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Detection of myxoid liposarcoma-associated *FUS–DDIT3* rearrangement variants including a newly identified breakpoint using an optimized RT-PCR assay

Martin P Powers[1,*], Wei-Lien Wang[2], Vivian S Hernandez[1], Kayuri S Patel[1], Dina C Lev[3,4], Alexander J Lazar[2,4] and Dolores H López-Terrada[1]

[1]*Division of Molecular Pathology, Department of Pathology, Baylor College of Medicine and Texas Children's Hospital, Houston, TX, USA;* [2]*Department of Pathology, MD Anderson Cancer Center, Houston, TX, USA;* [3]*Department of Cancer Biology, MD Anderson Cancer Center, Houston, TX, USA and* [4]*Department of Sarcoma Research Center, MD Anderson Cancer Center, Houston, TX, USA*

Myxoid/round cell liposarcoma is characterized by the recurrent translocations t(12;16)(q13;p11) and, less commonly, t(12;22)(q13;q12), which fuse *FUS* or *EWSR1*, respectively, to *DDIT3* on chromosome 12. Although a number of different variant breakpoints have been described, greater than 90% of all cases have one of the three different *FUS–DDIT3* fusions, which may have clinical significance. To identify the individual breakpoints, a sequence-specific assay such as reverse transcription-PCR (RT-PCR) is needed. In this study, we optimized primer design to develop an RT-PCR assay for the detection of the most common translocations in formalin-fixed paraffin-embedded tissue specimens. We compared our assay with primers previously published for testing formalin-fixed paraffin-embedded specimens and achieved the most consistent results with our primers. We obtained RNA from 32 MLS cases, of which 27 carried one of the three common *FUS–DDIT3* chimeric transcript types. Four of the negative cases were from very small biopsies with very low RNA concentration. One case was consistently negative by RT-PCR, but showed a *FUS* rearrangement by fluorescent *in situ* hybridization, suggesting that it may harbor one of the rarer *FUS–DDIT3* chimeric types. In addition to the common fusions, our assay also identified a novel *FUS–DDIT3* fusion between exon 9 of *FUS* and exon 3 of *DDIT3* in one of the cases.
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Myxoid/round cell liposarcoma represents ~10% of all adult sarcomas and ~33% of all liposarcomas.[1] It tends to affect the extremities of young adults, and is usually treated by surgical resection, beyond which few therapies are available. A higher grade, defined partially by an increasing percentage of round cell component, is associated with a worse prognosis.[2,3] Conventional myxoid liposarcomas have an 80–90% 12-year survival, whereas tumors with a significant round cell component have an ~50% 12-year survival.[4] Conventional myxoid liposarcoma classic histology consists of primitive nonlipogenic mesenchymal cells, chicken wire vasculature, and a myxoid background, but the morphological spectrum of tumors ranges from bland and hyalinized to a more chondroid stroma, and includes the higher-grade cellular round cell liposarcoma.[1] Lipoblasts may be seen, but are not required for diagnosis. The differential diagnosis includes other myxoid tumors including myxofibrosarcoma and myxoid chondrosarcoma, as well as lipoblastoma. The hypercellular round cell variants may be confused with other

Correspondence: Dr DH López-Terrada, MD, PhD, Division of Molecular Pathology, Department of Pathology, Texas Children's Hospital, MC 1-2261, 6621 Fannin, Houston, TX 77030, USA.
E-mail: dhterrad@texaschildrens.org
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*Current address: Departments of Pathology and Laboratory Medicine, University of California, San Francisco, CA, USA.
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cellular small round cell tumors, especially in smaller biopsies.

Myxoid/round cell liposarcoma is characterized by a recurrent and virtually diagnostic translocation and gene fusion, the t(12;16)(q13;p11), seen in over 90% of cases, which fuses the 5′ half of the *FUS* gene on chromosome 16 with the entire reading frame of the *DDIT3* gene on chromosome 12.[2,5,6] A much smaller fraction of myxoid liposarcoma cases harbor a similar variant translocation and gene fusion, the t(12;22)(q13;q12), which fuses the *EWSR1* gene to the *DDIT3* gene.[7] *FUS* and *EWSR1* are similar genes, ubiquitously expressed, with a transcriptional activation domain in their 5′ end that is fused to the entire coding region of *DDIT3*, which encodes an apparent DNA-binding and dimerization domain. This novel chimeric transcription factor is oncogenic for myxoid liposarcoma and inhibits adipocytic differentiation.[8–10] Translocations variably include portions of the RNA-binding domain of *FUS* or *EWSR1*, and the oncogenic gene fusion may alter RNA splicing.[11]

To date, at least 10 different mRNA breakpoints have been described for *FUS–DDIT3* (Figure 1) and at least 4 for *EWSR1–DDIT3*.[2,12,13] Most cases of myxoid liposarcoma are one of three different *FUS–DDIT3* chimeric types, including varying portions of *FUS*. The other variants are rare and most have only been reported once or twice. Two studies have looked at the clinical impact of the *FUS–DDIT3* fusion variants with conflicting results, and no strong clinical significance has been seen in either study.[2,12] However, recent data using a novel chemotherapeutic agent, trabectedin, suggest a possible role for gene fusion variant in predicting a response to therapy.

