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Comparison of Chromogenic In Situ Hybridization and Fluorescence In Situ Hybridization for the Evaluation of *MDM2* Amplification in Adipocytic Tumors

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Background: Atypical lipomatous tumor/well-differentiated liposarcoma (ALT-WDLPS) and dedifferentiated liposarcoma (DDLPS) are characterized cytogenetically by a 12q13–15 amplification involving the mouse double minute 2 (*MDM2*) oncogene. Fluorescence in situ hybridization (FISH) is used frequently to detect this amplification and aid with the diagnosis of these entities, which is difficult by morphology alone. Recently, bright-field in situ hybridization techniques such as chromogenic in situ hybridization (CISH) have been introduced for the determination of *MDM2* amplification status. *Methods:* The present study compared the results of FISH and CISH for detecting *MDM2* amplification in 41 cases of adipocytic tumors. Amplification was defined in both techniques as a *MDM2*/CEN12 ratio of 2 or greater. *Results:* Eleven cases showed amplification with both FISH and CISH, and 26 cases showed no amplification with both methods. Two cases had discordant results between CISH and FISH, and two cases were not interpretable by CISH. *Conclusion:* CISH is advantageous for allowing pathologists to evaluate the histologic and molecular alterations occurring simultaneously in a specimen. Moreover, CISH is found to be more cost- and time-efficient when used with automation, and the signals do not quench over time. CISH technique is a reliable alternative to FISH in the evaluation of adipocytic tumors for *MDM2* amplification. J. Clin. Lab. Anal. 29:462–468, 2015. © 2014 Wiley Periodicals, Inc.

Key words: adipose tissue; human gene amplification in situ hybridization chromogenic compounds; MDM2 protein; neoplasms

INTRODUCTION

Adipocytic tumors represent a heterogeneous group of neoplasms that may be difficult to differentiate based solely on histologic examination. In addition to their subtle histologic distinctions, these tumors are each associated with a different patient prognosis and treatment regimen, making an accurate diagnosis critically important. Liposarcomas represent the single most common type of soft tissue sarcoma, and they are classified by the World Health Organization (WHO) into four main categories: atypical lipomatous tumor/well-differentiated liposarcoma (ALT-WDLPS), dedifferentiated liposarcoma (DDLPS), myxoid liposarcoma, and pleomorphic liposarcoma (1). A particular diagnostic challenge is distinguishing ALT-WDLPS from benign adipocytic tumors. Similarly, DDLPS is often difficult to differentiate morphologically from other high-grade sarcomas. In these situations, ancillary studies are often required to arrive at a correct diagnosis. Fortunately, these adipocytic tumors harbor unique cytogenetic abnormalities that can be detected with molecular techniques (2).

ALT-WDLPS and DDLPS are characterized cytogenetically by a 12q13–15 amplification involving the mouse double minute 2 (*MDM2*) oncogene (3). The oncogenic properties of *MDM2* are explained by its ability to inhibit

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the *p53* tumor suppressor gene, thereby inhibiting apoptosis and enhancing cell survival (4). *MDM2* is abnormally upregulated in many human cancers, with an overall frequency of *MDM2* amplification estimated as 7%. The highest prevalence is documented in soft tissue tumors, followed by osteosarcoma and esophageal carcinoma (5).

Amplification of the *MDM2* locus is present in the majority of ALT-WDLPS and DDLPS, while it is not found in benign adipocytic tumors (2). This feature can thus be exploited with molecular techniques. Fluorescence in situ hybridization (FISH) has been commonly used to evaluate *MDM2* status in equivocal cases of ALT-WDLPS vs. benign lipomatous neoplasm. However, FISH requires specialized equipment to visualize fluorescence signals and needs to be interpreted with corresponding light microscopic sections. More recently, bright-field in situ hybridization techniques such as chromogenic in situ hybridization (CISH) and silver-enhanced in situ hybridization (SISH), which combine the general principles of immunohistochemical analysis and in situ hybridization, have been introduced for the determination of *MDM2* amplification status. These new techniques use a peroxidase enzyme-labeled probe with chromogenic detection, instead of a fluorescent-labeled probe, allowing results to be visualized by standard bright-field microscopy (6). They have been used successfully in the determination of amplification of other genes associated with human cancers, most notably for assessing *HER2* gene status in breast carcinoma (7).

