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ZFP64::NCOA3 GENE FUSION DEFINES A NOVEL SUBSET OF SPINDLE CELL RHABDOMYOSARCOMA

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Abstract

Spindle cell rhabdomyosarcoma represents a rare neoplasm characterized by monomorphic spindle cells with a fascicular architecture and variable skeletal muscle differentiation. Following incidental identification of a *ZFP64::NCOA3* gene fusion in an unclassified spindle cell sarcoma resembling adult-type fibrosarcoma, we performed a retrospective archival review and identified four additional cases with a similar histology and identical gene fusion. All tumors arose in adult males (28–71 years). The neoplasms were found in the deep soft tissues; two were gluteal, with one each arising in the thigh, abdominal wall, and chest wall. Morphologically the tumors were characterized by monomorphic spindle cells with a distinctive herringbone pattern and variable collagenous to myxoid stroma. The nuclei were relatively monomorphic with variable mitotic activity. Three tumors had immunoreactivity for MyoD1, and four contained variable expression of desmin and smooth muscle actin; all cases tested for myogenin, CD34, S100, pankeratin and epithelial membrane antigen were negative. Targeted RNA sequencing revealed a *ZFP64::NCOA3* fusion product in all five tumors. Three patients developed distant metastases, and two ultimately succumbed to their disease within two years of initial diagnosis. This study suggests *ZFP64::NCOA3* fusions define a novel subtype of rhabdomyosarcoma with a spindle cell morphology and aggressive clinical behavior. The potential for morphologic and immunohistochemical overlap with several other sarcoma types underscores the value of molecular testing as a diagnostic adjunct to ensure accurate classification and management of these neoplasms.

Keywords

ZFP64; NCOA3; soft tissue; spindle cell; rhabdomyosarcoma; fibrosarcoma; fusion

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INTRODUCTION

The widespread application of next-generation sequencing has facilitated the molecular subclassification of spindle cell rhabdomyosarcoma through the identification of various disease-defining alterations. These tumors are genetically diverse, and exhibit some variability based on patient age and anatomic location. They may harbor point mutations (e.g., *MYOD1*), particularly in older children and adults.[1,2] They may also contain various gene fusions, which are enriched in congenital tumors and those diagnosed within the initial years of life (e.g., *VGLL2::CITED2*, *SRF::FOXO1*, *SRF::NCOA1/2*, *TEAD1::NCOA2*, *VGLL2::NCOA2*).[3–6] Primary intraosseous rhabdomyosarcoma – which is less common, and often associated with a spindle cell morphology – also contains gene fusions (e.g., *MEIS1-NCOA2, FUS/EWSR1::TFCP2*).[7–10]

Herein we report five spindle cell rhabdomyosarcomas with a distinctive herringbone pattern resembling so-called adult-type fibrosarcoma. Immunohistochemistry, however, revealed patchy immunoreactivity for desmin and MyoD1 in most tumors, thereby favoring classification as rhabdomyosarcoma. Next-generation sequencing identified a *ZFP64::NCOA3* gene fusion in all cases. This molecular event is presumed to represent a genetic driver, and this observation suggests a genetically distinct subtype of spindle cell rhabdomyosarcoma.

MATERIALS AND METHODS

Case Selection

Following incidental discovery of a neoplasm with a *ZFP64-NCOA3* gene fusion in the course of routine diagnostic testing, retrospective pathology database reviews were performed to identify other tumors previously found to harbor fusions involving this gene (BCD, CRA). This study was performed with Research Ethics Board approval.

Immunohistochemistry

Staining was performed on a Dako OMNIS (Aligent, Santa Clara, CA) using standard techniques for CD34 (QBEnd/10; Roche), desmin (D33; Dako), epithelial membrane antigen (E29; Roche), keratin (AE1/AE3; Dako), MyoD1 (5.8A; Dako), myogenin (MyG007; Biocare), smooth muscle actin (1A4; Dako), S100 (polyclonal; Dako). On-slide positive controls were applied throughout.

