USP6 activation in nodular fasciitis by promoter-swapping gene fusions

Nimesh R Patel^{1,2}, John SA Chrisinger³, Elizabeth G Demicco³, Stephen F Sarabia^{1,2}, Jacquelyn Reuther^{1,2}, Erica Kumar^{3,8}, Andre M Oliveira⁴, Steven D Billings⁵, Judith VMG Bovée⁶, Angshumoy Roy^{1,2}, Alexander J Lazar^{3,7}, Dolores H Lopez-Terrada^{1,2} and Wei-Lien Wang^{3,7}

¹Department of Pathology, Texas Children's Hospital, Houston, TX, USA; ²Department of Pathology & Immunology, Baylor College of Medicine, Houston, TX, USA; ³Department of Pathology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA; ⁴Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN, USA; ⁵Department of Pathology, Cleveland Clinic, Cleveland, OH, USA; ⁶Department of Pathology, Leiden University Medical Center, Leiden, The Netherlands and ⁷Department of Translational Molecular Pathology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

Nodular fasciitis is a self-limited myofibroblastic lesion that can be misdiagnosed as a sarcoma as a result of its rapid growth, cellularity, and sometimes prominent mitotic activity. A recurrent translocation t(17;22) has been identified in nodular fasciitis, fusing the coding region of *USP6* to the promoter region of *MYH9*, and resulting in increased *USP6* expression. A subset of cases show *USP6* rearrangement without the typical fusion variants by RT-PCR, or any *MYH9* rearrangement by FISH. We sought to further characterize such tumors using molecular diagnostic assays. A novel RT-PCR assay was designed to detect the two known *MYH9–USP6* fusion types in formalin-fixed paraffin-embedded and frozen tissue, and a break-apart FISH assay was designed to detect *USP6* rearrangement. Twenty-six cases of nodular fasciitis diagnosed between 2002 and 2013 were retrieved from the pathology files of our institutions and were confirmed to be positive by FISH and/or RT-PCR. Seven samples showed *USP6* rearrangement by FISH but were negative for *MYH9–USP6* fusion by RT-PCR; these cases were subjected to a next-generation sequencing assay utilizing anchored multiplex PCR technology. This assay targets a single partner gene associated with fusions in bone and soft tissue tumors for agnostic detection of gene fusion partners. Novel fusion partners were identified in all seven cases and confirmed by RT-PCR. Structurally, all fusions consisted of the juxtaposition of the entire coding region of *USP6* with the promoter of the partner gene, driving increased *USP6* expression. This study confirms the neoplastic nature of nodular fasciitis, defines additional pathogenic fusion partners, and adds to the growing body of literature on *USP6*-associated neoplasia. Given the diagnostic challenges of these tumors, molecular assays can be useful ancillary tools; however, the prevalence of promoter swapping must be recognized when interpreting results.

Modern Pathology (2017) 30, 1577–1588; doi:10.1038/modpathol.2017.78; published online 28 July 2017

Nodular fasciitis is a self-limited myofibroblastic/fibroblastic tumor, which usually presents as a small but rapidly growing mass.^{1–4} Nodular fasciitis can occur in any age, with peak incidence between 20

and 40 years of age, and is found equally in males and females.^{4,5} It is most commonly identified in the upper extremities, head, neck, and trunk, but can be seen almost anywhere,^{6,7} including superficial presentations.⁸ Excision is typically curative and nodular fasciitis may spontaneously regress over time in the absence of treatment.^{1–3,9–12}

Histologically, these tumors are composed of plump fibroblasts and myofibroblasts¹³ that show a 'tissue culture' appearance² and are arranged in short, irregular bundles and fascicles. The cells contain oval nuclei with prominent nucleoli,^{1,2} but there is typically lack of pleomorphism/nuclear atypia and necrosis.¹ The overall appearance can vary, as these tumors can be cellular, hyalinized, or show a variably myxoid matrix.^{1–4,14} In addition,

Correspondence: Current address: Dr NR Patel, MD, Department of Pathology, Rhode Island Hospital and Alpert Medical School at Brown University, 167 Point Street, Coro East 3227, Providence, RI 02903, USA.

E-mail: nimeshrp3@gmail.com

⁸Current address: Department of Pathology, Regional Medical Laboratory, Tulsa, OK, USA.

A preliminary analysis of this work was presented as a poster at the 2012 Annual Meeting of the United States and Canadian Academy of Pathology in Vancouver, Canada.

Received 5 April 2017; revised 10 May 2017; accepted 11 May 2017; published online 28 July 2017

microcystic change, erythrocyte extravasation, lymphocytes, histiocytes, and multinucleated giant cells may be seen.^{1–3} Because of its rapid growth, cellularity, prominent mitotic activity,² and locally infiltrative growth pattern,^{1,3} nodular fasciitis can be misdiagnosed as a sarcoma.^{6,15}

Previously, nodular fasciitis was speculated to be a reactive process because 10–15% of patients report a history of trauma.¹⁴ However, Erickson-Johnson *et al*¹⁶ identified a balanced rearrangement involving the *USP6* gene in 44/48 cases, supporting the idea that nodular fasciitis represents a transient, often self-resolving neoplasm driven by overexpression of *USP6*. 5' RACE PCR and reverse transcription-PCR (RT-PCR) identified two fusion transcript types involving *MYH9* exon 1 and either *USP6* exon 1 or exon 2. However, break-apart fluorescence *in situ* hybridization (FISH) for *MYH9* showed balanced rearrangement in only 31/48 cases. Additional studies using FISH for *USP6* rearrangement have shown a sensitivity of 83–93%^{17–20} and a specificity of 100%.^{18,20} In contrast, RT-PCR yielded only 53–74% sensitivity.^{17,19} These findings suggest that other gene partners may be involved in a subset of these tumors.