Zhang et al. describe the development and validation of an automated bright field dual-color in situ hybridization assay to visualize both *MDM2* and chromosome 12 copies within the same tumor nuclei. The assay performance was evaluated on a cohort of 100 formalin-fixed, paraffin-embedded soft tissue specimens, and excellent sensitivity and specificity were reported (6). Thus far, a direct comparison has not been made between FISH and CISH results in the same tumor samples. An established effectiveness of CISH for use in lieu of FISH for detecting *MDM2* amplification would be beneficial for many institutions. The present study compared the results for 41 cases of adipocytic tumors that were tested for *MDM2* amplification by both FISH and CISH techniques.

MATERIALS AND METHODS

A total of 41 cases of adipocytic tumors with established histopathologic diagnoses were selected to be tested for *MDM2* amplification by FISH and CISH. Nineteen cases were benign, with a diagnosis of either lipoma or lipomatous neoplasm with minimal to moderate atypia. Twenty-two cases were malignant, with diagnoses including ALT-WDLPS, DDLPS, pleomorphic high-grade sarcoma, myxoid liposarcoma, mixed type (myxoid/round cell) liposarcoma, and spindle cell melanoma.

FISH for assessment of *MDM2* gene amplification was performed as follows. Four microns thick formalin-fixed, paraffin-embedded tissue slides were incubated at 56°C overnight followed by immersion in Hemo-De solution (10 min × 3 times) at room temperature. Slides were then treated with Lugol Iodine Solution for 5 min, and rinsed in 2.5% sodium thiocyanate until clear. The slides were incubated in 100% ethanol for 2 min. Slides immersed in citrate acid were microwaved for 4 min. Slides were placed in pepsin/HCl solution (prepared by mixing 0.20 g pepsin in 50 mM HCl, pH 2.0) for 1 h at 37°C before passing them through an alcohol series (70% EtOH for 1 min, 85% EtOH for 1 min, 100% EtOH for 10 min). Hybridization was performed with MDM2/CEP12 probes (Vysis, Downers Grove, IL) overnight at 37°C in a humidified chamber. Slides were washed with saline-sodium citrate buffer and counterstained with 4′,6-diamidino-2-phenylindole (DAPI) for interpretation using a fluorescent microscope. Scoring was performed by recording the total number of green (CEP12) signals and red (*MDM2*) signals for each cell, with a minimum of 100 cells evaluated per sample. A *MDM2*/CEP12 ratio was calculated for each case. Amplification of *MDM2* was defined as an *MDM2*/CEP12 ratio ≥2, whereas a ratio of less than 2 was defined as not amplified. In highly amplified cases, the numerous signals generate clusters which preclude quantitation of an exact numeric ratio. For these cases, the designation "amplified, cannot be quantitated (CBQ)" was used instead of an estimated ratio.

CISH for assessment of *MDM2* gene amplification was performed using the BenchMark® ULTRA automated slide processing system, with all materials and probes obtained from Ventana Medical Systems, Inc., Tucson, Arizona. The protocol was adapted from that used for Ventana's INFORM HER2 Dual ISH DNA Probe Cocktail, as described in the package insert (8). The formalin-fixed, paraffin-embedded tissue was cut at 4 μ. The slides were incubated at 60°C for 15 min, followed by application of EZ Prep Reagent for deparaffinization. Pretreatment included incubation at 82°C and application of Cell Conditioning Solution (CC2). ISH-Protease 3 was then applied and slides were incubated for 28 min. MDM2 DNP, and CEN12 DIG probes were applied and the slides were incubated for 8 min at 80°C. Hybridization was performed overnight at 37°C in a humidified chamber. Stringency washes (8 min × 3) were performed with Sodium Chloride Sodium Citrate buffer solution at 75°C. The metallic silver deposit for MDM2 ISH signal was developed using the ultraView SISH DNP Detection Kit, and the signal for CEN12 was visualized with a fast red and naphthol phosphate reaction using the ultraView Red ISH DIG Detection Kit, according to manufacturer's instructions. The

slides were then counterstained with Hematoxylin II and Bluing Reagent for interpretation by light microscopy.