RNA-Sequencing

Scrolls obtained from formalin-fixed paraffin-embedded tissue blocks were cut (4 at 10 microns) into Eppendorf tubes. RNA was extracted using ExpressArt FFPE Clear RNA Ready kits (Amsbio, Cambridge, MA). Cases 1–2, and 4: RNA-seq libraries were prepared with the TruSight RNA Fusion Panel (Illumina, San Diego, CA), as previously described.[11] Cases 3 and 5: libraries were prepared using the Archer FusionPlex™ assay (Enzymatics Inc, Beverly, MA), as previously described.[12]

RESULTS

A total of five tumors were identified; the demographic and clinical details are summarized in Table 1. The average patient age was 40 years (range, 28–71), and all were male. Patient 1 presented with pain in the gluteal region following exertion; a mass rapidly developed over the subsequent 6 weeks. Magnetic resonance imaging (MRI) identified a 12 cm tumor along the right gluteal fascia. A needle core biopsy was performed which was classified as an "Undifferentiated spindle cell sarcoma," at least grade II/III (Fédération Nationale des Centres de Lutte Contre le Cancer [FNCLCC]). The mass was widely excised approximately six weeks later, followed by adjuvant radiotherapy. Ten months after initial diagnosis the patient was found to have widespread metastases (soft tissue, peritoneum, and lungs). He received palliative chemotherapy and died as a result of disease 22 months after initial diagnosis. Patient 2 initially presented with a one-to-two year history of a hip mass. Computed tomography (CT) scan revealed a calcified soft tissue mass and a needle core biopsy was performed with a diagnosis of "Fibro-osseous lesion, consistent with myositis ossificans." The mass continued to progress over a five-year period, prompting a subsequent MRI that revealed a 12.8 cm mass within the right gluteus medius. A needle core biopsy was performed with a diagnosis of "Bland spindle cell neoplasm, suggestive of a low-grade sarcoma." The patient was treated with neoadjuvant radiotherapy and the mass widely resected five months later. Patient 3 presented with swelling over the right abdomen; MRI identified a 4.5 cm mass within the right rectus abdominus concerning for sarcoma. The patient underwent primary resection of the tumor with negative margins. Twelve months after initial diagnosis the patient developed bilateral lung metastases, for which he underwent a unilateral wedge resection. The patient is currently alive with disease over 16 months after initial diagnosis. Patient 4 presented with a thigh mass that was surgically resected. The tumor was classified as "Adult-type fibrosarcoma." Within two years the patient developed pulmonary metastases and ultimately succumbed to his disease. Patient 5 presented with an approximately 18 month history of a rib mass. Diagnostic imaging was subsequently reported to show an expansile lesion with mineralization. A 3.5 cm soft tissue mass with a broad base involving the underlying periosteum was subsequently resected. The patient is currently receiving adjuvant chemotherapy and is alive without disease six months after diagnosis.

Grossly, the tumors were all well demarcated, tan-pink and fleshy. Each was excised with negative margins. The average size was 10.1 cm (range, 3.5–16.6 cm). Morphologically they were composed of spindle cells with a herringbone-fascicular pattern (Figure 1–3). The cytoplasm was generally scant and eosinophilic with long bipolar processes. The nuclei were monomorphic and ranged from plump and ovoid to elongated and wavy. Mitotic activity was variable and ranged from 2 to > 50 mitoses per 10 high power fields (FD= 0.55 mm). The intervening stroma was focally collagenous to myxoid. In two cases (Case 1 and 3) there were occasional short/angulated capillary sized vessels surrounded by loose fibromyxoid stroma (Figure 3D). Necrosis was present in three cases. Case 1 additionally had focal areas with a round cell morphology; Case 2 had metaplastic bone, while Case 3 contained focal dystrophic calcification. Applying the FNCLCC criteria,[13] Cases 1 and 3–5 corresponded to a grade of III / III, while Case 2 was grade I/III.

Ancillary immunohistochemical staining revealed positivity for desmin in most cases tested, with variable expression of MyoD1 and smooth muscle actin (Table 2; Figure 1F, Figure 2D–E). However, none expressed myogenin. Targeted RNA sequencing identified a *ZFP64* exon 5 (of 6; NCBI Reference Sequence: NM_018197.3) fusion to *NCOA3* exon 14 (of 23; NCBI Reference Sequence: NM_181659.3) in patients 1–4 (Figure 4); patient 5 had a fusion between *ZFP64* exon 5 and *NCOA3* exon 15/16. The fusion products maintained the reading frame.

