Modern Pathology (2019) 32:764–773
https://doi.org/10.1038/s41379-019-0206-z

ARTICLE

NUTM1-rearranged neoplasia: a multi-institution experience yields novel fusion partners and expands the histologic spectrum

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Received: 6 November 2018 / Revised: 13 December 2018 / Accepted: 14 December 2018 / Published online: 5 February 2019

Abstract

Poorly differentiated neoplasms lacking characteristic histopathologic features represent a significant challenge to the pathologist for diagnostic classification. Classically, NUT carcinoma (previously NUT midline carcinoma) is poorly differentiated but typically exhibits variable degrees of squamous differentiation. Diagnosis is genetically defined by *NUTM1* rearrangement, usually with *BRD4* as the fusion partner. In this multi-institutional next-generation sequencing and fluorescence in situ hybridization study, 26 new *NUTM1*-rearranged neoplasms are reported, including 20 NUT carcinomas, 4 sarcomas, and 2 tumors of an uncertain lineage. *NUTM1* fusion partners were available in 24 of 26 cases. *BRD4* was the fusion partner in 18/24 (75%) cases, *NSD3* in 2/24 cases (8.3%), and *BRD3* in 1/24 (4.2%) cases. Two novel fusion partners were identified: *MGA* in two sarcomas (myxoid spindle cell sarcoma and undifferentiated sarcoma) (2/24 cases 8.3%) and *MXD4* in a round cell sarcoma in the cecum (1/24 cases 4.2%). Eleven cases tested for NUT immunoexpression were all positive, including the *MGA* and *MXD4*-rearranged tumors. Our results confirm that *NUTM1* gene rearrangements are found outside the classic clinicopathological setting of NUT carcinoma. In addition, as novel fusion partners like *MGA* and *MXD4* may not be susceptible to targeted therapy with bromodomain inhibitors, detecting the *NUTM1* rearrangement may not be enough, and identifying the specific fusion partner may become necessary. Studies to elucidate the mechanism of tumorigenesis of novel fusion partners are needed.

Introduction

NUT carcinoma (previously known as NUT midline carcinoma) is a rare, aggressive malignancy defined by rearrangement of the *NUTM1* (NUT midline carcinoma family member 1) gene on 15q14. Most commonly, *NUTM1* is fused with either the bromodomain-containing 4 (*BRD4)* gene on 19p13.1 or the bromodomain-containing 3 (*BRD3)* gene on 9q34, and less commonly with the nuclear receptor binding SET domain protein 3 *(NSD3*) gene. Typical histologic features associated with NUT carcinoma are those of a primitive, epithelial malignancy with variable "abrupt" squamous differentiation. While initially reported in midline structures in young patients, typically in the mediastinum or upper aerodigestive tract, NUT carcinoma is being increasingly recognized in adults and in non-midline anatomic locations such as the kidney, parotid, and thigh [1–3]. Prognosis is dismal, with a 6.7-month median overall survival [4]. Traditional chemotherapy and radiation treatments have shown little benefit in treating NUT carcinoma [4]. Bromodomain and extraterminal domain inhibitor therapies have been developed and are currently used in clinical trials.

About 1/3 of *NUTM1*-rearranged tumors are so-called "NUT variants", defined as cases harboring fusions of *NUTM1* to non-*BRD* genes, some of which are genes that do not code for or interact with bromodomain-containing proteins. This raises the possibility of some cases of

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NUTM1-rearranged neoplasia not responding to bromodomain and extraterminal domain inhibitor therapy [4, 5]. Therefore, identification of *NUTM1* fusion partners may be essential for appropriate clinical management.

Currently, NUTM1 immunohistochemistry and/or *NUTM1* fusion testing is typically limited to cases of poorly differentiated carcinoma with variable squamous differentiation. Recently, however, undifferentiated soft tissue tumors harboring *NUTM1* fusions have been identified [2, 3, 6]. Identification of novel histopathologic patterns and fusion partners in *NUTM1*-rearranged neoplasia not only broadens our understanding of these tumors but also potentially expands the number of patient candidates that may benefit from bromodomain and extraterminal domain inhibitor therapy. Therefore, we set out to describe a multi-institutional experience of *NUTM1*-rearranged neoplasia.

Materials and methods

Identification of cases

This study was approved by the Institutional Review Boards of all institutions involved. The databases of three institutions (The University of Nebraska Medical Center, Omaha, NE; Caris Life Sciences, Phoenix, AZ; University of Alabama at Birmingham) were searched for cases of *NUTM1*-rearranged neoplasia. A total of 26 cases were identified.

Thirteen cases (cases 1–13) were from University of Nebraska Medical Center, identified using both *NUTM1* break-apart fluorescence in situ hybridization (FISH) analysis, as well as fusion FISH analysis using two-color probe design with probes designed to span the *NUTM1* locus and *NUTM1* fusion gene loci including *MGA*, *MXD4*, *WHSC1L1*, *NSD3*, and *BRD4*.

Cases 14–16 were identified at the University of Alabama at Birmingham, and fusions were detected by next-generation sequencing with the Archer FusionPlex Solid Tumor panel (ArcherDX, Inc., Boulder, CO) at Caris Life Sciences.

Cases 17–25 were identified during routine systematic next-generation sequencing analysis of fusion genes in 14,107 solid malignancies submitted to Caris Life Sciences using the Archer FusionPlex Solid Tumor panel.