We examined a large series of nodular fasciitis cases to characterize alternative fusion genes and transcript variants. Samples were initially screened by FISH and RT-PCR. We then used a next-generation sequencing assay, the FusionPlex Sarcoma Panel (ArcherDx, Boulder, CO, USA), to identify novel fusion events in these samples. This assay performs targeted RNA sequencing using anchored multiplex PCR, which utilizes unidirectional gene-specific primers to detect fusion genes by targeting one of the fusion partners—*USP6* for this present series.²¹ Anchored multiplex PCR technology is particularly suitable for the identification of fusion genes when there is alternative splicing and the possibility of multiple partner genes, as in the case of nodular fasciitis. The FusionPlex Sarcoma Panel targets 127 exons in 26 genes for agnostic detection of gene fusions without *a priori* knowledge of the partner gene. We sought to determine whether this assay would detect fusion transcripts not identified by our RT-PCR assay (which is focused on the known *MYH9–USP6* fusion transcripts).

Materials and methods

Tumor Samples

With institutional review board approval, we searched the pathology archives of three institutions (University of Texas MD Anderson Cancer Center, Texas Children's Hospital, and Leiden University) and identified 26 cases of nodular fasciitis diagnosed between 2002 and 2013, with available formalin-fixed, paraffin-embedded or frozen (case 5) tissue (Table 1). Cases were reviewed by bone and soft

tissue pathologists (WLW, AJL, ED, SB, and JVMGB). Patient age and sex, tumor location, and available clinical information were obtained. One case (case 15) had histological features in keeping with nodular fasciitis but was located in the hand/finger and had a differential diagnosis of cellular fibroma. Seven cases were previously published in a study on superficial/dermal nodular fasciitis,⁸ but were further characterized and re-analyzed for this present study.

USP6 FISH

Break-apart FISH was performed on interphase nuclei for detection of *USP6* rearrangement. A customized probe was developed using direct-labeled bacterial artificial chromosome DNA clones, as previously described.⁸ Briefly, clones RP11-794K10 and RP11-124C16 (telomeric to *USP6*) were both labeled using SpectrumGreen dUTP (Abbott Molecular; Abbott Park, IL, USA). Clones RP11-1022O13 and RP11-457I18 (centromeric to *USP6*) were labeled with SpectrumOrange dUTP (Abbott Molecular). All bacterial artificial chromosome clones were obtained from the BAC-PAC Resource at Children's Hospital Oakland Research Institute (<http://bacpac.chori.org/>).

The probe mixture consisted of 0.5 μ l of each labeled DNA combined with 1 μ l of nuclease-free water and 5 μ l LSI/WCP Hybridization Buffer (Abbott Molecular). Four-micrometer-thick tumor sections were prepared for hybridization using the Paraffin Pretreatment Reagent Kit II (Abbott Molecular), per manufacturer recommendations; however, protease treatment was increased to 32 min. Hybridization was performed at 37 °C for up to 72 h in a ThermoBrite System (Abbott Molecular). Post hybridization, slides were washed in 2× saline-sodium citrate buffer (SSC) with 0.3% IGEPAL at 70 °C for 2 min, followed by two additional washes with 2× SSC with 0.3% IGEPAL at room temperature for 1 min each. Vectashield Mounting Media with 4,6-diamino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA) was used for counter stain and for anti-fading effects. FISH analysis was performed with a multi-filtered fluorescence microscope (Olympus BX51; Olympus; Center Valley, PA, USA) and the CytoVision imaging system (version 7.4, Leica Microsystems Inc.; Buffalo Grove, IL, USA), following standard procedures. FISH signals were scored by two pathologists (DL-T and NRP), and 200 interphase nuclei were scored for fused or split orange and green signals. Cases in which 20% of interphase nuclei had split signals were considered positive for *USP6* rearrangement (Figure 1d).