DISCUSSION

Spindle cell rhabdomyosarcoma is a relatively recently characterized subtype of rhabdomyosarcoma. These tumors are genetically diverse and can be further subdivided into discrete molecular subtypes. Here we report the clinical, morphologic, immunohistochemical attributes of a novel molecular subtype of spindle cell rhabdomyosarcoma characterized by a *ZFP64*::*NCOA3* gene fusion.

The World Health Organization first recognized spindle cell / sclerosing rhabdomyosarcoma as a distinct entity in 2013.[14] Sclerosing and spindle cell rhabdomyosarcoma in older children and adults often share recurrent *MYOD1* mutations leading to their unification as a single entity.[2] Since then, with the widespread application of next-generation sequencing, yet more genetic drivers have been identified leading to the recognition of additional molecular subtypes. Gene fusions are emerging as important molecular drivers in spindle cell rhabdomyosarcoma. Many of these events are extremely rare and found on the order of case reports; however, some genes have a recurring presence in this category. For example, fusions involving *NCOA1* or *NCOA2*, with multiple potential partner genes, are regularly identified in either infantile or skeletal variants of spindle cell rhabdomyosarcomas;[3,6,15,16] however, to date, there have been no prior reports of this tumor type harboring *NCOA3* rearrangement. The three members of the p160 steroid receptor coactivator (SRC) family – *NCOA1*, *NCOA2*, and *NCOA3* – have homologous domains and share significant sequence overlap;[17] as a result, one might naturally assume fusions involving *NCOA3* may likewise be possible in this context.

Following incidental identification of a *ZFP64*::*NCOA3* gene fusion in a tumor with a herringbone pattern suggestive of adult-type fibrosarcoma, but showing skeletal muscle differentiation immunohistochemically, we performed a retrospective review that resulted in the identification of four additional tumors within this clinical, histopathologic, and molecular spectrum. The tumors were all deep-seated and arose in males. Three of the patients developed metastatic disease within two years of diagnosis, with two ultimately succumbing to their disease. Each tumor was composed of monomorphic spindle cells with a herringbone-fascicular arrangement and variable amounts of collagenous stroma. In none of the cases were rhabdomyoblasts with convincing cross striations identified. Of note, two cases contained foci of either metaplastic bone or dystrophic calcification. The immunophenotypes included patchy staining for desmin in four cases and MyoD1 in the three cases tested. Each tumor harbored a *ZFP64::NCOA3* gene fusion, thereby suggesting a novel molecular subset of spindle cell rhabdomyosarcoma. Admittedly, in the absence of prototypic rhabdomyoblasts and limited skeletal muscle marker immunoexpression (the

tumors were completely negative for myogenin, and often showed only focal MyoD1 and/or patchy desmin staining) a myofibroblastic derivation remains a possibility.

Spindle cell sarcomas with a prominent herringbone pattern can be diagnostically challenging since these tumors are rare, overlap morphologically with several other entities (e.g., fibrosarcoma, malignant peripheral nerve sheath tumor, synovial sarcoma, spindle cell rhabdomyosarcoma), and possess a null or non-specific immunophenotype. In contrast to the subset of adult-type fibrosarcomas with *NTRK3* or *RET* rearrangements, *ZPF64::NCOA3* fusion-positive rhabdomyosarcoma does not express CD34 and/or S100.[18,19] Likewise fibrosarcomatous dermatofibrosarcoma protuberans often has at least focal CD34 expression, which can be a useful discriminator. Malignant peripheral nerve sheath tumor, with adequate sampling, may show focal staining for S100 and/or SOX10. Differentiating these tumors from monophasic synovial sarcoma can be a challenge – calcification is likewise common in synovial sarcoma[20] – but synovial sarcoma usually has focal expression of epithelial membrane antigen and/or keratins. The relationship between these tumors and other subtypes of rhabdomyosarcoma harboring *NCOA1*[6,21] or *NCOA2*[3,16,22,23] rearrangements remains to be established. Ultimately all of the aforementioned entities can be rigorously classified using ancillary molecular diagnostic techniques. As more definitive classification becomes increasingly important for patient management and clinical trials, molecular confirmation is expected to become imperative in the future. Targeted RNA sequencing is particularly efficient in this regard; furthermore, because *ZFP64* and *NCOA3* are located in relatively close proximity on chromosome 20, there is a potential risk a false-negative result by fluorescence in situ hybridization. As more definitive classification becomes increasingly important for patient management and clinical trials, molecular confirmation is expected to become imperative in the future. For example, *NCOA3* has recently been identified as a potential therapeutic target in melanoma,[24] hence it is conceivable that this might likewise extend to patients with *ZFP64::NCOA3*-associated spindle cell rhabdomyosarcoma.