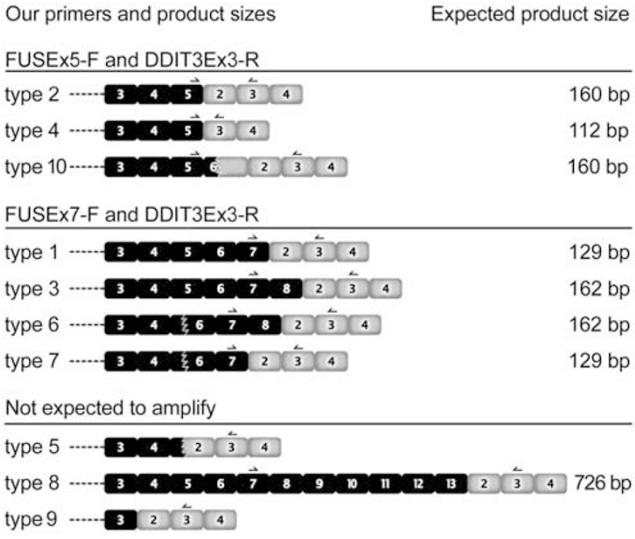

Figure 1 Primer location and expected PCR product size. The top half of this figure shows the location of our FUSEx5-F primers and expected amplicon size from the type 2, 4, and 10 *FUS-DDIT3* fusions. The middle panel shows where our FUSEx7-F primers bind and the expected amplicon size from types 1, 3, 6, and 7. Note that the types 3 and 6 and 1 and 7 give the same product. The bottom panel shows the previously reported *FUS–DDIT3* fusions not expected to amplify with our primers. Types 5 and 9 do not have a binding site for one of our forward primers. Type 8 fusion could theoretically amplify with our primers, but a 726 bp product is unlikely to be amplified from FFPE tissue.

Trabectedin (ET-743) is a compound isolated from a sea squirt, which binds to the minor groove of DNA, and may act by modifying gene expression or nucleotide excision repair.[14] Many patients with myxoid liposarcoma have responded to this drug, and it was noted that patients treated with tumors carrying a type 3 *FUS–DDIT3* chimeric transcript showed no response to this agent.[15] In addition, an *in vitro* study of the drug on myxoid liposarcoma cell lines showed that treatment with trabectedin lead to dissociation of the *FUS–DDIT3* protein product from chromatin and promotes gene expression consistent with terminal adipogenesis, but this response was only seen in the cell lines with the type 1 *FUS–DDIT3* fusion and not in a cell line with the rarer type 8 gene fusion.[16] Of note, both the type 3 and type 8 gene fusions have more of the RNA-binding domain from *FUS* included in the gene fusion. Although these data are incomplete, it suggests that as new therapies are developed, the specific gene fusion type may become relevant, which highlights the need for assays that can differentiate the gene fusion type in the clinical setting.

Formalin-fixed paraffin-embedded tissue is the most commonly available diagnostic specimen in pathology. Routine cytogenetic analysis requires fresh tissue and would not subtype the fusion type. Fluorescent *in situ* hybridization (FISH) probes are commercially available for *DDIT3*, *FUS*, and *EWSR1* genes, and can be performed on formalin-fixed paraffin-embedded tissue, but provide no information about the specific fusion partner or mRNA breakpoints. Reverse transcription-PCR (RT-PCR) can be carried out on formalin-fixed paraffin-embedded tissues, but because of RNA degradation, amplicon sizes are small and controls for RNA integrity are needed to avoid false-negative results. Two studies have carried out RT-PCR on formalin-fixed paraffin-embedded tissue in myxoid liposarcoma.[12,17] In this study, we sought to develop a clinically robust RT-PCR assay that could be used on formalin-fixed paraffin-embedded samples for the most common translocations seen in myxoid liposarcoma. We evaluated the primers in the literature and used computational tools to analyze the previously published primers and to design our own, more efficient, PCR primers and assay conditions, which were needed in developing a clinical test.

Materials and methods

Cases

A total of 34 unique histologically confirmed myxoid liposarcoma cases were obtained from the