RT-PCR for *MYH9–USP6* Fusion Transcript

RT-PCR was performed as previously described.⁸ Briefly, total RNA was extracted from formalin-fixed,

Table 1 Clinical–pathologic features

Case no.	Age	Sex	Location	Largest dimension (cm)	Additional history
1	41	Male	Psoas	11.0	Lumbar pain × 2 months
2	14	Male	Cheek	2.0	
3	42	Female	Arm	2.2	
4	12	Female	Left axilla	3.0	
5	13	Male	Left upper arm	3.1	Painless mass × 6 weeks
6	34	Female	Left nasolabial fold	1.0	Painless mass × 3 months (history of radiation to right side of face as infant for hemangioma)
7	18	Male	Right arm	3.3	Painless mass × 3 weeks
8	46	Male	Right hand	2.0	Slow-growing painless mass × 2 months
9	27	Male	Back	3.0	
10	31	Female	Right buccal	2.0	Painless mass × 6 weeks
11	65	Male	Right anti-tragus	1.0	Painless mass × 2 months
12	19	Male	Left parotid gland	2.1	Neck mass × several months
13	21	Female	Neck	2.5	
14	38	Male	Left subscapula	4.0	Incidental mass in patient followed for lymphoma, found to be enlarging over 2 months
15	55	Male	Left hand, third to fourth tendons	3.0	Numbness and tingling in hand for 3 months, decreased range of motion
16	45	Female	Orbit	1.5	
17	19	Male	Skin, right lower back	N/A	
18	60	Male	Skin, right back	N/A	
19	N/A	N/A	N/A	N/A	
20	N/A	N/A	N/A	N/A	
21	6	Male	Left arm	2.0	
22	12	Female	Soft tissue, submandibular	1.0	
23	28	Male	Right palm	5.5	Enlarging mass × 6 months, intermittent pain
24	51	Male	Pelvic lymph node	2.2	History of lymphoma, stem cell transplant; restaging revealed PET-avid inguinal adenopathy
25	19	Male	Right pelvis	5.2	Incidental mass found in patient presenting with abdominal pain, nausea, and vomiting
26	40	Female	Right palm	3.1	Sharp pain after mopping, mass × 1 month

N/A: not available.

paraffin-embedded tissue scrolls using the Ambion RecoverAll Total Nucleic Acid Isolation Kit (Ambion, Life Technologies, ThermoFisher; Austin, TX, USA), as per the manufacturer's instructions. Reverse transcription of RNA to cDNA was performed using the Invitrogen Super Script III Reverse Transcriptase Kit (Invitrogen, Life Technologies; Carlsbad, CA, USA), according to the manufacturer's instructions, with random hexamers as primers. One forward primer (MYH9-For: 5'-GCACGGAAGGCT AAGCAAG-3') and two reverse primers (USP6-RS: 5'-GGATGTGGATGTGAACTGCG-3' and USP6-RL: 5'-CGGTGTCCCTTGTCATACTTC-3') were used. Primers were designed to detect the two known fusion types¹⁶ (Figure 1a–c). PCR was performed with an annealing temperature of 55 °C.

PCR products were resolved on 2% agarose gel by electrophoresis with ethidium bromide staining for visualization. Bidirectional Sanger sequencing of the PCR products was performed to confirm amplicon identity. Analysis was performed using web-based alignment tools and gene databases (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>, <http://www.ncbi.nlm.nih.gov/gene>) with mRNA reference sequences for *MYH9* (NM_002473.5) and *USP6* (NM_004505.3).

Amplification of the housekeeping gene beta-actin was used as an RNA integrity control. Positive controls for *USP6–MYH9* fusion consisted of clonally

amplified plasmids derived from sequence-confirmed cDNA from positive patient samples. In addition, negative controls were tested with each set of reactions.

Targeted RNA Sequencing by Anchored Multiplex PCR

Total RNA was extracted from nine formalin-fixed, paraffin-embedded nodular fasciitis specimens and quantified using the Qubit RNA assay (ThermoFisher Scientific; Waltham, MA, USA). Next-generation sequencing libraries were prepared with anchored multiplex PCR-based methodology as previously described,²¹ using the FusionPlex Sarcoma Panel. Libraries were generated according to the manufacturer's protocol using 100 ng of total RNA. Amplifiable cDNA quality was assessed using the PreSeq RNA QC assay. Final libraries were quantified using the KAPA Library Quantification Kit (Kapa Biosystems, Roche; Wilmington, MA, USA) for Illumina Platforms. Illumina paired-end indexed libraries were sequenced 8-plex on a MiSeq (2 × 150 bp, v2 chemistry) and data analyzed on a vendor-provided virtual-machine-based analysis pipeline with custom-developed output scripts. On average, sequencing with the Archer FusionPlex Sarcoma Panel generated a total of 1.07 million

Figure 1 (a) Schematic of alternative *MYH9-USP6* fusion types with corresponding design of RT-PCR primers. (b) Schematic of the resulting fusion transcripts, showing the juxtaposition of *MYH9* exon 1 (encoding the 5'-UTR) to either exon 1 or 2 of *USP6*. Both fusion transcripts contain the entire coding sequence of *USP6*. Fusion type 1 may be detected with both brown (RS) and blue (RL) reverse primers, while fusion type 2 can only be detected using the blue (RL) reverse primer. (c) Case 5, electropherograms showing the type 1 (top) and type 2 (bottom) fusion transcripts with in-frame fusion of *MYH9* to *USP6*. (d) Case 2, break-apart FISH for *USP6* showing rearrangement with splitting of the green and orange signals.

paired-end reads with ~28 980 unique RNA reads per sample. Sequencing quality was assessed by % purity-filtered reads, % bases >Q30, total number of reads, total number of unique RNA reads, and percent of aligned reads with high mapping quality. Eight of nine cases yielded optimal sequencing metrics, with one case, case 10, showing low unique RNA read counts (12 751 unique RNA reads). Therefore, eight out of nine cases proceeded forward to analytic processing and fusion detection. All detected fusion genes were confirmed using RT-PCR, followed by Sanger sequencing and gel electrophoresis, as described above but with specifically designed PCR primers.