Nuclear receptor coactivator 3 (NCOA3) is one of three members of the steroid receptor coactivator p160/SRC family, and implicated in the pathophysiology of various cancers through the modulation of gene expression.[25–27] Fusions involving *NCOA* family members have been documented in various mesenchymal tumors, including: biphenotypic sinonasal sarcoma,[28] low-grade spindle cell sarcoma of the genitourinary tract,[29] mesenchymal chondrosarcoma,[30] Mullerian adenosarcoma,[31] *PRRX*-rearranged fibrous tumor,[32] rhabdomyosarcoma (alveolar and spindle cell),[3,6,8,33] and uterine tumor resembling ovarian sex-cord tumor.[34,35] Zinc finger proteins – with roles in cell proliferation, apoptosis, immune function, and tumorigenesis – represent the largest group of sequence-specific DNA-binding proteins in the human genome.[36] *ZFP64* is a coactivator of Notch1 and through this pathway has been demonstrated *in vitro* to mediate osteoblastic differentiation.[37] Gene fusions involving zinc finger proteins are exceedingly uncommon. In mesenchymal neoplasms gene fusions involving *ZFP36::FOSB* have been reported in epithelioid hemangioma;[38] however, to our knowledge, fusions involving *ZFP64* have not previously been described. Further studies are required to elucidate the pathophysiology of this novel fusion event and determine whether permutations arising from fusions with

NCOA1/2, and/or other *NCOA* partners, may provide greater molecular diversity for these neoplasms.

In conclusion, we report a cohort of highly aggressive sarcomas characterized by a herringbone morphology and *ZFP64-NCOA3* gene fusion. Three of the patients developed distant metastases within two years of diagnosis underscoring the malignant biologic potential of these neoplasms. Most of the tumors contained immunohistochemical evidence of rhabdomyoblastic differentiation, suggesting a novel molecular subtype of spindle cell rhabdomyosarcoma. Future studies are needed to characterize the spectrum of clinical, morphologic and molecular findings possible in these neoplasms. Additional studies are likewise also necessary to evaluate the downstream mechanisms of this fusion event as it relates to sarcomagenesis and its potential as a therapeutic target.

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Data Availability Statement:

