 immunohistochemical staining is from case 7 (200X).

Figure 4. A. NGS reads aligning to *CIC*. Perfectly aligned reads (no variants) are in gray, while nucleotides not corresponding to the *CIC* reference sequence are colored (denoted as "soft clipped reads"). **B.** Soft clipped reads align to exon 1 of *DUX4*.

Table 2. Pertinent IHC results are included in table. Additional immunohistochemical stains which were variably performed and were uniformly negative including: S100 protein, Sox10, Melan A, HMB45, cytokeratins (unless specified in table), myogenin, caldesmon, CK20, CEA, PAX8, TTF1, AE1/3, Ber-EP4, STAT6, MDM2, DOG1, androgen receptor, ER, CD34, CD10, FactorXIIIa, FLI-1, glypican-3, PD-1L, NapsinA, CD68 and CD45. Nuclear INI-1 expression was retained. Case 3 showed positive staining for CD33, CD117, and focal Pax-5; however

CD3, CD20, CD30, CD34, CD43, CD45, TdT, MPO and lysozyme staining was negative. Vimentin and PAS (cytoplasmic) were positive when tested.

Table 3. Molecular results from cases in series. Break apart fluorescence in situ hybridization (FISH) assays for rearrangements involving *EWSR1* and *FUS* were negative in Cases 1-4 and 6. In case 2, *WWTR1-CAMTA1* gene fusion was also negative by FISH. In case 1, FISH testing for *CIC* rearrangements was negative. In cases 3-4. FISH testing was positive for *CIC* rearrangement. In case 1-2 and 5-8, our custom-designed Archer® FusionPlex® targeted sequence laboratory developed test was utilized to probe for fusions of 34 known sarcoma-associated rearrangements, and revealed a positive t(4;19)(q35;q13) *CIC-DUX4* fusion. Results were confirmed with RT-PCR in Cases 1 and 2.



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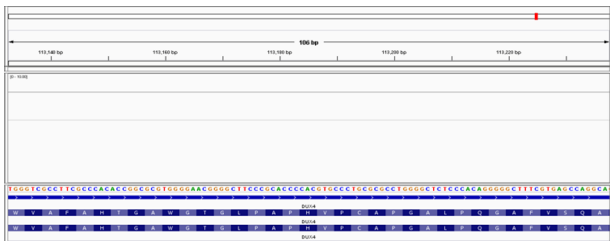

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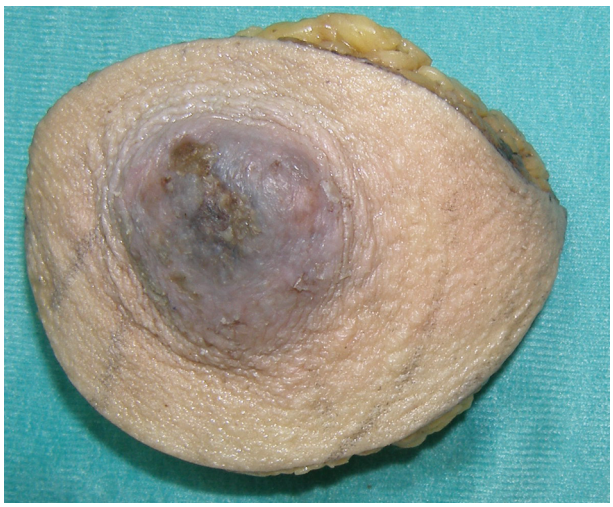

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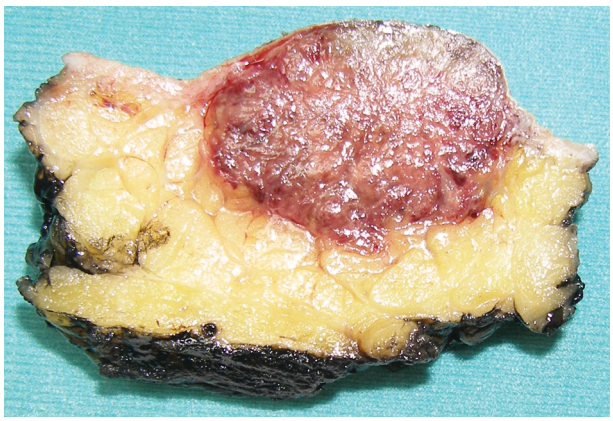