Table 1 Cases and summary of all results

Case no.	*Year*	*Histology*	*Specimen*	*RNA (ng/μl)*	*β-Actin*	*FUS-CHOP RT-PCR result*	*DDIT3 FISH*	*Comments*
1	2000	Classic	Resection	64	FAILED	N/A	ND	
2	2000	Classic	Biopsy	10	FAILED	N/A	ND	
3	2001	Classic	Resection	128	POS	Negative	POS	FUS FISH positive
4	2002	Classic	Resection	75	POS	Type 1	POS	
5	2002	Mixed	Resection	251	POS	Type 2	POS	
6	2003	Classic	Resection	172	POS	Type 1	POS	
7	2003	Classic	Biopsy	9	POS	Type 1	ND	
8	2004	Classic	Resection	275	POS	Type 3	POS	
9	2004	Classic	Resection	51	POS	Type 11[a]	POS	
10	2004	Classic	Biopsy	5	FAILED	N/A	ND	
11	2004	Classic	Resection	48	POS	Type 2	ND	
12	2004	Classic	Biopsy	13	POS	Type 2	ND	
13	2004	Classic	Resection	62	POS	Type 3	POS	
14	2004	Classic	Resection	74	POS	Type 2	ND	
15	2005	Round	Resection	420	POS	Type 3	ND	
16	2005	Classic	Biopsy	6	POS	Type 2	ND	
17	2005	Classic	Biopsy	6	POS	Type 1	ND	
18	2005	Classic	Resection	128	POS	Type 3	POS	
19	2006	Classic	Resection	37	POS	Type 3	POS	
20	2006	Classic	Biopsy	4	POS	Negative	ND	
22	2006	Classic	Resection	18	POS	Negative	POS	Known EWSR1–DDIT3 case
23	2006	Classic	Resection	12	POS	Negative	ND	
24	2006	Classic	Resection	5	POS	Negative	ND	
25	2007	Classic	Resection	115	POS	Type 2	ND	
26	2007	Mixed	Resection	121	POS	Type 2	ND	
27	2007	Classic	Resection	64	POS	Type 2	POS	
28	2008	Mixed	Resection	9	POS	Type 2	ND	
29	2008	Classic	Resection	235	POS	Type 1	POS	
32	2008	Classic	Resection	11	POS	Type 2	ND	
34	2008	Classic	Resection	85	POS	Type 3	ND	
35	2008	Classic	Biopsy	3	POS	Negative	ND	
36	2008	Classic	Resection	5	POS	Type 2	POS	
37	2008	Classic	Resection	35	POS	Type 2	POS	
38	2008	Classic	Resection	60	POS	Type 2	POS	

N/A, not applicable; ND, not done; POS, positive.
[a]New variant, proposed type 11.
Case numbers are not sequential because multiple blocks were received from the same case, but only one was tested.

archives at MD Anderson Cancer Center (Table 1) with IRB approval. One case, a known cytogenetically positive t(12;16) case from Texas Children's Hospital was used as a positive control. Most cases showed classic histology. One case was primarily a round cell liposarcoma, and three had a mixed classic and round cell histology. In all, 8 samples were from needle biopsies and 26 samples were from larger resections. All histology samples were reviewed (AJL and WLW) and confirmed as myxoid/round cell liposarcoma. One formalin-fixed paraffin-embedded block was obtained from each case and a 40-μm thick scroll was cut from it; this was followed by H and E staining.

Molecular Methods

RNA was extracted from formalin-fixed paraffin-embedded tissue scrolls using the Ambion RecoverAll kit, as per the manufacturer's instructions (Ambion, Applied Biosystems, Austin, TX, USA). RT was carried out using the Invitrogen Superscript III reverse transcriptase and random hexamers as primers (Invitrogen, Carlsbad, CA, USA). Each RT reaction had 2 μl RNA, 10.1 μl DEPC water, 2.2 μl 5 $\times$ RT buffer, 2.2 μl of 10 mM dNTPs, 2.2 μl of 0.1 M DTT, 1.1 μl of random hexamers (50 ng/ml), 0.5 μl of RNase out (40 U/ml), and 0.5 μl of reverse transcriptase. RT was performed at 37°C for 1 h and 95°C for 5 min to inactivate the reaction. cDNA (5 μl) was used in subsequent PCR reactions. Each PCR reaction included 13.875 μl DEPC water, 2.0 μl of PCR buffer, 1.75 μl of dNTPs (10 mM), 2.5 μl of DTT (0.1 M), 0.5 μl of each primer (10 μM working stock each), and 0.125 μl of AmpliTaqGold (Life Technologies, Carlsbad, CA, USA). The following PCR conditions were used an initial denaturation at 94°C for 10 min, 40 cycles at 94°C (30 s), 55°C (60 s), 72°C (2 min), and one final extension at 72°C for 7 min. PCR conditions and the primers used were the same as described in previously published papers and the reagents used are those described in this paper.[12,17] The Invitrogen One-Step RT-PCR kit was used for one-step RT-PCR, as instructed. Amplification of β-actin (234 bp) was used as an

RNA integrity control.[18] PCR products were visualized on a 2% agarose gel using ethidium bromide staining and sequenced with forward and reverse primers in the ABI PRISM 3100-Avant Genetic Analyzer (Life Technologies, Carlsbad, CA, USA). Electropherograms were interpreted using Chromas Software (Technelysium, Tewantin, QLD, Australia) and the different breakpoints were analyzed using Specialized BLAST Multiple Alignment tool (NCBI).