Results

Clinical and Pathologic Features of Samples

Clinical and pathological parameters are summarized in Table 1. Mean age was 31.5 years (range =

6–65), with a male to female ratio of 2:1. The most common tumor sites included the upper extremities and head and neck, with additional locations including pelvis and pelvic lymph node. One case was initially diagnosed as sarcoma. Tumors ranged in size from 1.0 to 11.0 cm, with the largest sample located within the psoas muscle (case 1). In all, 10 samples were biopsies, while 16 were excision specimens (Table 2). Histologically, the samples showed a combination of various patterns consistent with nodular fasciitis, including hypercellular, sclerotic, myxoid degeneration, and microcystic change (Figure 2). Notable cases include the 11.0 cm tumor, which is larger than usual for nodular fasciitis (case 1), and another case located in the tendon of the hand (case 15). In both cases, the histological findings were consistent with the diagnosis of nodular fasciitis (Figure 3). Follow-up information was available for 12 cases, with a mean follow-up time of 12.5 (1–33) months. In 10 cases, the tumor was excised without recurrence. In the remaining 2 cases, the tumor was not excised, showing regression in 1 case and lack of significant growth in the other.

USP6 FISH

FISH was successfully performed in 20 cases (Table 2), yielding positive results for *USP6* locus rearrangement in all cases. Case 1 showed a variant pattern consisting of two copies of fused signals and one extra orange signal, suggestive of copy gain with an unbalanced translocation and retention of the 3'-portion of *USP6*. For the remainder of cases, testing was not performed or was not successful due to insufficient tissue or inadequate quality due to pre-analytical variables.

RT-PCR for *MYH9-USP6* Fusion Transcript

RT-PCR was successfully performed in 20 cases and fusion transcripts were identified in an overlapping set of 13/20 samples (Table 2). Five cases showed only fusion type 1, while 8 cases showed the presence of both the type 1 and type 2 transcripts, likely as a result of alternative splicing.^{16,22} All RT-PCR-positive cases were positive by FISH, when results were available. RT-PCR was also positive in 5 cases in which there was insufficient or unavailable tissue for FISH. In contrast, 2 cases were insufficient for RT-PCR testing, but yielded positive results by FISH. Of the 7 negative RT-PCR cases, 6 were positive by FISH, while FISH was not performed in the remaining case.

Targeted RNA Sequencing by Anchored Multiplex PCR

Paired-end sequencing of target-enriched RNA libraries prepared from eight nodular fasciitis cases

Table 2 Summary of molecular results

Case no.	Specimen type	USP6 FISH	MYH9-USP6 RT-PCR	Fusion type	FusionPlex
1	Excision	Positive/unbalanced	Negative	1	<i>RRBP1-USP6</i>
2	Excision	Positive	Positive	1	N/A
3	Excision	Positive	Positive	1	N/A
4	Excision	Positive	Positive	1 and 2	<i>MYH9-USP6</i>
5	Biopsy	Positive	Positive	1 and 2	N/A
6	Excision	Positive	Positive	1 and 2	N/A
7	Excision	Positive	Positive	1 and 2	N/A
8	Excision	Positive	Positive	1 and 2	N/A
9	Biopsy	Positive	Positive	1 and 2	N/A
10	Biopsy	Positive	N/A	N/A	N/A
11	Excision	Positive	N/A	N/A	N/A
12	Excision	Positive	Negative	3	<i>CALU-USP6</i>
13	Biopsy	Positive	Negative	1 and 2	<i>CTNNB1-USP6</i>
14	Excision	Positive	Negative	1	<i>MIR22HG-USP6</i>
15	Excision	Positive	Negative	2	<i>SPARC-USP6</i>
16	Excision	Positive	Negative	1 and 2	<i>THBS2-USP6</i>
17	Excision	Positive	N/A	N/A	N/A
18	Biopsy	Positive	N/A	N/A	N/A
19	Excision	Positive	N/A	N/A	N/A
20	Biopsy	Positive	N/A	N/A	N/A
21	Excision	N/A	Negative	1 and 2	<i>COL6A2-USP6</i>
22	Excision	N/A	Positive	1	N/A
23	Biopsy	N/A	Positive	1	N/A
24	Biopsy	N/A	Positive	1	N/A
25	Biopsy	N/A	Positive	1 and 2	N/A
26	Biopsy	N/A	Positive	1 and 2	N/A

N/A: not available.

using the FusionPlex Sarcoma kit yielded an average of 1.07×10^6 paired-end reads and 28 980 unique RNA reads per sample. Analysis of FASTQ files using the Archer Analysis pipeline for fusion gene detection revealed *USP6* fusions in eight of eight (100%) cases, including a control case that was also positive by *USP6* FISH and *MYH9-USP6* RT-PCR. In the remaining seven cases, *USP6* fusions were detected with novel 5'-gene partners for nodular fasciitis, including *RRBP1* (NM_001042576.1), *CALU* (NM_001219.4), *CTNNB1* (NM_001098210.1), *MIR22HG* (NR_028502.1), *SPARC* (NM_003118.3), *THBS2* (NM_003247.3), and *COL6A2* (NM_001849.3). FusionPlex data are summarized in Table 3. All novel fusions were confirmed using RT-PCR, followed by Sanger sequencing. RT-PCR and FusionPlex data were re-analyzed using public databases and the fusion transcripts identified in each case are summarized in Table 4.