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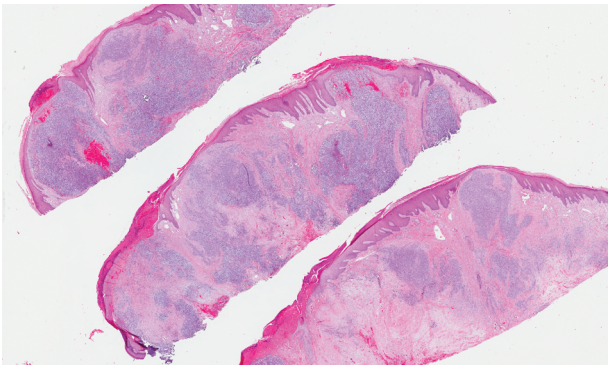

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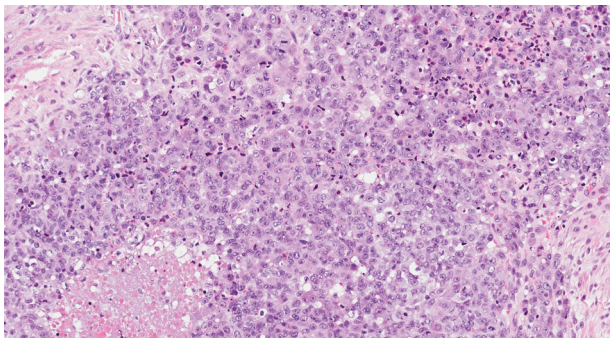

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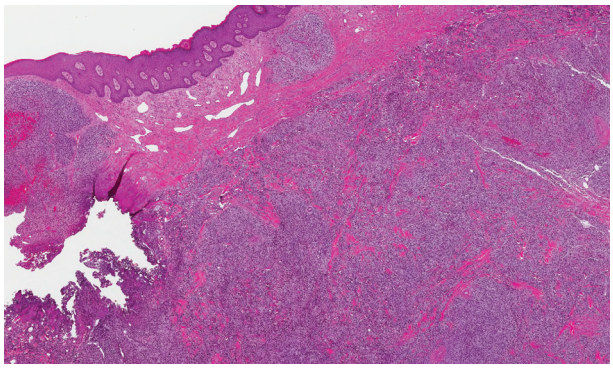

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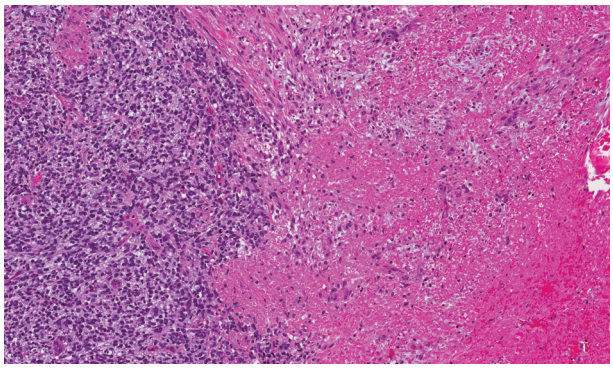

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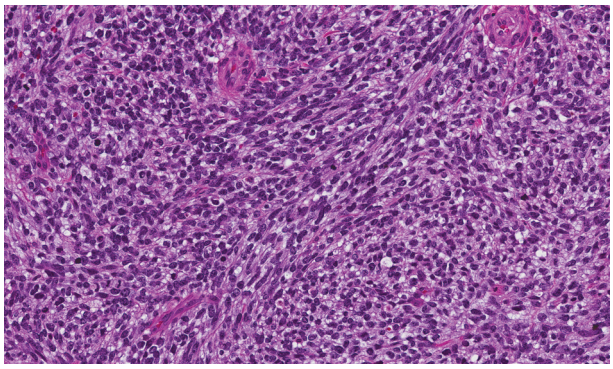