Primer Design

Three different bioinformatic tools were used to design optimized primers for the detection of the most common *FUS–DDIT3* translocations. Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=NcbiHomeAd) was used with the *FUS–DDIT3* fusion exon5 to exon 2 and exon 7 to exon 2 cDNA sequences to select 20 candidate primer pairs with an amplicon size of less than 200 bp including all breakpoints, and an annealing temperature of ~55°C.[19] These primer pairs were then submitted to BLAST, and any primer matching greater than 75% of its length to anywhere else in the genome was discarded.[20] Lastly, the remaining primer pairs were analyzed for any dimer formation using PerlPrimer.[21] Using 57°C as the annealing temperature, only primers with dimerization energy less than 1 kcal/mole were retained. Those with an amplicon size closest to 150 bp were chosen as the final primer pairs (Figure 1). Because of amplicon size and primer location, our primers that were specific for the type 1 and 3 fusions would also give an identical product with the type 7 and 6 fusions, respectively (Figure 1). Primers published in previous papers on myxoid liposarcoma studied in formalin-fixed paraffin-embedded tissues were also analyzed in with the same tools.[12,17] Our final primers were FUSEx5-F: 5′-GCAGAACCAGTACAACAGCA-3′, FUSEx7-F: 5′-GGTGGCTTCAATAAATTTGG-3′, *DDIT3*Ex3-R: 5′-GGAGAAAGGCAATGACTCAG-3′.

FISH

Sections (5 μm) of available cases were tested for rearrangements using *DDIT3* and *FUS* break-apart FISH probes and the LSI *DDIT3–CHOP* and LSI *FUS–TLS* commercially available break-apart probes and the recommended protocols (Vysis/Abbott Molecular, Downers Grove, IL, USA). Briefly, slides were baked at 56°C overnight. The slides were deparaffinized with Hemo-De for 10 min, twice, and with 100% ethanol for 1 min, twice. Protease digestions were carried out by immersing the slide in the pretreatment solution for 10 min at 80°C, washing with purified water at room temperature for 3 min, in protease solution for 20 min at 37°C, and in purified water for 3 min. A volume of 10 μl of the probe mixture (1 μl probe, 7 μl hybridization buffer, and 2 μl water) was hybridized to an ~1 cm² area of the slide. Hybridization was carried out in a HyBrite chamber at 80 °C for 5 min and 37°C overnight. Slides were washed four times in 2× SSC/0.3% NP-40 and visualized. Slides were analyzed using a fluorescence microscope (Olympus BX5, Applied Imaging, San Jose, CA, USA) and Cytovision Genus 3.7 software (Applied Imaging). A minimum of 100 cells was scored for the presence of rearranged signals in a blinded manner (Figure 2).

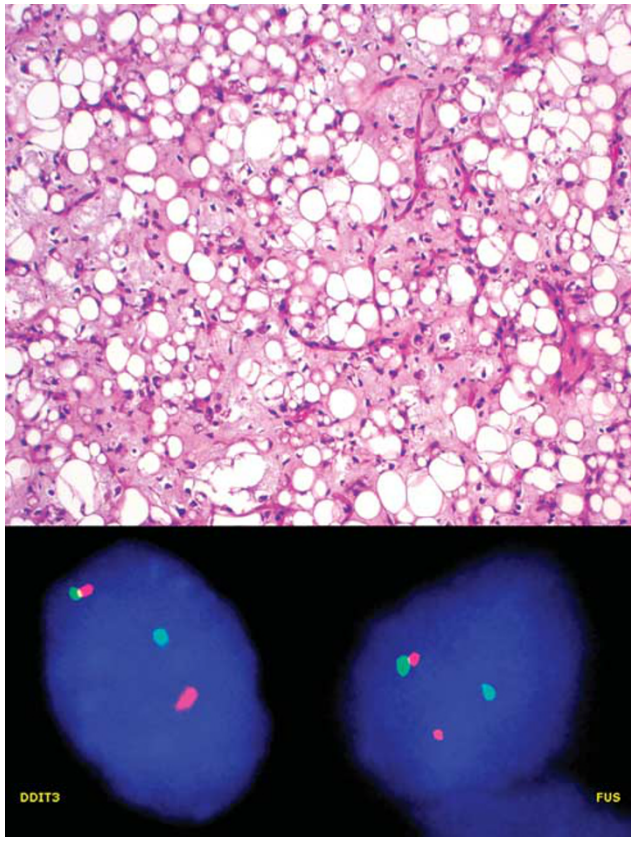

Figure 2 Case no. 3. The top panel shows the histology of case 3, consistent with a classic myxoid liposarcoma. This case was repeatedly negative by RT-PCR with ours and the published primers. The bottom left panel shows the break-apart FISH for *DDIT3*, and the bottom right the break-apart FISH for *FUS* in this case, indicating that this case most likely has a *FUS–DDIT3* translocation.