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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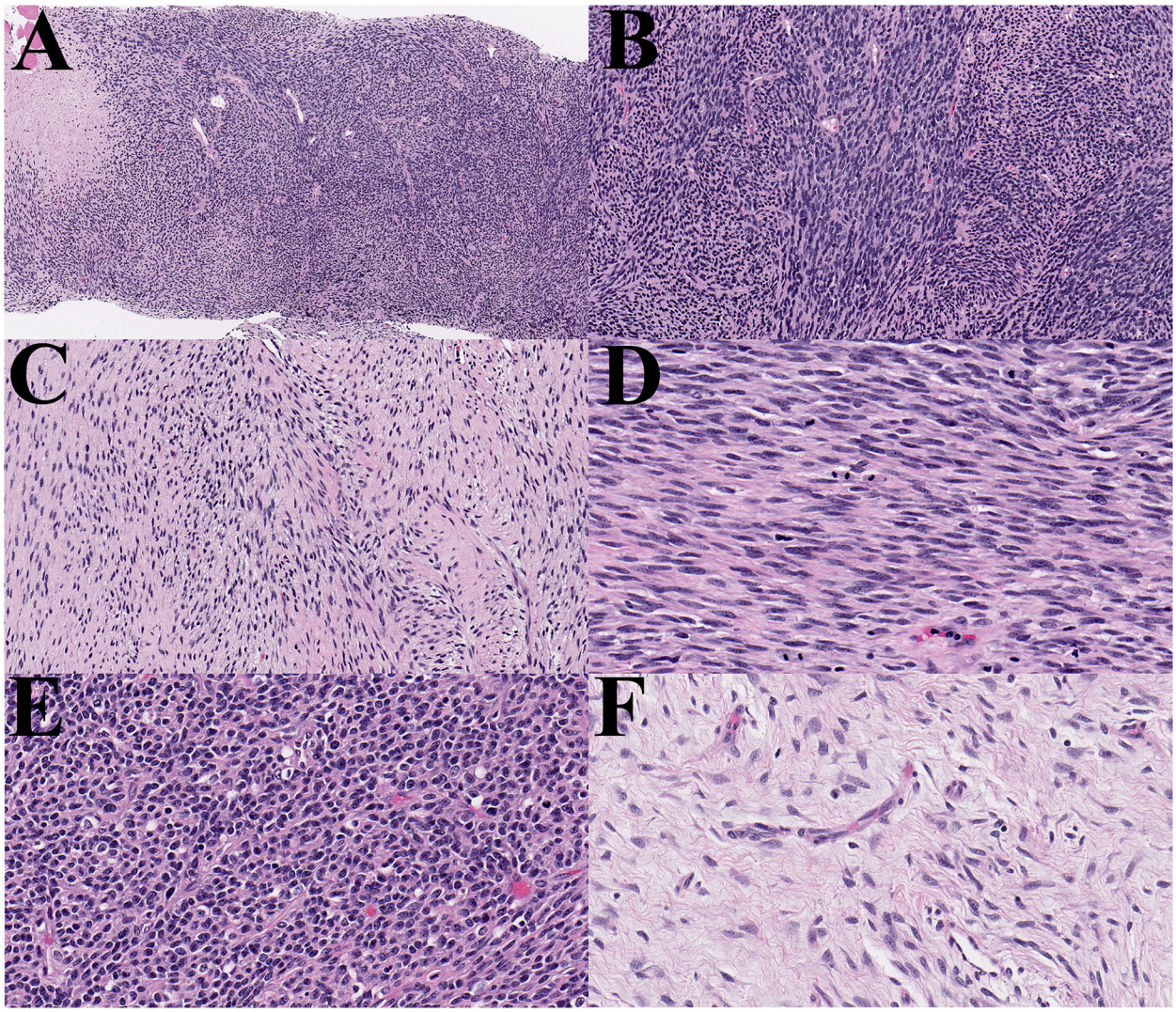

Figure 1:

Case 1: A. Cellular spindle cell neoplasm with occasional thin-walled hyalinized vessels and patchy necrosis (H&E, x100). B. Cellular spindle cells with a herringbone architecture (H&E, x200). C. Intermediate cellularity with spindle-stellate cells with a herringbone architecture (H&E, x200). D. Monomorphic wavy nuclei with conspicuous mitotic activity (H&E, x400). E. Focally the tumor was comprised of sheets of round-epithelioid cells (H&E, x400). F. Hypocellular regions with loose fibromyxoid stroma occasionally associated with small angulated vessels.