Cells were examined using a 100× objective under oil immersion. Slide adequacy was verified before enumerating each slide, using the criteria established by Ventana in the "Interpretation Guide" for the INFORM HER2 Dual ISH DNA Probe Cocktail Assay (9). The first criterion for adequacy is the presence of internal positive control staining in non-neoplastic cells including normal adipocytes, endothelial cells, and inflammatory cells. The non-neoplastic nuclei should contain one or two copies of both CEN12 (red signals) and *MDM2* (black signals). Next, staining within the neoplastic cells must be enumerable. Due to truncation in the plane of sectioning, it is likely that not every neoplastic cell will contain signals, but the slide must contain an acceptable region of lesional tissue that is enumerable, or else the slide is considered inadequate for interpretation. Lastly, background staining must not interfere with enumeration. Once slide adequacy was confirmed, neoplastic cells were examined for the presence of red (CEN12 copies) and black (*MDM2* copies) signals. For the first ten cases analyzed, two separate counts with 50 and 100 cells were performed and evaluated for equivalence. No significant difference was observed between results for counting 50 cells vs. 100 cells (see Results below), so for the remainder of cases, 50 cells were counted. Interpretation was accomplished according to Ventana's "Interpretation Guide" for the INFORM HER2 Dual ISH DNA Probe Cocktail Assay (9). The overall ratio of black to red signals was calculated for each case. An *MDM2*/CEN12 ratio ≥2 was defined as amplified, while a ratio of less than 2 was defined as not amplified. As with FISH, in highly amplified cases containing signal clusters, the result is reported as "amplified, cannot be quantitated (CBQ)" instead of an estimated ratio.

RESULTS

All data are reported as *MDM2*/CEN12 ratios. In the analysis of ten cases for MDM2 amplification by CISH, the ratios calculated from scoring 50 cells and 100 cells were not significantly different ($t = -0.759$, $P = 0.47$) (Table 1).

Table 2 summarizes all of the tumors analyzed, including the patient demographics, histopathologic diagnosis, and results of *MDM2* amplification by FISH and CISH. Overall, 11 cases showed amplification with both FISH and CISH, 26 cases showed no amplification with both methods, two cases showed discordant results between FISH and CISH, and two cases were noninterpretable by CISH. Table 3 displays the correlation of FISH and CISH results among the 39 interpretable cases. Results from 37 out of the 39 cases (95%) were concordant. The concordant nonamplified cases included 15 benign lipomatous neoplasms, three lipomas with borderline atypical features, five myxoid liposarcomas, two ALT-WDLPS, and one mixed type (myxoid/round cell) liposarcoma (Fig. 1). The concordant amplified cases included seven ALT-WDLPS, three DDLPS and one pleomorphic high-grade sarcoma (Fig. 2). One case (undifferentiated pleomorphic high-grade liposarcoma) showed amplification by FISH only, and one case (ALT-WDLPS) showed amplification by CISH only. Six cases displayed weak or absent signals following application of the CISH methodology. CISH was repeated in these cases, with interpretable results in four cases while two cases remained noninterpretable.