Results

Of the 34 cases extracted (Table 1), β-actin amplicons were obtained in 31, indicating adequate RNA quality. Of these 31 cases, 26 amplified with our PCR primers as expected. The positive control was consistently positive for type 2 fusion. One known *EWSR1–DDIT3*-positive case was negative, as expected, confirming the specificity of our primers. Of our 25 amplified cases, 13 had type 2 fusion, 6 type 1 (or 7) fusion, and 5 type 3 (or 6) fusion. We also identified one novel variant fusion (discussed

below). *DDIT3* rearrangements were confirmed by FISH in all positive cases (Table 1).

Of the five cases that did not undergo amplification using our primer pairs, four were from very small biopsies that yielded a very small amount of RNA. An alternative explanation for the negative amplification results is that these samples may carry a variant *FUS–DDIT3* or and *EWSR1–DDIT3* gene fusion that would not be detected with our primers. Because of the small amount of tissue, we were unable to test either hypothesis using FISH. One case, MLS-3 had an adequate amount of RNA and amplified β-actin, but failed to amplify with our primers. Histology was consistent with myxoid liposarcoma (Figure 2). Break-apart FISH for *DDIT3* and *FUS* indicated that both genes were rearranged, suggesting a *FUS–DDIT3* rearrangement (Figure 2). It may be possible that this case has one of the rare variant translocations or a novel and not previously described *FUS–DDIT3* fusion that would not amplify with our primers. However, this was the oldest case of our series that showed β-actin amplification (Table 1), and perhaps, not enough *FUS–DDIT3* mRNA was sufficiently preserved.

Primers previously published were also examined, but were found to be less sensitive. The nested primers from Hisaoka *et al*[17] were designed toward exons 5 and 2 of the *FUS–DDIT3* fusion transcripts and would, therefore, produce an amplicon larger than 200 bp from the type 1 and 3 chimeric transcripts (Figure 1 and Table 2). A total of 11 cases were studied using these primers and with the conditions published in their paper, and only three amplified giving a ladder-type product. Only type 2 fusions were amplified, and not all type 2 fusions tested were positive using this assay (Figure 3 and Table 1). No type 1 or 3 fusions were amplified by these primers. In addition, our primer pairs gave a stronger PCR product, indicating a more robust amplification (Figure 3). When Hisaoka *et al*'s primers were analyzed using Primer-BLAST, NCBI-BLAST, and PerlPrimer, both of their primer pairs, the internal and the external, showed significant nonextensible dimer formation at greater than 1 kcal/mole (Table 2). This type of dimer formation would not be expected to consume excess primer, but may decrease PCR efficiency.

The primers from Bode-Lesniewska *et al*[12] target both exons 7 and 5 of *FUS* and have products that would be expected to amplify genes from formalin-fixed paraffin-embedded tissue (Table 2). However, we had very weak signals from the cases that were type 2 fusions, and no amplification was specified from the type 1 or 3 fusions carried out using the Invitrogen one-step RT-PCR kit and cycling conditions in their paper (Figure 3). Primer-BLAST, BLAST, and PerlPrimer analysis of the Bode-Lesniewska *et al* primers showed that the primers of exons 5 and 7 in *FUS* have significant overlap with other transcripts, which may reduce their efficiency, and the *FUS* exon 7 primer and the reverse primer have significant nonextensible dimer formation, which could also be a possible cause of decreased PCR efficiency (Table 2).

In the course of our investigations, one case, MLS-9, repeatedly showed an unexpected size product of about 210 bp (Figure 4). None of the previously published translocations between *FUS* and *DDIT3* would be expected to generate this amplicon size. This case had characteristic myxoid liposarcoma histology (Figure 4). The cDNA product was sequenced and found to be a new *FUS–DDIT3*

Table 2 Bioinformatic analysis of published and new primers

Published FFPE primers		*Expected product sizes*			*BLAST*	*PerlPrimer*
Bode-Lewniewska et al						
Exon 5 FUS forward	Type 1	394 bp	Type 3	427 bp	Cross reacts with HNRNPAB	
Exon 3 DDIT3 reverse	Type 2	143 bp	Type 4	95 bp	No significant interaction	GTG repeat
Exon 7 FUS forward	Type 1	121 bp	Type 3	154 bp	Cross reacts with DNAJB8	Significant dimer formation
Exon 3 DDIT3 reverse	Type 2	NA	Type 4	NA	No significant interaction	
Hisaoka et al (nested PCR)						
Outer primers						
Exon 5 FUS forward	Type 1	451 bp	Type 3	484 bp	No significant interaction	Significant nonextensible dimer formation
Exon 3 DDIT3 reverse	Type 2	197 bp	Type 4	149 bp	No significant interaction	
Inner primers						
Exon 5 FUS forward	Type 1	379 bp	Type 3	412 bp	No significant interaction	Significant nonextensible dimer formation
Exon 2 DDIT3 reverse	Type 2	103 bp	Type 4	NA	No significant interaction	
Our primers						
Exon 5 FUS forward	Type 1	436 bp	Type 3	469 bp		
Exon 3 DDIT3 reverse	Type 2	160 bp	Type 4	112 bp		
Exon 7 FUS forward	Type 1	129 bp	Type 3	162 bp		
Exon 3 DDIT3 reverse	Type 2	NA	Type 4	NA		

NA: no expected amplification.