Identification of a *NUTM1*-rearranged case with some histologic overlap with extraskeletal myxoid chondrosarcoma (case 20) prompted one of us (MMM) to screen a previously constructed tissue microarray composed of 31 cases previously diagnosed as extraskeletal myxoid chondrosarcoma for NUT protein expression by immunohistochemistry. This microarray was constructed using previously described [7] methods. This led to the identification of case 26.

FISH studies

FISH studies were performed on 4-μm unstained sections of representative formalin-fixed, paraffin-embedded tissue using a dual-color probe set designed to flank the *NUTM1* gene locus (break-apart probe design) and a dual-color probe set designed to span the *NUTM1* and *NUTM1* fusion gene loci including *MGA*, *MXD4*, *WHSC1L1*, *NSD3*, and *BRD4* (fusion probe design).

Both the custom *NUTM1* break-apart probe set and the *NUTM1* fusion probe sets utilized cocktails of BAC clones that were selected based on their location in the University of California Santa Cruz Human Genome Browser Gateway (http://genome.ucsc.edu) and were obtained from the BACPAC sources of Children's Hospital of Oakland Research Institute (Oakland, CA) (http://bacpac.chori.org). Probes were directly labeled by nick translation with either Spectrum Green or Spectrum Orange-dUTP utilizing a modification of the manufacturer's protocol (Abbott Molecular Inc., Des Plaines, IL) as previously described [8].

Before hybridization, the slides were deparaffinized and then pretreated at room temperature in 0.2 N HCl for 20 min, washed in water for 3 min, incubated at 80 °C for 30 min in VP 2000 Pretreatment Reagent (Abbott Molecular Inc.), and then washed again in H_2O for 3 min. Subsequently, the slides were incubated for 30 min at 37 °C in protease solution (25 mg of protease in 50 mL of protease solution) (Abbott Molecular Inc.), washed in 1 × phosphate-buffered saline at room temperature for 5 min, and then dehydrated in gradient ethanol (75%, 85%, and 100%) at room temperature for 1 min each and air-dried. After the cells and probes were codenatured at 80 °C for 5 min and incubated overnight at 37 °C using the ThermoBrite system (Abbott Molecular Inc.), posthybridization washing was performed in 2 × saline-sodium citrate (SSC)/0.3% NP-40 at 72 °C for 2 min, followed by 2 × SSC/0.3% NP-40 at room temperature for 2 min. The slides were then counterstained with 4', 6-diamidino-2-phenylindole (Abbott Molecular Inc.).

Hybridization signals were assessed in 100 interphase nuclei with strong, well-delineated signals for the *NUTM1* break-apart and each individual dual fusion probe set, respectively. Only nonoverlapping tumor nuclei with a complete set of signals were scored. As controls, each probe set was also hybridized to metaphase cell preparations of karyotypically normal peripheral blood lymphocytes to confirm correct mapping, optimal signal intensity, and lack of cross-hybridization, before proceeding with analysis of the patient sample. Images were acquired using the Cytovision Image Analysis System (Applied Imaging, Santa Clara, CA). The cutoff level for scoring aberrations was 15% abnormal nuclei for both the dual-color *NUTM1* break-apart and dual-color fusion probe systems, based on related in-house validation studies.

Fusion detection

For cases submitted to Caris Life Sciences for gene fusion detection, anchored multiplex PCR was performed for targeted RNA sequencing using the ArcherDx fusion assay (Archer FusionPlex Solid Tumor panel). The formalin-fixed paraffin-embedded tumor samples were microdissected to enrich the sample to ≥ 20% tumor nuclei, and mRNA was isolated and reverse transcribed into complementary DNA (cDNA). Unidirectional gene-specific primers were used to enrich for target regions, followed by next-generation sequencing (Illumina MiSeq platform [Illumina, Sand Diego, CA]). Targets included 52 genes known to be associated with various malignancies. The full list of genes can be found at http://archerdx.com/fusionplex-assays/solid-tumor. Reads and contigs that were matched to a database of known fusions and other oncogenic isoforms (Quiver database, ArcherDx), as well as those novel isoforms or fusions with high reads (> 10% of total reads) and high confidence after bioinformatic filtering, were analyzed. Samples with < 4000 unique RNA reads were reported as indeterminate and excluded from analysis, and all the analyzed fusions were in-frame. Fusions among the > 11,000 fusions known to be found in normal tissues were excluded. The detection sensitivity of the assay allows for detection of a fusion that is present in at least 10% of the cells in the samples tested.

NUT immunohistochemistry

NUT protein expression was evaluated using Leica Bond automation with the AR2 epitope retrieval buffer (25 min). Rabbit monoclonal NUTM1 antibody clone C52B1 (Cell Signaling Technologies Inc., Danvers, MA) was diluted 1:50. Given the multi-institutional nature of this study, only 11 cases had material available for NUT immunohistochemistry. The additional immunohistochemistry data listed in Table 1 were obtained from the outside pathology reports, when available.

Tissue microarray

Immunohistochemistry screening for NUT was performed on the previously constructed tissue microarray composed of 31 cases previously diagnosed as extraskeletal myxoid chondrosarcoma. The slides were both immunostained for NUT and screened for *NUTM1* rearrangement using a *NUTM1* break-apart FISH probe.

Results

The 26 cases of *NUTM1*-rearranged tumors are summarized in Table 1. *NUTM1* gene rearranged tumors were identified across a wide age range in adolescents and adults (mean age: 44.7 years; range, 12–74; 17 males, 9 females).