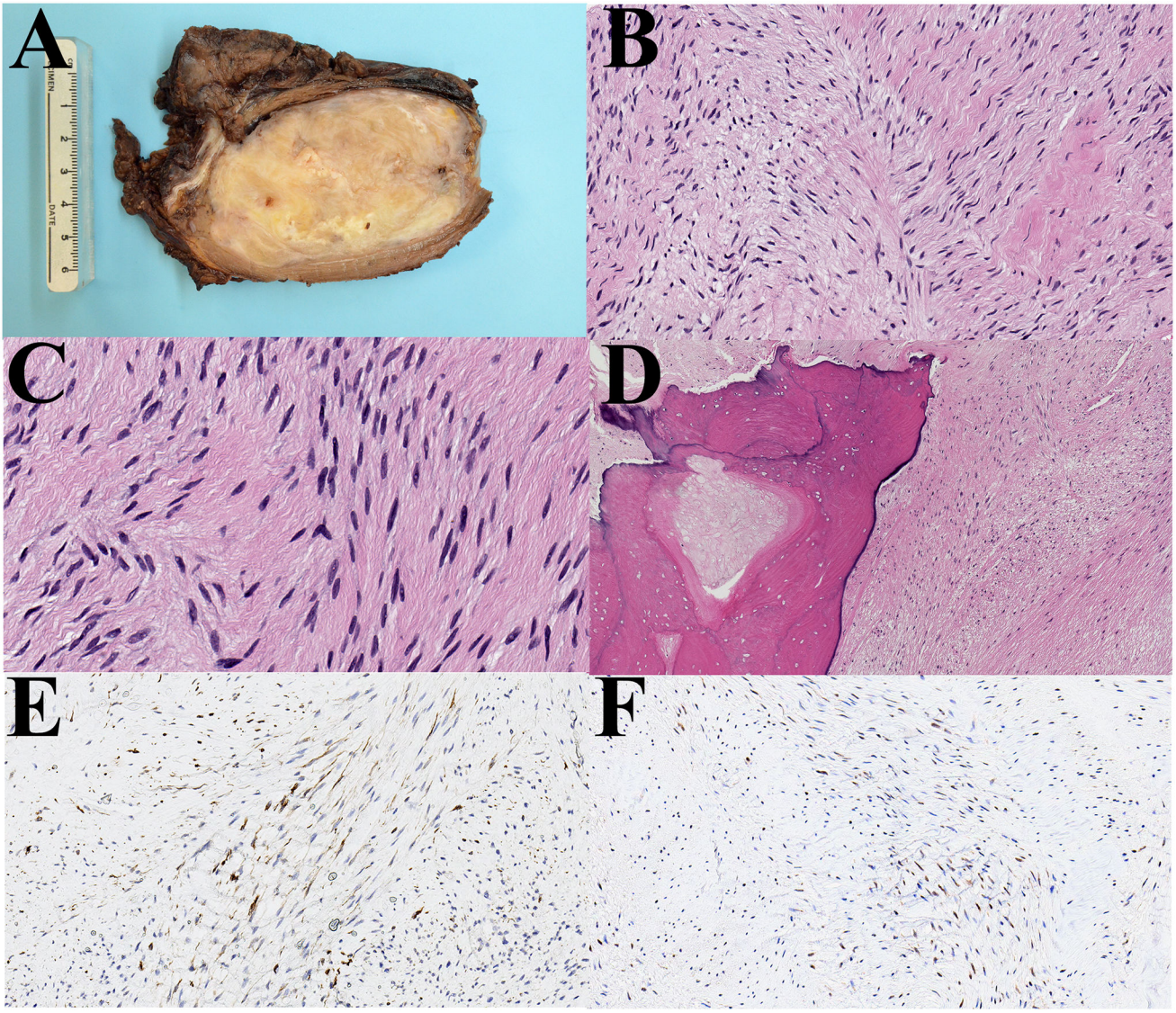

Figure 2:

Case 2: A. Gross photograph following neoadjuvant radiotherapy. The tumor is deep and well-demarcated with minimal objective response. B. Intermediate cellularity with spindle-stellate cells with a herringbone architecture and myxo-collagenous stroma (H&E, x200). D. Monomorphic ovoid nuclei with inconspicuous mitotic activity (H&E, x400). E. Patchy dystrophic calcification (x100). F. There was focal immunoreactivity for desmin (x200). G. There was focal immunoreactivity for MyoD1 (x200).