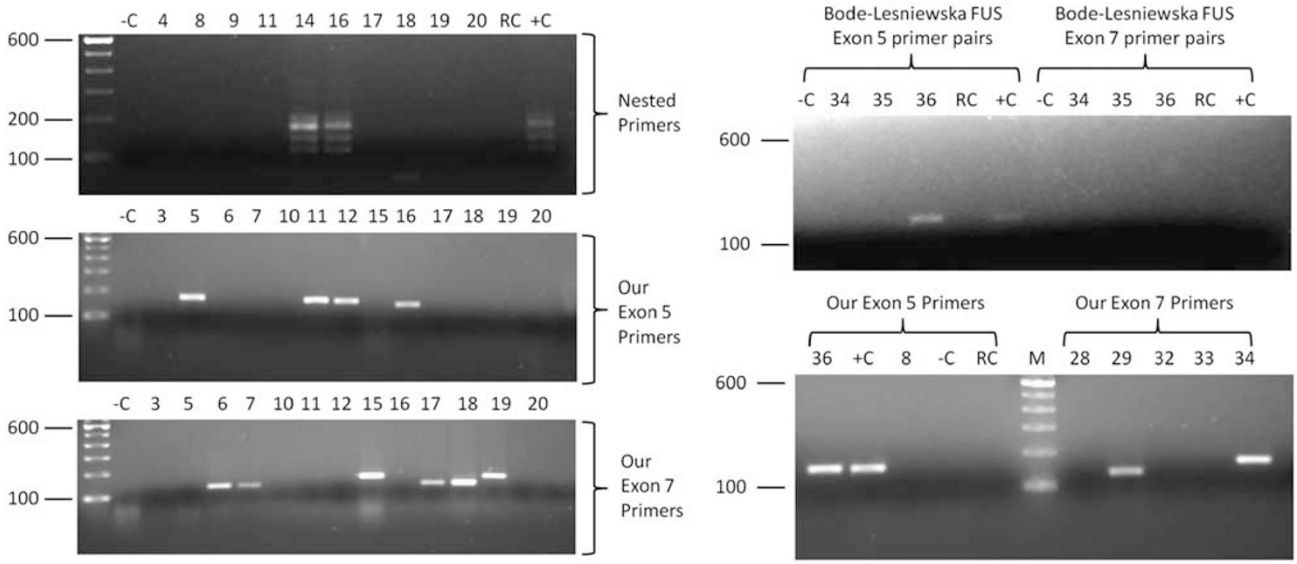

Figure 3 Representative PCR results. The upper left of this figure shows the amplification using the nested primers from Hisaoka *et al*. Only three of the four type 2 fusions amplified and gave a confusing ladder-like amplification. Case 11, which is strongly amplified by our primers (middle left panel), is not amplified by these primers, and none of the cases with type 1 or 3 fusions were amplified by the nested primers (cases 4,8,17,18, and 19), but were robustly amplified by our primers (bottom left panel for cases 17,18, and 19 shown here). The upper right panel is representative of the results we obtained from the primers published in Bode-Lesniewska *et al*. Only the type 2 fusions amplified and gave very weak bands on the gel. These same cases (cases 36 and +C) gave strong results with our primers (bottom right panel). In addition, none of the type 1 or 3 fusions were amplified with the Bode-Lesniewska primers (case 34 shown here), whereas the same cases were strongly amplified with our primers (bottom right). The right half of the bottom right panel also shows the size difference between a type 1 fusion (129 bp; case 29) and a type 3 fusion (162 bp; case 34).

fusion involving the end of exon 9 of *FUS* to the fifteenth nucleotide of the third exon of *DDIT3* (Figure 4). The frame was maintained from the end of exon 9 to the start ATG codon of *DDIT3*, consistent with the previously reported *FUS–DDIT3* chimeric transcripts. If the 3′ end of exon 9 in *FUS* were fused to the 5′ end of the third exon of *DDIT3*, the frame would not be retained. Therefore, the first 14 bp of *DDIT3* must be removed to produce a functional fusion protein. Whether this results because of a DNA breakpoint right at or near these nucleotides or because of alternative RNA splicing is unknown. The two immediate nucleotides upstream of the *DDIT3* breakpoint are AG (the canonical 3′ splice site), suggesting that this chimeric mRNA results from alternative splicing of the primary fusion transcript. On the basis of the previously published *FUS–DDIT3* fusion transcripts, we propose that this be called the type 11 fusion transcript (Figure 5).