NUTM1 fusion genes identified by fusion FISH and the ArcherDx fusion assay

NUTM1 fusion partners were available in 24 of 26 cases. Twenty-one cases (21/24) contained previously described *NUTM1* fusion partners. *BRD4* was the most common partner, identified in 18 cases (18/24, 75%). Two cases harbored *NSD3-NUTM1* fusions (2/24, 8.3%). *BRD3-NUTM1* was identified in one case (1/24, 4.2%).

Two of the *BRD4-NUTM1* fusion cases (cases 19 and 21) and one *NSD3-NUTM1* fusion case (case 23) identified by ArcherDx Fuson Assay were confirmed by fusion FISH analysis. A single patient (case 23) with paired primary (lung) and metastatic (liver) tumor showed an identical *NSD3-NUTM1* fusion in both sites.

The fusion partner was not available in two cases (only break-apart FISH results were available on cases 3 and 26).

Novel *MGA-NUTM1* and the only recently reported *MXD4-NUTM1* were identified by the ArcherDx Fusion Assay (Fig. 1). The *MGA-NUTM1* fusion was detected in two cases (2/24, 8.3%; cases 20 and 25) and the *MXD4-NUTM1* fusion was detected in one case (1/24, 4.2%; case 22). In cases 20 and 25, the Archer panel detected highly expressed, in-frame *MGA-NUTM1* fusions with 162 and 192 unique cDNA start sites, respectively, resulting in exon 21 of *MGA* (NM_001080541) joined to exon 2 of *NUTM1* (NM_175741). *MGA* and *NUTM1* are located 7.4 Mb apart, in opposite orientations on chromosome 15. Therefore, this fusion may have arisen via an inversion. The *MXD4-NUTM1* gene fusion in case 22 was in-frame, with exon 5 of *MXD4* (NM_006454) joined to exon 2 of *NUTM1* (NM_175741). Both fusions preserve the functional domains of *MGA* and *MXD4* and only remove the C-termini of the proteins.

One *MGA-NUTM1* fusion case (case 20) was confirmed by fusion FISH analysis.

NUT immunohistochemistry and *NUTM1* break-apart FISH on tissue microarray

The extraskeletal myxoid chondrosarcoma tissue microarray identified one case (1/31, 3.2%) with strong nuclear NUT expression by immunohistochemistry. One other case showed focal weak nuclear NUT immunostaining, whereas the rest of the cases in the microarray were completely negative for NUT protein expression.

The case with strong NUT nuclear staining (Table 1, case 26) demonstrated a rearrangement of the *NUTM1* locus in 94 of 100 cells evaluated by *NUTM1*-break-apart FISH, indicating that this case is a *NUTM1*-rearranged tumor

Table 1 Summary of 26 cases of *NUTM1*-rearranged neoplasia

Case	Submitting diagnosis	Final diagnosis	Age, sex	Clinical primary site	Gene fusion[a]	NUT IHC	IHC positive	IHC negative
1	Poorly differentiated neoplasm, favor large cell neuroendocrine carcinoma	NUT carcinoma with large cell neuroendocrine carcinoma-like features	39, F	Paranasal sinuses	*BRD4-NUTM1*	NT	CAM5.2, SYN	Pankeratin
2	Poorly differentiated carcinoma, non-small cell type	NUT carcinoma	46, M	Mediastinum	*BRD4-NUTM1*	NT	p63, CK7	TTF1, CHR, SYN, B72.3, CD5
3	Metastatic squamous cell carcinoma	NUT carcinoma	13, M	Bone (proximal humerus)	X-*NUTM1*	NT	n/a	n/a
4	Large cell neuroendocrine carcinoma	NUT carcinoma, with large cell neuroendocrine carcinoma-like features	36, M	Lung and chest	*BRD4-NUTM1*	NT	p63, CK5/6 focal, SYN	CHR, TTF1, Napsin A, Pankeratin, S100, CD56, CK20
5	Undifferentiated malignant neoplasm	NUT carcinoma	12, M	Soft tissue (neck)	*BRD4-NUTM1*	NT	AE1/AE3, CK5/6, CAM5.2, CK19, patchy p63, SYN, CD99	CHR, desmin, SMA, myogenin, GFAP, CD34, HMB45, MelanA, CD20, CD3, CD79a, CD30, ALK1, TDT, INI-1 retained
6	Poorly differentiated squamous cell carcinoma	NUT carcinoma	49, M	Frontal sinus	*BRD4-NUTM1*	NT	p63, CK5/6, CK7, p16	TTF1, SYN, CHR
7	Favor NUT midline carcinoma	NUT carcinoma	48, M	Ethmoid sinus, orbit	*BRD4-NUTM1*	NT	AE1/AE3, EMA	SYN, CHR, TTF1, GFAP
8	Nonkeratinizing squamous cell carcinoma	NUT carcinoma	30, M	Floor of mouth, glottis	*BRD4-NUTM1*	NT	AE1/AE3, p63, CK5/6, SYN, p16	CHR, OCT3/4
9	Undifferentiated carcinoma with abrupt squamous differentiation	NUT carcinoma	49, M	Sinonasal	*BRD4-NUTM1*	NT	PanCK, p63, CD99	CHR, SYN, S100
10	Poorly differentiated carcinoma with squamous and glandular differentiation	NUT carcinoma with squamous and glandular differentiation	44, M	Lung	*BRD4-NUTM1*	NT	PanCK, TTF1 (patchy weak), p63, CK5/6, p16	Napsin A, CK20
11	Poorly differentiated/high-grade carcinoma	NUT carcinoma	32, F	Ovary and lung	*BRD4-NUTM1*	NT	AE1/AE3	PLAP, CD30, S100, CHR, SYN, Calretinin, inhibin, MelanA
12	Undifferentiated carcinoma	NUT carcinoma	67, F	Nasal and maxillary sinus	*BRD4-NUTM1*	NT	p63, SYN	CHR, S100, CD99, CK7, CK5/6, Desmin, CD34
13	Undifferentiated malignant neoplasm	Malignant neoplasm of uncertain lineage with *BRD4-NUTM1*	47, F	Sphenoid sella	*BRD4-NUTM1*	NT	None	p40, EMA, CK5/6, SYN, CHR, PLAP, OCT3/4, Melan-A, CD34, desmin, FLI1, CD68, CD30
14	Poorly differentiated non-small cell carcinoma with squamous features	NUT carcinoma	44, M	Pleura	*BRD4-NUTM1*	Pos	p63, patchy TTF1, CK5/6	Napsin A
15	Poorly differentiated squamous cell carcinoma	NUT carcinoma	56, F	Skull Base	*BRD4-NUTM1*	Pos	CAM5.2	CHR, SYN, CD56