Analysis of the novel fusion transcripts reveals that they are structurally identical to the *MYH9-USP6* fusion (Figure 4). Each case shows fusion of the 5'-UTR of the upstream gene to either exon 1 (5'-UTR) or exon 2 (one base upstream of the ATG translation initiation codon) of *USP6*. Some cases show multiple fusion types, which show the same *USP6* fusion sites as the type 1 and type 2 *MYH9* fusions (Table 4). The fusions result in promoter swap, such that the promoter and associated regulatory sequences of the 5'-partner gene are fused to the complete coding region of *USP6*. Activation of the 5'-gene promoter should therefore drive *USP6*

expression. In the FusionPlex assay, each of the fusion genes was supported by moderate-to-high unique split read counts (range: 92–1220 reads), with the fusion-to-wild-type fraction ranging from 20 to 100%, suggestive of elevated *USP6* expression from the rearranged loci.

Seven of the eight cases, including the *MYH9-USP6* fusion, show inter-chromosomal rearrangements, with one case (case 14) characterized by an intra-chromosomal rearrangement between *MIR22HG* and *USP6*, which are located 3.4 MB apart on chromosome 17. The *MIR22HG-USP6* gene fusion within case 14 is the only fusion identified in our study that occurs between a non-coding RNA gene and *USP6*. Furthermore, an additional case (case 12) has an insert within the fusion transcript, which maps to a portion of intron 1 of the 5'-gene (*CALU*). Case 1, which was 11 cm in greatest dimension, showed *RRBP1-USP6* fusion, confirming the diagnosis of nodular fasciitis. No significant association was identified between the fusion variants and either clinical behavior or histologic features of the tumors.

Discussion

USP6 is a hominoid-specific gene primarily expressed in testicular tissue that is located on chromosome 17p13 and is part of a family of de-ubiquitinating enzymes.²³ Rearrangements involving the *USP6* gene were first identified and characterized in aneurysmal bone cysts by Oliveira *et al*^{24,25}

Figure 2 Representative histological features of nodular fasciitis cases seen in this series. (a) Vaguely circumscribed tumor involving skeletal muscle ($\times 1$, H&E). (b) Cellular areas with tissue culture-like pattern and myxoid areas. (c) Myofibroblastic cytomorphology with extravasated red blood cells and lymphocytes. (d) Mitotic figures can be present. (e) Some tumors had prominent myxoid 'pseudocystic' areas. (f) Others had thick hyalinized bundles reminiscent of keloid collagen.

Subsequently, multiple fusion partners for *USP6* were discovered in these tumors.²⁶ Similar to nodular fasciitis, aneurysmal bone cyst is a benign tumor that grows rapidly. It was demonstrated that these tumors arise as a result of a promoter-swapping

mechanism that drives transcriptional upregulation of *USP6* (refs 26,27) (a mechanism of transformation seen in many tumor types). Subsequently, *MYH9-USP6* fusion was identified in nodular fasciitis and it was shown that increased expression of *USP6* could

Figure 3 (a) Case 1. Cross section of nodular fasciitis, which was unusually large, measuring 11.0 cm in longest dimension (specimen sectioned perpendicular to longest dimension). This tumor had classic histological features (b) and was positive for *RRBP1-USP6* fusion transcripts. The patient had no evidence of disease at 33 months' follow-up. (c) Case 12. Low power of a 3.0 cm tumor involving the tendons of the finger with histological features of nodular fasciitis (d). This tumor was found to harbor *SPARC-USP6* fusion transcripts. The patient had no evidence of disease at 7 months' follow-up.

induce formation of a tumor histologically and clinically similar to nodular fasciitis in xenografts.¹⁶

In our study, FISH, RT-PCR, and targeted RNA sequencing assays were developed and utilized for the detection of *USP6* rearrangement in nodular fasciitis. For clinical laboratories, several factors must be considered when implementing various methodologies to detect genomic events associated with tumors. For the detection of fusion transcripts, RT-PCR can be an ideal method when there are a limited number of fusion variants. However, RNA extracted from formalin-fixed, paraffin-embedded tissue is often fragmented to < 300 bases in length and this fragmentation increases with storage time²⁸ and more harsh conditions. Assays that are not appropriately designed may result in false negatives.

On the basis of the previously described fusion types,¹⁶ we designed an RT-PCR assay to detect both types 1 and 2. Several cases in our study harbored both fusion transcripts in the same sample. Alternative splicing has been well documented in fusion

genes associated with bone and soft tissue tumors,^{22,29,30} and this likely explains the findings in our cases and in similar cases in the literature¹⁶ (Figure 1). Such an assay is highly specific, but it cannot detect other fusion partners. Moreover, if there is alternative splicing, amplicons that are larger or have different fusion sites may not be detected.