Discussion

In this study, we developed a set of primers that can reliably detect the most common *FUS–DDIT3* translocations seen in myxoid/round cell liposarcoma, and discovered a new *FUS–DDIT3* variant translocation. Our assay readily distinguishes the three most common breakpoints in *FUS–DDIT3*, types 1, 2, and 3 translocations. In our study, as the relevant literature would indicate, we most often identified the type 2 translocation, which fuses the fifth exon of *FUS* to the second exon of *DDIT3*.[2,12] However, although our assay differentiates whether exon 8 or 7 of *FUS* is fused to exon 2 or 3 of *DDIT3*, these primers would not distinguish the splice variants that are missing a portion of exons 5 and 6, (fusion types 6 and 7) (Figure 5), given the limitations of RT-PCR from paraffin-embedded tissue testing. Therefore, the exact variants reported by us and others may be limited by primer choice. To detect these transcripts, primers would be required upstream of exon 5, which would amplify a product larger than that is routinely amplifiable from formalin-fixed paraffin-embedded tissue. In addition, no known significance has been attached to these variant transcripts. In five publications totaling 149 positive cases, 97 (65%) were mentioned to be the exon 5–exon 2 fusions (presumptive type 2), 33 (22%) as exon 7–exon 2 fusions (presumptive type 1), and 13 (9%) exon 8–exon 2 fusions (presumptive type 3).[2,12,17,22,23] The other cases were either a rare novel variant or one of four (3%) cases of a *EWSR1–DDIT3* fusion. However, another study using frozen tissue and longer-range PCR from the first four exons of *FUS* revealed that a number of the exon 7 and exon 8 translocations to *DDIT3* often had a portion of exons 5 and 6 spliced out (the type 6 and 7 fusions).[24] Also seen in this paper was the fact that many of the tumors had more than one transcript present, with type 6 and type 2 seen simultaneously in a number of cases and one case with type 1, type 6, and type 7 transcripts. Therefore, it is possible that cases

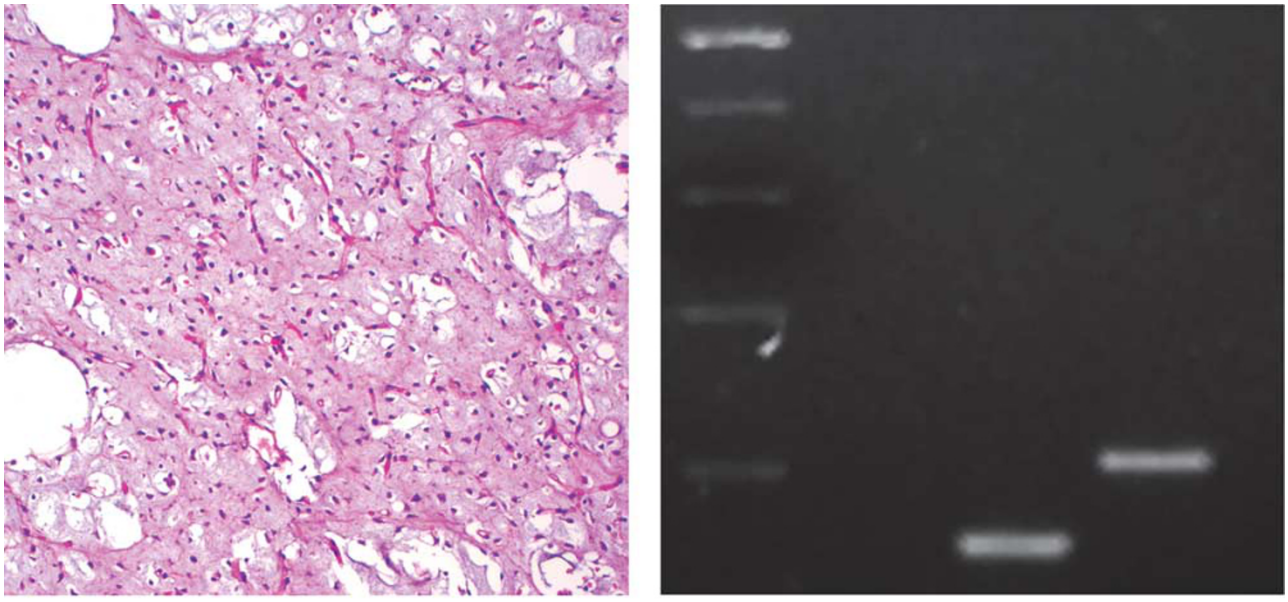

Figure 4 Case no. 9. The upper left panel shows the classic myxoid liposarcoma histology in this case. The upper right panel shows the PCR result for this case (far right lane), at an unexpected size of ~210 bp. Case 8, 162 bp, a type 3 fusion, is immediately to the left of case 9. The PCR product from case 9 was sequenced (bottom panel), and the end of exon 9 (last 5 codons shown in blue text), was continuous with nucleotide 15 of exon 3 of *DDIT3* (black text) and in-frame with the ATG start codon of *DDIT3* (red text), as seen in all other reported *FUS–DDIT3* fusions.

determined to be type 1 or type 3 using either our primers or those previously published may also have splice variants that are either type 7 or 6 fusion, respectively, and both splice variants may be seen in the same tumor. Regardless, our assay can reliably determine whether exon 5 of *FUS* is fused to *DDIT3* (the most common type 2 fusion), or whether exons 7 or 8 of *FUS* are fused to *DDIT3*.