Table 1 (continued)

Case	Submitting diagnosis	Final diagnosis	Age, sex	Clinical primary site	Gene fusion[a]	NUT IHC	IHC positive	IHC negative
16	Squamous cell carcinoma	NUT carcinoma	56, F	Lacrimal sac	*BRD4-NUTM1*	Pos	CK5/6, p63	BER-EP4
17	Basaloid carcinoma	NUT carcinoma	36, M	Lung (hilar)	*NSD3-NUTM1*	NT	p63	TTF1, SYN, CHR
18	Poorly differentiated carcinoma	NUT carcinoma	35, M	Lung	*BRD3-NUTM1*	Pos	n/a	n/a
19	Poorly differentiated carcinoma	NUT carcinoma	45,M	Lung	*BRD4-NUTM1*	Pos	n/a	n/a
20	Spindle cell sarcoma with features of myxoid chondrosarcoma metastatic to liver	NUT sarcoma with features of myxoid chondrosarcoma metastatic to liver	63, F	Lung primary	*MGA-NUTM1*	Pos	n/a	n/a
21	Small round blue cell malignancy	NUT rearranged tumor with a small round cell pattern	38, F	Lung	*BRD4-NUTM1*	NT	SYN	CHR, CAM5.2, STAT6, CD99, CD45, desmin, SMA, S100, CD34
22	Extraosseous osteosarcoma	NUT sarcoma with a small round/epithelioid cell pattern, and scant fibrous matrix, high-grade	65, F	Colon (cecum)	*MXD4-NUTM1*	Pos	Vimentin	AE1/AE3, EMA, CK19, CK20
23	Squamous cell carcinoma	NUT carcinoma	74, M	Lung, mets to liver	*NSD3-NUTM1*	Pos	CK5/6, p63, CD56	CK7,NapsinA,TTF1
24	NUT midline carcinoma	NUT Carcinoma	29, M	Mediastinum	*BRD4-NUTM1*	Pos	AE1/3, Cam5.2, CK7, CK20, EMA, p63	n/a
25	NUT midline carcinoma	NUT sarcoma with a round-oval cell pattern and fibrous matrix	61, M	Chest wall/pleural	*MGA-NUTM1*	Pos	Weakly positive for CD34 in a subset of cells	Pankeratin, CD45, CD43, calretinin, desmin, myogenin, S100, AFP, CD30, CD43, CD45, CD68, PLAP
26	Extraskeletal myxoid chondrosarcoma, solid variant	NUT sarcoma with features of extraskeletal myxoid chondrosarcoma with a solid pattern	48, M	Foot (lung metastasis 4 years later)	X-*NUTM1*	Pos	Estrogen receptor, minimal dot-like keratin positivity (AE1/AE3 cocktail)	GFAP, S100, SMA

NT not tested, *n/a* not available, *IHC* immunohistochemistry, *SYN* synaptophysin, *CHR* chromogranin, *PanCK* pan-cytokeratin, *SMA* smooth muscle actin, *X* unknown partner

[a]Cases 1–13: fusions identified by FISH

[a]Cases 14–25: fusions identified by Archer

[a]Case 26: NUT break-apart FISH only. Identified by NUT IHC screening of a tissue microarray composed of cases previously diagnosed as extraskeletal myxoid chondrosarcoma

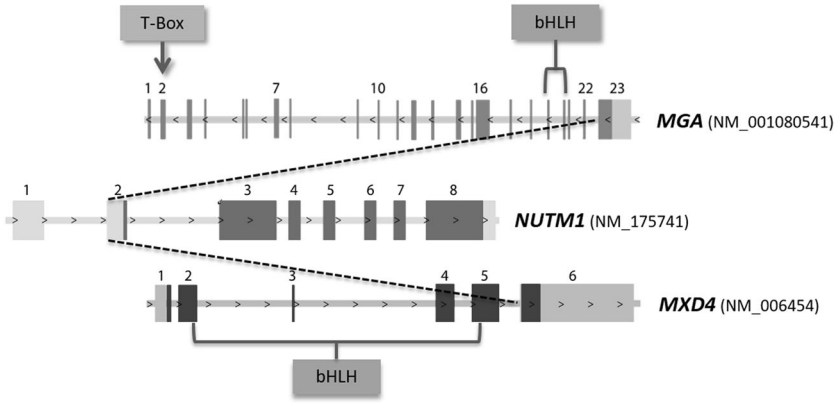

Fig. 1 Novel *NUTM1* fusions. T-Box (*MGA*) and bHLH domains (*MGA* and *MXD4*) are located before the breakpoints in the *MGA-NUTM1* and *MXD4-NUTM1* fusions