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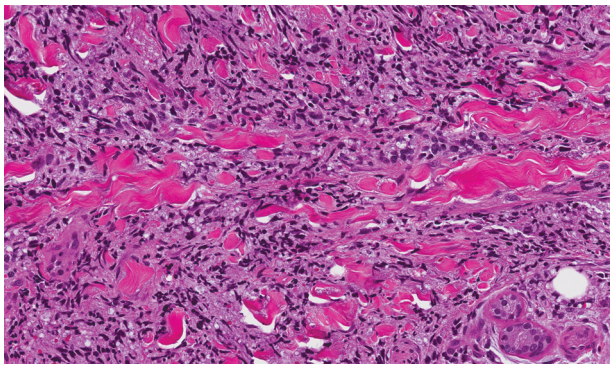

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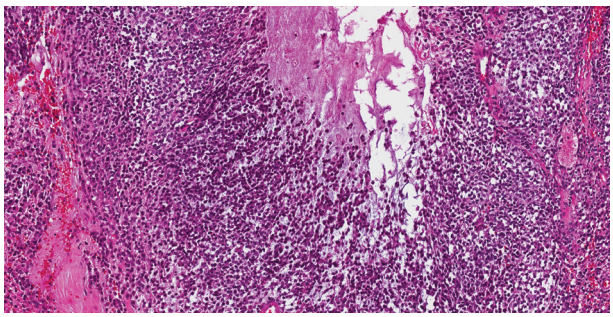

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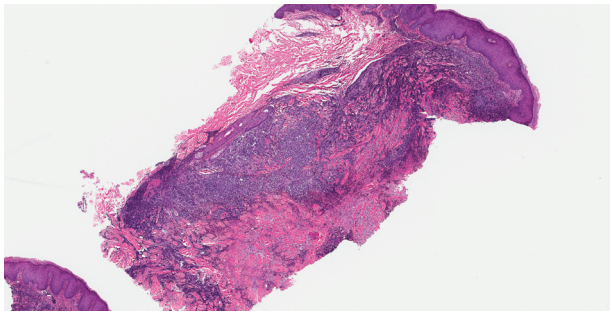

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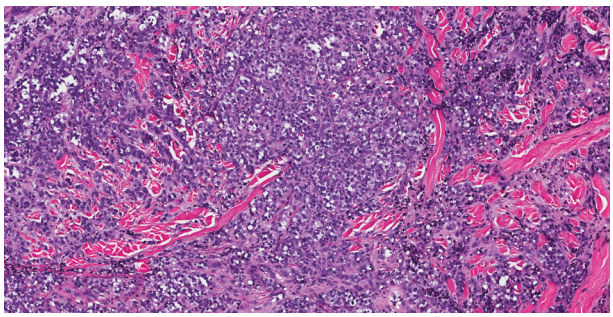

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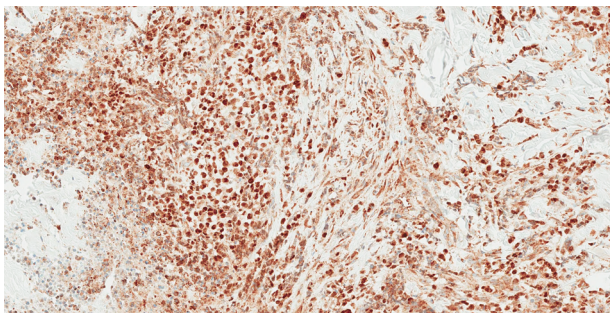

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Superficial Sarcomas with *CIC* Rearrangement Are Aggressive Neoplasms: A Series of Eight Cases

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Running title: Superficial CIC-rearranged sarcoma

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