In this study, we designed a set of primers using different computational tools to increase our primer efficiency. If an RT-PCR assay, which determines *FUS–DDIT3* fusion type, is going to be used clinically on formalin-fixed paraffin-embedded tissue, the most efficient and consistent amplification would be needed, as RNA integrity is impaired by formalin fixation and processing. It is often difficult to amplify RNA over 200 bp obtained from formalin-fixed paraffin-embedded tissue, and we include a 234 bp β-actin mRNA control for RNA integrity. We also compared our primers with two previously published primer sets used on formalin-fixed paraffin-embedded tissue.[12,17] Although the previously published primers often worked in most cases, amplification was both more robust and consistent using our newly designed primers. The nested primers from Hisaoka *et al*[17], also would amplify a number of fusions at greater than 200 bp and may miss some of the splice variants or variant fusions in samples with more degraded RNA. We also found

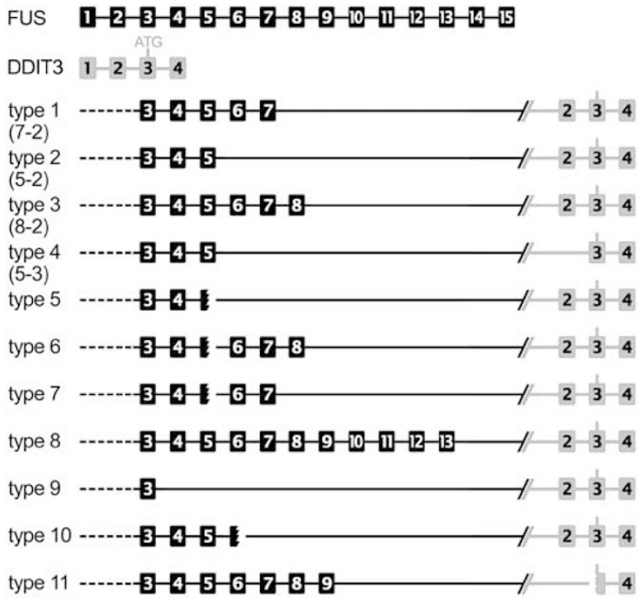

Figure 5 Classification of *FUS–DDIT3* fusions. Shown on the top of this figure are the exon structures of the wild-type *FUS* and *DDIT3* genes. The ATG start codon of *DDIT3* is shown with a hatch above exon 3. The exon structure of each of the different *FUS–DDIT3* fusions is shown below. For the type 1–4 fusions, they are sometimes reported in the literature by the exons fusion (that is, 7–2, 5–3). Our new fusion is proposed as type 11, which fuses the 3′ end of exon 9 to a sequence internal to exon 3 of *DDIT3*, but still upstream of *DDIT3*'s start codon.

some predicted dimerization that may reduce their efficiency. The primers from Bode-Lesniewska *et al*[12] would be expected to amplify the most common fusions from formalin-fixed paraffin-embedded tissue; however, they failed to amplify a number of the cases used in this study. Our analyses suggested a number of variables that may have reduced their efficiency. However, we did not perform the assay with precisely the same reagents indicated in their paper. The subtle change of reagents may have made a difference in efficiency as well. Given the computational tools we used to reduce dimer formation and to reduce off target effects, we expect that our primers would work under varying conditions, and we have shown here that they yield highly reproducible results.

In the course of our studies, we also identified a new *FUS–DDIT3* variant, reported never before, between exon 9 of *FUS* and exon 3 of *DDIT3*. We are proposing this be called as the type 11 variant of *FUS–DDIT3* (Figure 5). This is based on the nine variants published by Panagopoulous *et al*.[13] Since that publication, another variant has been reported between exon 6 of *FUS* and exon 2 of *DDIT3*, which we are proposing should be designated as type 10 variant.[2,22] Even though other variants have only been reported in rare cases in the literature, the new variant described here and elsewhere highlight the different possible *FUS* and *DDIT3* fusions seen in myxoid liposarcoma. Whether any of these variants have any clinical significance is not known at this time, highlighting the importance of identifying and reporting cases carrying rare variants. Our variant case showed no unusual histology, and previous publications have not identified any histological differences for any of the variant translocations, common or rare.[22]

Although still uncertain, the translocation breakpoint variants may have clinical significance. One variant may be more aggressive than the others, or may respond better to chemotherapy such as trabectedin.[2,12,15,16] As newer chemotherapeutic and other treatment strategies are developed for myxoid liposarcoma, confirmation of both the diagnosis and subtyping of the translocation may become part of routine clinical practice and of future research studies to identify the best treatment for each patient in this era of personalized medicine. In conclusion, we have developed a clinically robust RT-PCR assay that can identify and determine all known breakpoints in the most common subtypes of myxoid liposarcoma, and have identified a new *FUS–DDIT3* mRNA breakpoint, which needs to be considered in future myxoid liposarcoma studies.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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