TABLE 1. Comparison of Ratios From Scoring 50 Cells vs. 100 Cells

Case No.	Ratio 50 Cells	Ratio 100 Cells	Difference (Ratio 100 Cells-Ratio 50 Cells)
1	1.1	1.21	0.11
2	1.87	1.84	−0.03
3	1.08	1.12	0.04
4	0.96	0.90	−0.06
5	0.99	0.97	−0.02
6	1.06	1.01	−0.05
7	1.07	1.03	−0.04
8	1.03	0.98	−0.05
9	1.1	1.05	−0.05
10	1.03	1.05	0.02

DISCUSSION

Accurate diagnosis of the various adipocytic tumors is challenging and critically important due to the impact on patient management. ALT-WDLPS has been classically defined based on histologic aspects alone, but it is often difficult to distinguish morphologically this tumor from its benign counterparts, especially in the context of a lipomatous neoplasm in a deep seated location. The lower grade ALT-WDLPS in particular has a very close resemblance to normal fat. Similarly, the histological complexity of DDLPS leads to a large differential diagnosis including myxofibrosarcoma, pleomorphic rhabdomyosarcoma, malignant mesenchymoma, and poorly differentiated sarcomas such as malignant fibrous histiocytoma, fibrosarcoma, and malignant hemangiopericytoma (3). Additionally, the increased use of minimally invasive biopsies for adipocytic tumors may further complicate the matter by providing limited diagnostic material. Fortunately, the presence of a unique cytogenetic abnormality in ALT-WDLPS and DDLPS can be exploited by supplementary diagnostic tools such as immunohistochemistry, FISH, comparative genomic hybridization

TABLE 2. Summary of Tumors Tested for MDM2 Amplification Status by FISH and CISH

Case No.	Age	Gender	Diagnosis	Tumor Category	FISH Ratio	Status by FISH	CISH Ratio	Status by CISH
1	26	F	Lipoma	Benign	1.01	Not amplified	0.92	Not amplified
2	44	M	Lipoma	Benign	0.98	Not amplified	0.98	Not amplified
3	38	M	Lipoma	Benign	0.95	Not amplified	0.99	Not amplified
4	54	M	Lipoma	Benign	1.03	Not amplified	1.04	Not amplified
5	71	F	Lipoma	Benign	0.96	Not amplified	1.02	Not amplified
6	42	M	Lipoma	Benign	0.97	Not amplified	Unknown	Unknown
7	81	F	Benign lipomatous tumor	Benign	0.97	Not amplified	0.95	Not amplified
8	47	F	Benign lipomatous tissue	Benign	0.98	Not amplified	1.01	Not amplified
9	43	F	Hibernoma	Benign	0.92	Not amplified	0.95	Not amplified
10	68	F	Intramuscular lipoma	Benign	1.02	Not amplified	1.05	Not amplified
11	60	M	Mature fibroadipose tissue	Benign	1.10	Not amplified	0.95	Not amplified
12	46	F	Myolipoma	Benign	0.94	Not amplified	1.03	Not amplified
13	25	F	Well differentiated lipomatous tissue	Benign	1.02	Not amplified	0.97	Not amplified
14	50	M	Well differentiated lipomatous tumor with overlap features of spindle cell lipoma and cellular angiofibroma	Benign	0.99	Not amplified	1.01	Not amplified
15	51	F	Lipomatous areas with minimal atypia	Benign	1.01	Not amplified	1.03	Not amplified
16	51	F	Lipomatous neoplasm, minimal cytologic atypia	Benign	1.02	Not amplified	1.01	Not amplified
17	48	M	Atypical lipoma	Benign	1.02	Not amplified	0.98	Not amplified
18	75	M	Lipomatous tumor, scattered atypical cells	Benign	1.00	Not amplified	1.05	Not amplified
19	43	F	Lipomatous tumor, scattered moderate nuclear atypia	Benign	1.10	Not amplified	1.03	Not amplified
20	61	F	Myxoid LPS	Malignant	1.01	Not amplified	1.21	Not amplified
21	40	F	Myxoid LPS	Malignant	0.95	Not amplified	1.12	Not amplified
22	62	F	Myxoid LPS	Malignant	0.98	Not amplified	0.90	Not amplified
23	65	F	Myxoid LPS	Malignant	1.04	Not amplified	0.95	Not amplified
24	37	F	ALT/Well differentiated myxoid liposarcoma	Malignant	1.00	Not amplified	0.99	Not amplified
25	75	M	Liposarcoma, mixed type (myxoid and high grade round cell)	Malignant	0.70	Not amplified	0.88	Not amplified
26	80	F	ALT-WDLPS	Malignant	1.01	Not amplified	1.84	Not amplified
27	64	M	ALT-WDLPS	Malignant	1.00	Not amplified	0.96	Not amplified
28	62	F	ALT-WDLPS	Malignant	1.45	Not amplified	CBQ	Amplified
29	58	F	ALT-WDLPS	Malignant	CBQ	Amplified	CBQ	Amplified
30	60	M	ALT-WDLPS	Malignant	CBQ	Amplified	CBQ	Amplified
31	68	F	ALT-WDLPS	Malignant	CBQ	Amplified	CBQ	Amplified
32	58	M	ALT-WDLPS	Malignant	CBQ	Amplified	CBQ	Amplified
33	62	M	ALT-WDLPS	Malignant	CBQ	Amplified	CBQ	Amplified
34	62	F	ALT-WDLPS	Malignant	CBQ	Amplified	CBQ	Amplified
35	81	M	ALT-WDLPS	Malignant	CBQ	Amplified	CBQ	Amplified
36	54	M	DDLPS	Malignant	CBQ	Amplified	CBQ	Amplified
37	53	M	DDLPS	Malignant	CBQ	Amplified	CBQ	Amplified
38	57	F	Liposarcoma with low-grade dedifferentiation	Malignant	CBQ	Amplified	CBQ	Amplified
39	52	M	Pleomorphic high-grade sarcoma	Malignant	CBQ	Amplified	CBQ	Amplified
40	55	M	Undifferentiated pleomorphic high-grade liposarcoma	Malignant	CBQ	Amplified	1.05	Not amplified
41	50	F	Spindle cell melanoma	Malignant	0.9	Not amplified	Unknown	Unknown