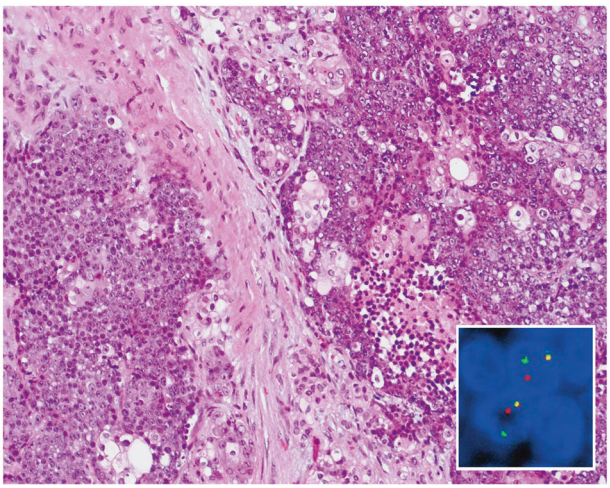

Fig. 2 *NUTM1*-rearranged neoplasia. Typical examples of NUT carcinoma consist of primitive cells with variable foci of "abrupt" keratinization (hematoxylin and eosin, ×100). Break-apart *NUTM1* FISH probes show split green and orange signals in case 26, indicating a *NUTM1* gene rearrangement (inset)

mimicking extraskeletal myxoid chondrosarcoma. In contrast, there was no evidence of a *NUTM1* gene rearrangement by FISH in the other cases in the microarray, including the weak NUT expressing case.

Histologic findings

The histomorphology of the *NSD3*- and most of the *BRD4-NUTM1* neoplasms was similar in appearance to what has been previously described in NUT carcinomas. As such, they were composed of sheets of primitive epithelioid cells with a high mitotic rate and variable evidence of squamous differentiation (Fig. 2). However, two poorly differentiated *BRD4-NUTM1* fusion tumors (cases 13 and 21) could not be definitively classified as carcinomas, mainly due to insufficient evidence of epithelial differentiation.

The metastatic tumor previously diagnosed as an extraskeletal myxoid chondrosarcoma (case 26) was composed of sheets of round cells (Fig. 3a) and showed nuclear expression of NUT (Fig. 3b) and rearrangement of the *NUTM1* gene locus by FISH (Fig. 2, inset). The fusion partner of case 26 could not be determined in the absence of RNA of sufficient quality. Case 26 was classified as a sarcoma based largely on its histologic features.

The two cases with novel *MGA-NUTM1* fusions were classified as sarcomas. One *MGA*-fused case (case 20, a lung primary, with liver metastasis) was composed of bland, spindled cells with tapered nuclei in an abundant myxoid matrix (Figs. 4a, b). The other *MGA*-rearranged case (case 25, chest wall/pleural mass) showed sheets of primitive, round to oval cells with vesicular chromatin and 1–2 prominent nucleoli and scant, nondescript cytoplasm (Figs. 4c, d). The neoplastic cells in case 25 appeared more mesenchymal than epithelial and were associated with foci of hyalinized stroma.

The sarcoma with *MXD4-NUTM1* fusion (case 22) demonstrated sheets of high-grade epithelioid to plasmacytoid cells with moderate amounts of eosinophilic cytoplasm and focal dense fibrous matrix (Figs. 3c, d), prompting original microscopic interpretation as extraskeletal osteosarcoma. Case 22 was negative for epithelial membrane antigen and pan-cytokeratin, supporting the morphologic impression of sarcoma.

The available immunophenotypic results of these cases are presented in Table 1. Note: given the multi-institutional nature of this study, the immunostains used on the cases in this study were not able to be standardized across all cases. However, a few noteworthy examples are noted here. Six of 14 (6/14; 42.8%) cases showed synaptophysin expression, whereas 0 of 12 cases (0/12) were positive for chromogranin. Of eight cases studied, two (2/8; 25%) expressed TTF1, including both lung and pleural examples.

Immunohistochemistry for NUTM1 protein

Immunohistochemistry results for the NUTM1 protein were available in 11 cases. All 11 cases showed nuclear