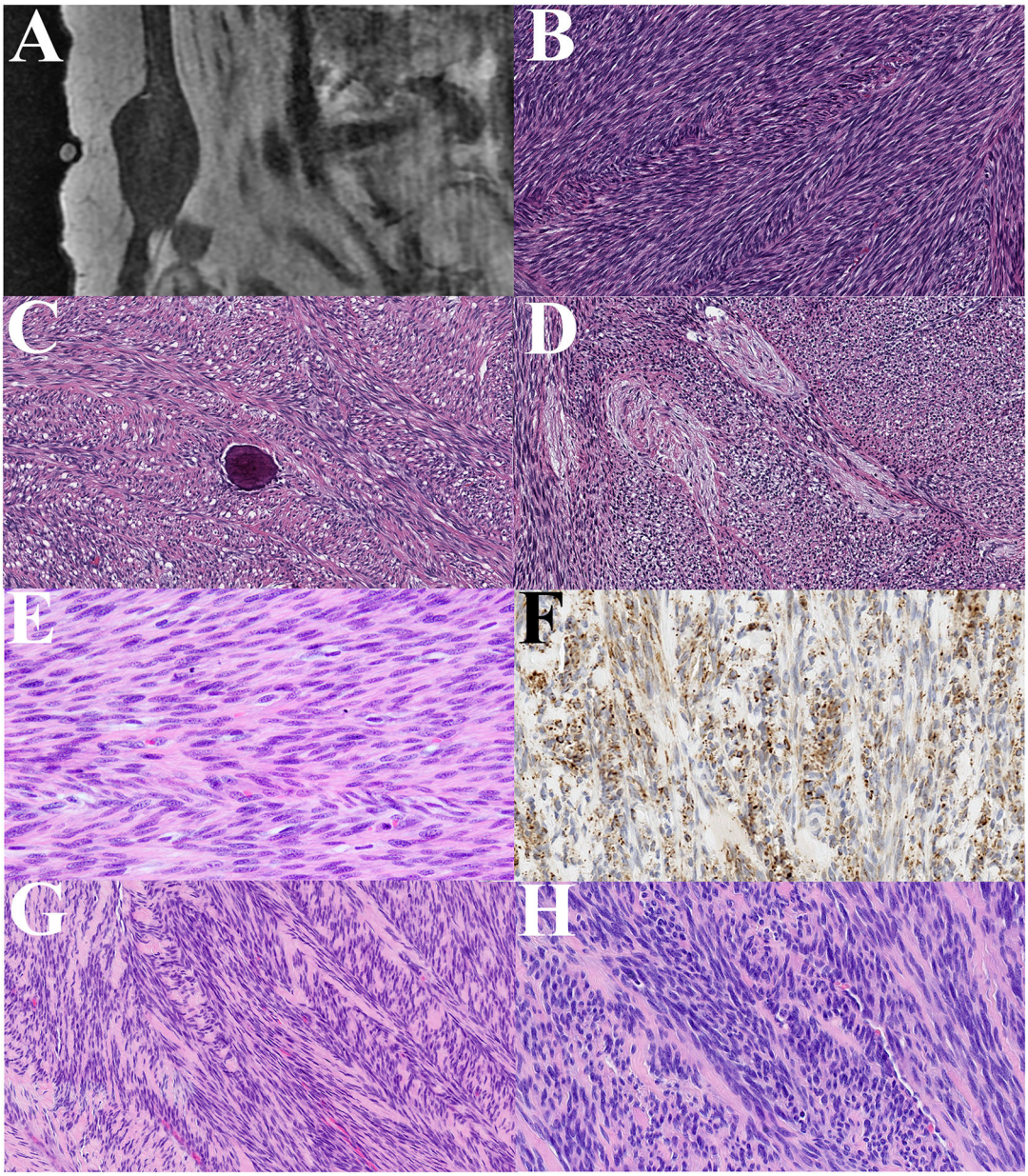

Figure 3:

Case 3: A. MRI demonstrating an intramuscular mass (below vitamin E marker). B. Cellular spindle cell neoplasm with a herringbone architecture (H&E, x200). C. Focal dystrophic calcification (H&E, x200). D. Less cellular region with loose fibromyxoid stroma and occasional small angulated vessels. Case 4: E. Cellular spindle cell neoplasm with a herringbone architecture (H&E, x400). F. Patchy immunoreactivity for desmin (x200). Case 5: G. Spindle cells with a herringbone architecture and broad collagen bands (H&E, x200). H.Higher magnification highlighting fibrillary nature of collagen bands (H&E, x200).