A break-apart FISH assay is especially useful when there is a single gene fused with a variety of partner genes, as in the case of aneurysmal bone cyst or, now, nodular fasciitis. *USP6* rearrangement has also been identified in a variety of other tumors, including cellular fibroma of tendon sheath³¹ and giant cell reparative granulomas of the hands and feet (a subset of aneurysmal bone cyst),³² allowing for the implementation of a single assay across these diseases. Occasionally, unusual patterns may be identified and it can be difficult to determine the clinical significance. The pattern identified in case 1 was also described in one of six cases analyzed by Papp *et al*,³³ consisting of two fused signals and one extra

Table 3 Summary of FusionPlex results

Case no.	Fusion	5'-gene	5'-transcript	5'-gene GRCh37 fusion position	5'-fusion point location	3'-gene	3'-transcript	3'-gene GRCh37 fusion position	3'-fusion point location
1	RRBP1-USP6	RRBP1	NM_001042576	Chr20: 17660644	Exon: 2 (5'-UTR)	USP6	NM_004505	Chr17: 5033231	Exon: 1
4	MYH9-USP6	MYH9	NM_002473	Chr22: 36783852	Exon: 1 (5'-UTR)	USP6	NM_004505	Chr17: 5033231	Exon: 1
12	CALU-USP6	CALU	NM_001219	Chr7: 128381146	Intron: 1 (5'-UTR)	USP6	NM_004505	Chr17: 5033282	Exon: 1
13	CTNNA1-USP6	CTNNA1	NM_001098210	Chr3: 41241161	Exon: 1 (5'-UTR)	USP6	NM_004505	Chr17: 5033231	Exon: 1
14	MIR22HG-USP6	MIR22HG	NR_028502	Chr17: 1619424	Exon: 1 (ncRNA exonic)	USP6	NM_004505	Chr17: 5033231	Exon: 1
15	SPARC-USP6	SPARC	NM_003118	Chr5: 151066426	Exon: 1 (5'-UTR)	USP6	NM_004505	Chr17: 5033662	Exon: 2
16	THBS2-USP6	THBS2	NM_003247	Chr6: 169653819	Exon: 2 (5'-UTR)	USP6	NM_004505	Chr17: 5033231	Exon: 1
21	COL6A2-USP6	COL6A2	NM_001849	Chr21: 47518087	Exon: 1 (5'-UTR)	USP6	NM_004505	Chr17: 5033231	Exon: 1
								Chr17: 5033662	Exon: 2

3'-signal. In our experience, normal cases may show split signals in a low percentage of cells. When interpreting results, it is important to adhere to strict criteria regarding number of cells scored, distance between the orange and green signals, and validated cutoff for percentage of split cells in positive cases.

The discrepancy between FISH and RT-PCR results in our study suggested the possibility of alternative fusion types or partner genes. Alternative fusion partners have already been described in aneurysmal bone cyst and Guo *et al*³⁴ identified PPP6R3-USP6 amplification in a case they termed 'malignant nodular fasciitis.' Anchored multiplex PCR is a powerful method for identifying alternative fusion partners, although it is not completely unbiased, as one partner gene must be targeted. During the composition of this manuscript, several other groups published studies using the Archer FusionPlex Sarcoma Panel to characterize USP6 rearrangement partners in aneurysmal bone cyst³⁵ and cellular fibroma of tendon sheath.³¹

Of 26 total positive cases in our study of nodular fasciitis, 7 showed novel fusion partners, expanding the spectrum of genes known to be fused with USP6 in this tumor. In addition to encoding a ubiquitin-specific protease, the USP6 protein also contains a TBC (Tre-2/Bub2/Cdc16) domain implicated in GTPase regulation and trafficking.²³ The two MYH9-USP6 fusion types show retention of both the TBC and UBP (or USP, ubiquitin-specific peptidase) domains.²⁰ Similar to MYH9 fusion, some of the novel fusions showed alternative splicing, with the simultaneous presence of both fusion transcripts (as detected by next-generation sequencing and/or RT-PCR; Table 4). In all cases, the entire coding sequence of USP6 is retained. USP6 functions in remodeling of the cytoskeleton and extracellular matrix, inflammatory response, cell signaling, cellular trafficking, and protein turnover,^{23,27,36-44} but its specific role in tumorigenesis is not entirely clear. More recently, Quick *et al*⁴⁵ reported that Jak1-STAT3 signaling has an essential role in USP6-related tumorigenesis, while Madan *et al*⁴⁶ suggested a role for Wnt signaling.