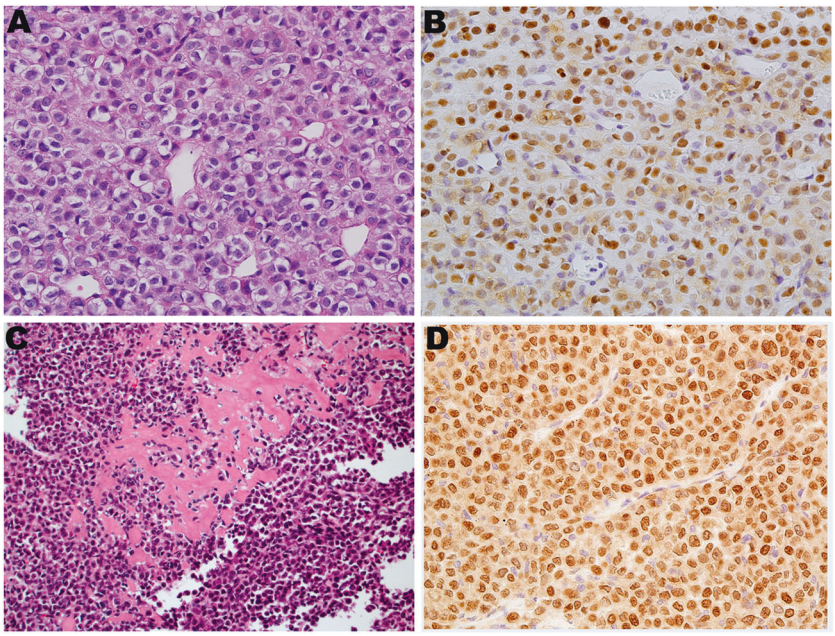

Fig. 3 *NUTM1*-rearranged tumors with variant morphology. **a**, **b** Case 26 showed undifferentiated round cells along with NUT expression (NUT IHC in **b**). This case harbored *NUTM1* gene break by break-apart FISH, leading to a diagnosis of *NUT*-rearranged tumor. **c** Case 22 showed sheets of epithelioid to plasmacytoid cells with production of dense extracellular collagen type material and harbored an *MXD4-NUTM1* fusion. **d** Case 22 showed nuclear expression of NUT. (**a**, **c**, hematoxylin and eosin, ×400 and ×200, respectively; **b**, **d**, ×400)

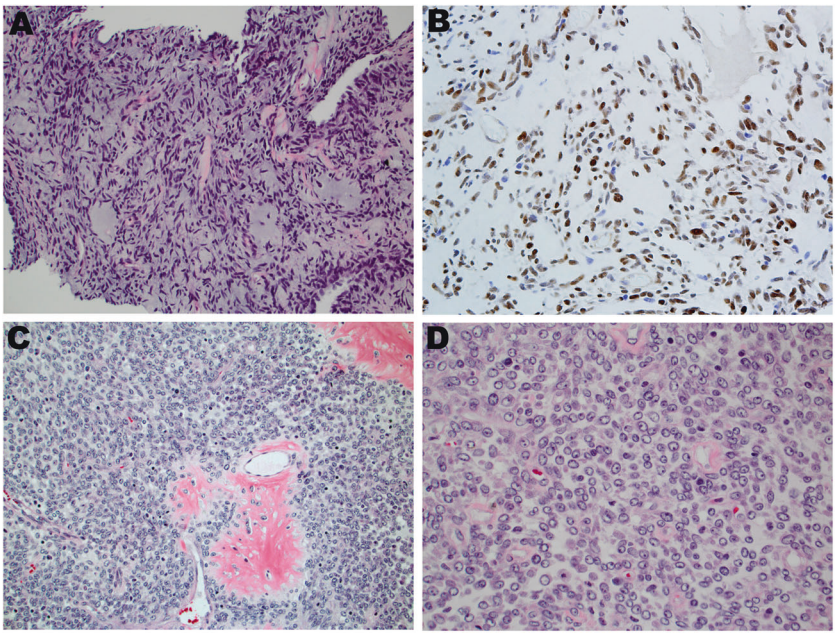

Fig. 4 *MGA-NUTM1*-rearranged cases. **a** An *MGA-NUTM1* case (case 20) showed spindled cells with tapered ends set in an abundant myxoid stroma. **b** Case 20 also showed speckled pattern of nuclear NUT expression. **c**, **d** The other *MGA-NUTM1* case (case 25) showed oval to round primitive cells with vesicular nuclei and prominent nucleoli, imparting a more primitive and mesenchymal appearance. (**a**, **c**, **d**, hematoxylin and eosin, ×100, ×200, and ×400, respectively; b, ×200)

expression, and often times a dot-like pattern could be discerned within the nuclear staining. This included the two novel *MGA-NUTM1* fused cases and the *MXD4-NUTM1* fused case.

Discussion

The NUTM1 protein, a protein of largely unknown function, is normally only expressed to any significant degree in the testis, where it shuttles between the nucleus and cytosol. However, when fused to the bromodomain proteins BRD4 or BRD3 in NUT carcinomas, NUTM1 becomes trapped in the nucleus [5, 9]. This is because the bromodomain proteins bind and become localized to acetylated lysine residues on histone proteins. As the NUTM1 protein binds to p300, a histone acetyltransferase, there is sequestration of p300 to sites of BRD4/3-NUTM1 complexes, leading to localized hyperacetylation of histone proteins. This leads to further recruitment of BRD4/3-NUTM1 complexes in a feed-forward mechanism, ultimately forming large contiguous expanses measuring up to two megabases in size

that are co-occupied by BRD4/3-NUTM1, p300 and active histone acetylation, so-called "megadomains" [10]. Because acetylation of lysine residues on histone tails is associated with open chromatin and bromodomains are critical in transcription elongation, the fusion protein is preferentially tethered to transcriptionally active DNA resulting in increased transcription of progrowth genes [9]. In contrast, areas away from the megadomains become hypoacetylated, resulting in transcriptional repression of prodifferentiation genes [11, 12]. The BRD4/3-NUTM1 megadomains override the preexisting histone code leading to increased expression of key oncoproteins such as MYC, the stem cell marker SOX2 and TP63 [12–14]. The importance of MYC overexpression in NUT carcinoma is evidenced by studies showing that when MYC is force expressed in NUT carcinoma cell lines, differentiation arrest occurs even when BRD4-NUTM1 is knocked down by small interfering RNA [15]. Moreover, bromodomain and extraterminal domain inhibitor therapy represses MYC expression [16]. BRD4-NUTM1 has been shown to drive expression of SOX2, which in turn induces NUT carcinoma cell lines to form stem cell-like spheres with cellular transformation [14]. The increased expression of TP63, a negative regulator of TP53, allows NUT carcinoma cells to evade TP53 [12]. Therefore, NUT carcinomas have a reprogrammed epigenome [4] leading to increased proliferation and arrest of differentiation.