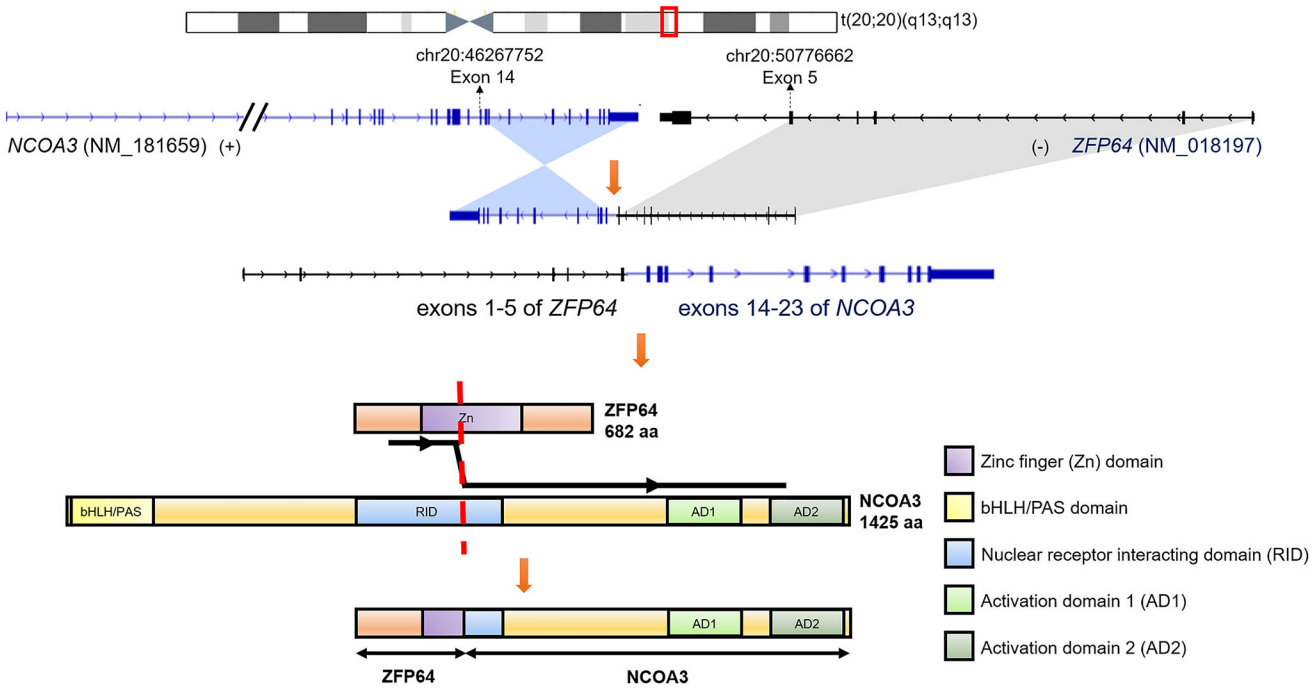

Figure 4:

Diagram illustrating of *ZFP64::NCOA3* gene fusion highlighting the involved exons and direction of transcription (top), and components of the translated protein domains (bottom).

TABLE 1.

Summary of clinical characteristics of patients with *ZFP64::NCOA3*-rearranged sarcoma.

Patient	Age/sex	Tumor site	Size (cm)	Metastases	Treatment	Progression (mo)	Follow-up (mo)	Status at last follow-up
1	30 M	Buttock	14.7	Yes	S, R, P-C	10	18	DOD
2	41 M	Gluteus medius	16.6	No	S, R	N/A	66	AND
3	71 M	Rectus abdominus	5.3	Yes	S, M	12	15	AWD
4	28 M	Thigh	10.6	Yes	S*	N/A	24	DOD
5	29 M	Chest wall	3.5	No	S, C	N/A	6	AND

Abbreviations: AND (alive with no evidence of disease), AWD (alive with disease), C (chemotherapy), DOD (dead of disease), M (metastasectomy), P-C (palliative chemotherapy), R (radiotherapy), S (surgery),

*incomplete details of subsequent management.

TABLE 2.

Summary of immunohistochemical staining in spindle cell rhabdomyosarcoma with *ZFP64::NCOA3* fusions.

Patient	Immunohistochemistry							
	Desmin	**Myogenin**	**MyoD1**	**SMA**	**CD34**	**S100**	**Keratin**	**EMA**
1	P/W	−	F/W	P/W	−	−	−	−
2	P	−	F/W	P	−	−	−	−
3	−	−	N/A	−	N/A	−	−	−
4	P	−	N/A	F/W	−	−	N/A	−
5	+	−	+	P	−	−	−	−

Abbreviations: - (negative); + (positive); F (focal); P (patchy); SMA (smooth muscle actin); W (weak). Patient 1: additionally negative for H-caldesmon and SOX10; H3K27me3 was intact. Patient 2: additionally negative for STAT6 and MUC4. Patient 3: additionally negative for CD56 and SOX10. Patient 4: additionally negative for H-caldesmon and STAT6. Patient 5: additionally positive for BCOR; negative for ALK, TLE1, SOX10; H3K27me3 was intact.