In the majority of nodular fasciitis cases, USP6 fuses to MYH9, which is located on chromosome 22q12.3-q13 and encodes for a non-muscle myosin protein involved in cytokinesis, cell motility, maintenance of cell shape, and disassembly of the actin network in crawling cells.¹⁶ In the body, it is normally present at low levels, except in fibroblasts, endothelial cells, macrophages, leukocytes, and certain renal cells. Mutations have been associated with macrothrombocytopenias,⁴⁷ and MYH9 has been reported to be fused to ALK in a case of anaplastic large cell lymphoma.⁴⁸

We identified several novel fusion partners for USP6 in nodular fasciitis. COL6A2 encodes for the collagen type VI alpha 2 chain. Mutations in COL6A2 are associated with inherited muscular dystrophies.⁴⁹ Collagen VI is enriched in the

Table 4 Fusion types

Case no.	Fusion type	5'-gene	Fusion position (exon/intron)	Fusion position (GRCh37)	5'-gene fusion point sequence	Insert position (exon/intron)	Insert coordinates (GRCh37)	Insert sequence	3'-gene	Fusion position (exon/intron)	Fusion position (GRCh37)	3'-gene fusion point sequence
1	1	RRBP1	End of exon 2	Chr20: 17660644	GCAGACCTCCTA				USP6	Within exon 1	Chr17: 5033231	GAAACTGGGCAT
4	1	MYH9	End of exon 1	Chr22: 36783852	ATCCAGGTTTCAG				USP6	Within exon 1	Chr17: 5033231	GAAACTGGGCAT
4	2	MYH9	End of exon 1	Chr22: 36783852	ATCCAGGTTTCAG				USP6	Start of exon 2	Chr17: 5033662	GATGACATGCT
12	3	CALU	End of exon 1	Chr7: 128379486	CTAGGAGCAAG	CALU intron 1	Chr7:128381122-128381146	GTATACCAAGGA TATTGAAAGACA	USP6	Within exon 1	Chr17: 5033282	AGACCTCTGGTG
13	1	CTNNB1	End of exon 1	Chr3: 41241161	TCTTCCCTGAG				USP6	Within exon 1	Chr17: 5033231	GAAACTGGGCAT
13	2	CTNNB1	End of exon 1	Chr3: 41241161	TCTTCCCTGAG				USP6	Start of exon 2	Chr17: 5033662	GATGACATGCT
14	1	MIR22HG	End of exon 1	Chr17: 1619424	ACCCAGCACCCAG				USP6	Within exon 1	Chr17: 5033231	GAAACTGGGCAT
15	2	SPARC	End of exon 1	Chr5: 151066426	CTTGCCACTGAG				USP6	Start of exon 2	Chr17: 5033662	GATGACATGCT
16	1	THBS2	End of exon 2	Chr6: 169653819	TTGGCAAAACCAG				USP6	Within exon 1	Chr17: 5033231	GAAACTGGGCAT
16	2	THBS2	End of exon 2	Chr6: 169653819	TTGGCAAAACCAG				USP6	Start of exon 2	Chr17: 5033662	GATGACATGCT
21	1	COL6A2	End of exon 1	Chr21: 47518087	CCTGGGACCCAG				USP6	Within exon 1	Chr17: 5033231	GAAACTGGGCAT
21	2	COL6A2	End of exon 1	Chr21: 47518087	CCTGGGACCCAG				USP6	Start of exon 2	Chr17: 5033662	GATGACATGCT

pericellular matrix of tendon fibroblasts and has an important role in tendon repair.⁴⁹

THBS2 encodes for a member of the thrombospondin family of extracellular proteins, which function in cell-to-cell and cell-to-matrix communication.⁵⁰ Thrombospondin-2 (TBS-2) has also been shown to inhibit angiogenesis.⁵⁰ In Ewing sarcoma, the *EWSR1-FLI* fusion gene downregulates TBS-2.⁵¹

Case 12 showed *CALU-USP6* fusion, with the fusion transcript containing an insertion that maps to *CALU* intron 1. The inclusion of a cryptic exonic sequence in the fusion transcript occurs commonly in other tumors, and we previously showed this is a recurrent event in epithelioid hemangioendotheliomas.²² This may be related to alterations in splice sites or splicing enhancers and silencers as a result of chromosomal translocation.²² Calumenin, encoded by *CALU*, is a calcium-binding protein belonging to the CREC protein family.⁵² Different isoforms are localized to different parts of the secretory pathway, including the endoplasmic reticulum, Golgi apparatus, and extracellular medium. Calumenin is thought to have a role in inhibiting tumor metastasis, and its expression levels have been reported to be altered in cancer of the head and neck, endometrium, liver, pancreas, lung, and colon.⁵³ *CALU* expression was demonstrated to be increased in the tumoral stroma (cancer-associated fibroblasts) and epithelium of colorectal cancer samples.⁵⁴

Activating mutations in *CTNNB1* have been associated with desmoid fibromatosis,⁵⁵ indicating a role in fibroblastic tumors. *CTNNB1* encodes beta-catenin, which interacts with APC and has a well-characterized role in the Wnt signaling pathway in most cell types and cellular adhesion in epithelial cells. Furthermore, *CTNNB1-PLAG1* fusion in pleomorphic adenomas of the salivary gland has been shown to result in activation of *PLAG1* by a promoter-swapping mechanism.^{56,57} Of note, *CTNNB1-USP6* fusion was recently identified in a single case of aneurysmal bone cyst,³⁵ another member of the biological spectrum of *USP6*-induced neoplasms.²⁰