Bromodomain and extraterminal domain inhibitors have been developed that reduce the binding of acetylated histones to BRD4/BRD3, deplete megadomains, and cause cellular differentiation in vitro and in mice [11]. In addition, treatment of NUT carcinoma cell lines with histone deacetylation inhibitors can restore global acetylation, leading to squamous differentiation and arrested growth [11]. At least three clinical trials for bromodomain and extraterminal domain inhibitors are underway as of the writing of this article, and there is a clinical trial for histone deacetylation inhibitors for patients who fail bromodomain and extraterminal domain inhibitor therapy [17]. Indeed, patients with NUT carcinoma have been successfully treated with vorinostat, a Food and Drug Administration-approved histone deacetylation inhibitor [11] and an oral bromodomain and extraterminal domain inhibitor [10]. *MYC* inhibition therapy with OmoMyc is also being explored in NUT carcinoma [15].

The *NSD3* gene, the *NUTM1* fusion partner in 10–20% of NUT carcinoma cases [16], encodes a histone methyltransferase protein. Although not a bromodomain protein, NSD3 associates with the extraterminal domain of BRD4 and forms a BRD4-NUTM1-like complex attached to chromatin [10]. In addition, NSD3-NUTM1 containing cells have shown growth arrest and differentiation in response to the bromodomain and extraterminal domain inhibitor JQ1 and therefore the NSD3-NUTM1 fusion protein likely alters epigenetic programming via a similar mechanism as BRD4/3-NUTM1 fusion proteins [16]. Thus, there is rational for bromodomain and extraterminal domain inhibitor treatment in *NSD3-NUTM1* cases [10].

In the current study, most NUTM1-rearranged tumors were carcinomas with focal squamous cell differentiation, and *BRD4* was the most common fusion partner (18/24, 75%). Many NUT carcinomas in this study were synaptophysin-positive indicating neuroendocrine differentiation, only occasionally reported in NUT carcinomas [18]. Two NUT carcinomas with *NSD3* fusions and one case with a *BRD3* fusion were also identified. Moreover, an *MXD4-NUTM1* and novel *MGA-NUTM1* fusions were identified in one and two cases, respectively. Interestingly, MAX dimerization protein 4 (*MXD4*) and MAX gene-associated protein (*MGA*) are both members of the MAX-interacting transcription factor network, which includes proteins that engage MAX as a cofactor for DNA binding and control of gene expression. Heterodimerization of MXD4 or MGA with MAX result in transcriptional repression of E-box target DNA sequences [19]. Immunohistochemical staining of our *MXD4-NUTM1* and *MGA-NUTM1* fusion cases with NUTM1 antibody showed distinct nuclear foci of NUTM1 protein accumulation, not unlike those seen in *BRD4-NUTM1* fusion cases [20], suggesting that *NUTM1*-rearranged tumors with *MXD4* and *MGA* fusion partners may share similar oncogenic mechanisms as *BRD3/BRD4-NUTM1* cases. In contrast, no NUTM1 immunoexpression was observed in a single *MXD1-NUTM1* fusion positive NUT carcinoma reported by Dickson et al. utilizing the same antibody as used in our study. These authors, however, did demonstrate high levels of mRNA expression of *NUTM1* and accordingly hypothesized that a posttranscriptional mechanism may be responsible for the lack of NUTM1 immunostaining [2].

Given that only the C-termini of both MXD4 and MGA are lost as a result of the fusion resulting in the functional domains of both proteins (T-Box in MGA and bHLH in MGA and MXD4) remaining intact (Fig. 1), a possible mechanism of bringing MXD4-NUTM1 and MGA-NUTM1 fusion proteins to specific areas of the nucleus would be through heterodimerization with MAX and binding of the heterodimers to the DNA. This would tether NUTM1 to specific areas of the nucleus where it could attract p300 resulting in acetylation of neighboring histones. This would imply a bromodomain and extraterminal motif family-independent mechanism of NUTM1 recruitment to the nucleus, which has thus far not been described in the literature. Studies of novel NUTM1 fusion proteins such as ZNF532-NUTM1 and NSD3-NUTM1 have found interactions between these proteins and BRD4 [20, 21] but to the best of our knowledge, no interactions have been described

between the MAX-interacting transcription factor network and BRD4 complexes or between the MAX protein and BRD4 complexes. Additional studies are needed to elucidate if MXD4-NUTM1, MGA-NUTM1 and the recently reported MXD1-NUTM1 and BCORL1-NUTM1 fusion proteins [2] act through a bromodomain and extraterminal motif family-independent mechanism. If so, this would have treatment implications as bromodomain and extraterminal domain inhibitors may not have the same effect on malignancies harboring these novel, bromodomain and extraterminal motif-independent fusions [22].

There were four *NUTM1*-rearranged tumors that were classified as sarcomas. Three of these tumors featured either *MGA-NUTM1* (two cases) or *MXD4-NUTM1* (one case) fusions. One of the *MGA-NUTM1* cases (case 20) showed spindled cells in a myxoid stroma, with resemblance to extraskeletal myxoid chondrosarcoma. The other *MGA-NUTM1* case (case 25) and the *MXD4-NUTM1*-rearranged cecal tumor showed small round cell features, and were keratin negative. Recently, Tamura et al. reported an *MXD4-NUTM1* fusion in a fatal ovarian sarcoma that featured small round cells with abundant eosinophilic cytoplasm, not unlike our *MXD4-NUTM1*-rearranged sarcoma that arose in the cecum (case 22) [23]. Our *MXD4-NUTM1* cecal tumor also shared histologic features with the gastric tumor that harbored an *MXD1-NUTM1* fusion as reported in Dickson et al. [2].

In addition, neoplasms with sarcomatous features have been reported in association with *NUTM1* fusions with traditional partners such as *BRD4*. Den Bakker et al. [3] reported a *BRD4-NUTM1*-rearranged tumor in the parotid gland that showed carcinoma, which transitioned into mesenchymal and chondroid areas, the latter of which were negative for keratins and p63. Dickson et al. [2] recently reported on soft tissue tumors harboring *NUTM1* rearrangements, at least four of which could potentially be considered sarcomas. Patient 4 of that study harbored a *BRD4-NUTM1* fusion but was negative for multiple epithelial markers and histologically showed spindled cells organized in a reticular pattern set in a myxoid stroma, not unlike our case 20. Patient 5 also harbored a *BRD4-NUTM1* fusion, was located in the kidney, showed only focal weak expression of keratins and histologically featured sheets of round to rhabdoid cells. Patient 2 was intramuscular, contained a *BCORL1-NUTM1* fusion, contained areas with chondromyxoid stroma not too different from our case 20 (see Fig. 2d of Dickson et al. [2]) and was negative for multiple keratins. Finally, Patient 3 had a gastric tumor that carried a *MXD1-NUTM1* fusion and histologically showed sheets of eosinophilic rhabdoid cells, which judging by Figs. 3a-c of Dickson et al. is not too dissimilar from our case 22, which arose in the cecal area and contained an *MXD4-NUTM1* fusion. Additional experience with *NUTM1*-rearranged neoplasia may shed light on a possible association between *NUTM1*-rearranged gastrointestinal tumors and *MXD* genes as fusion partners. Furthermore, recent descriptions of highly aggressive *CIC-NUTM1* sarcomas have been described involving the central nervous system, bone, soft tissue and viscera, usually in younger patients [24, 25]. Microscopically they feature monomorphic cells, mostly round, but spindled, epithelioid, and plasmacytoid/rhabdoid cytology can be seen. Tumor cells may grow in sheets with vague lobularity, nests, trabecular, and/or reticular architectures. Stroma is typically scanty in *CIC-NUTM1* sarcomas but can be myxoid. Thus, *CIC-NUTM1* sarcomas show histologic overlap with *CIC-DUX4* sarcoma, NUT carcinomas, and malignant myoepithelial tumors. Interestingly, however, using transcriptomics data, *CIC-NUTM1* sarcomas are distinct from NUT carcinomas but are indistinguishable from other CIC-fused sarcomas. Like NUT carcinomas, *CIC-NUTM1* sarcomas have shown consistent staining for NUT using the C52B1 clone, whereas *CIC-DUX4* sarcomas, *BCOR*-rearranged sarcomas, and Ewing sarcomas are negative for this antibody [24].

The identification of a *MGA-NUTM1* fusion-positive neoplasm in the current study that morphologically resembled extraskeletal myxoid chondrosarcoma (case 20) prompted the staining of a previously constructed tissue microarray composed of 31 cases previously diagnosed as extraskeletal myxoid chondrosarcoma for the NUT immunostain. Notably, 1 of the 31 cases exhibited strong nuclear NUT immunoexpression and was subsequently confirmed to have a rearrangement of the *NUTM1* locus by FISH. These findings, as well as those of Dickson et al. [2] support an expanded histologic spectrum of *NUTM1*-rearranged neoplasia.

Case 11 is the first NUT carcinoma, to our knowledge, to involve the ovary. However, given widespread disease in this patient, it was not clear if the lung or ovary was the primary site in this case. This case and cases 3, 20, 22, 25, and 26 reinforce the concept that *NUTM1*-rearranged neoplasia may be located outside of the anatomic midline.

In summary, most NUTM1-rearranged tumors are carcinomas containing BRD4-NUTM1 or NSD3-NUTM1 fusions, and they often feature focal squamous cell and sometimes neuroendocrine differentiation. The novel *NUTM1* fusion partner *MGA* and the only recently reported *MXD4* fusion partner were identified in tumors showing sarcomatous morphology. Prior to molecular assessment, only 4 of the 26 cases were initially suspected to be *NUTM1*-rearranged tumors emphasizing the value of advanced molecular techniques in solidifying diagnostic classification and providing treatment opportunities such as bromodomain and extraterminal domain inhibitor therapy or related appropriate clinical trial. NUTM1 immunostaining for suspect cases, including those outside the classic

presentation of a midline poorly differentiated carcinoma with "abrupt" squamous differentiation should be considered [18]. If positive, molecular confirmation to include identification of the specific fusion partner by FISH and/or next-generation sequencing is necessary, since it is currently not known if the novel fusion partners respond to bromodomain and extraterminal motif inhibition.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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