MIR22HG is a long non-coding RNA that is downregulated in lung adenocarcinoma⁵⁸ and has been shown to be a responder to chemical stress,⁵⁹ microgravity,⁶⁰ and hypoxia.⁶¹ It serves as host gene for miR-22, which functions as a tumor suppressor by post-transcriptional regulation of p21 and represses cancer progression by inducing cellular senescence.⁶⁰

SPARC encodes a cysteine-rich acidic matrix-associated protein. It is a member of a family of proteins that modulate interactions between cells and their environment by regulating growth factor signaling and extracellular matrix assembly and deposition.⁶² *SPARC* functions as both a secreted glycoprotein and an intracellular and membrane-associated protein that regulates cellular apoptotic pathways.⁶² It shows increased expression in

Figure 4 Schematic of fusion transcripts. The top pane shows the two *MYH9*–*USP6* fusion types, both of which include the entire coding sequence of *USP6* (transcription start site indicated by an arrow). The TBC and USP (UBP) domains of the *USP6* protein are also indicated. In the remaining panes, the other novel fusion transcripts identified in this study are shown. Case 12 shows the inclusion of a cryptic exon from *CALU*, while cases 13, 16, and 21 show the presence of two fusion transcripts, with identical *USP6* fusion sites as those seen in *MYH9* fusions.

epithelial cells from tissues with a high turnover rate during tissue injury and inflammation, as well as during abnormal tissue growth associated with neoplasia.⁶² Interestingly, this fusion was found in a case occurring in the tendon of the hand (case 15). The histological features were in keeping with nodular fasciitis, but given the location, a differential diagnosis of cellular fibroma could also be entertained. Recently, Carter *et al*³¹ described *USP6* gene rearrangements in six of nine cellular fibromas of tendon sheath by FISH. No *MYH9* rearrangements or *MYH9*–*USP6* and *CDH11*–*USP6* fusion transcripts were detected by RT-PCR assays. Given the

overlapping features, they postulate that some cellular fibromas of tendon sheath may in fact be tenosynovial nodular fasciitis. Our case also highlights the similarities between these entities, both histologically and molecularly, and now with a fusion partner of *SPARC*.

All of the fusion partners for *USP6* likely provide promoters that lead to increased transcription over what would be seen if the *USP6* promoter was left in place. Some of these fused promoters could represent genes that are activated specifically within the cellular context of the myofibroblasts that comprise nodular fasciitis—a conjecture that requires further investigation.

To our knowledge, we also report the largest nodular fasciitis case with molecular confirmation in the literature. Although most cases are small, tumors up to 9 cm have been previously described.^{63,64} Case 1 in our study was 11 cm and showed an unusual FISH pattern with unbalanced translocation and *RRBP1*–*USP6* fusion by next-generation sequencing. The association between the phenotype (ie, large size) and fusion variant is not certain; however, the patient was noted to be alive with no evidence of disease (recurrences or metastases) 10 years post excision. Ribosome-binding protein 1 (*RRBP1*) encodes for a coiled-coil protein that has a role in microtubule binding and in the interaction between the endoplasmic reticulum and ribosomes.⁶⁵ *RRBP1* is highly expressed in cells such as fibroblasts and is necessary for secretory activity.⁶⁶ Overexpression of *RRBP1* is associated with poor prognosis in breast and colorectal cancer, and *RRBP1*–*ALK* fusion has been identified in several cases of epithelioid inflammatory myofibroblastic sarcoma.⁶⁵

As previously mentioned, Guo *et al*³⁴ described a patient who had multiply recurrent nodular fasciitis over 10 years before developing a metastasis.³⁴ This is the first reported case of nodular fasciitis with malignant behavior, and the tumor was found to harbor a novel fusion transcript *PPP6R3*–*USP6*. This fusion was not seen in our series, and no differences were seen in the behavior and histological features in the tumors of various fusion transcripts and types.

In summary, we identified seven novel fusion partners for *USP6* in nodular fasciitis, highlighting the importance of *USP6* expression and promoter-swapping fusions in the etiology of this neoplasm. The partner genes normally have a role in the interaction between the cell and extracellular matrix, inflammation, myo/fibroblastic activity, or stress response. The association of the coding sequence of *USP6* with the promoter of these genes results in increased expression of *USP6* within the milieu that gives rise to nodular fasciitis. This may account for the histologic features of inflammation and myofibroblastic proliferation associated with these tumors. Molecular testing for *USP6* rearrangement can be a useful ancillary tool, though pathologists need to be cognizant of the limitations of

each technique to detect multiple partners. Recently, a similar study examining aneurysmal bone cysts by anchored multiplex PCR expanded the spectrum of associated partner genes³⁵ and, together, this provides additional evidence for the neoplastic nature of USP6-associated tumors. Further studies must be performed to elucidate the biology and phenomenon of transient neoplasia inherent to nodular fasciitis.

Acknowledgments

We thank Karen Prince and Kim-Anh Vu for their help with the preparation of the images, as well as Hadi Sayeed, Kayuri Patel, Vijetha Kumar, Angela Major, and E Faith Hollingsworth for assistance in experimental procedures.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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