CBQ, cannot be quantitated.

TABLE 3. Correlation of MDM2 Amplification Status by FISH and CISH

		FISH		
		Amplified	Nonamplified	Total
CISH	Amplified	11	1	12
	Nonamplified	1	26	27
	Total	12	27	39

(CGH), and most recently CISH. The increased emphasis placed on these molecular characteristics is reflected by the current WHO classification of liposarcomas, which is based on both morphological and genetic features (1).

MDM2 is amplified in close to 100% of ALT-WDLPS and DDLPS and is not amplified in benign lipomas (10). Myxoid liposarcomas also do not show *MDM2* amplification; instead they are associated with a classical t(12;16)(q13;p11) or t(12;22)(q13;q12) translocation (11). The results of the present study are consistent with these observations. The eleven amplified cases included seven ALT-WDLPS, three DDLPS and one pleomorphic high-grade sarcoma. An additional ALT-WDLPS was amplified by CISH only. The nonamplified cases included 18 lipomas, five myxoid liposarcomas, two ALT-WDLPS and one mixed type (myxoid/round cell) liposarcoma.

The high concordance between CISH and FISH establishes the clinical utility of CISH testing for *MDM2* in order to classify adipocytic tumors. CISH provides several advantages over FISH. First, CISH slides are viewed using a conventional light microscope. The crisp chromogenic signals developed in dual color CISH are supported by a hematoxylin counterstain to enhance morphological features, allowing for pathologists to evaluate the tissue architecture and molecular alterations simultaneously in a specimen. Furthermore, tumor heterogeneity is more easily appreciated by CISH (12). CISH signals are not subject to rapid fading and the slides can therefore be easily archived; in contrast, FISH slides have stricter storage requirements and are subject to quenching of the fluorescent signal. Cellular and extracellular proteins can contribute to a dull, generalized, autofluorescence that often obscures FISH signals in paraffin sections (13). Moreover, CISH is found to be more cost- and time-efficient than FISH when used with automation (12).

The results from two cases were discordant between FISH and CISH. One tumor, an undifferentiated pleomorphic high-grade liposarcoma, showed amplification by FISH but not by CISH. The CISH signals were strong and distinct, so a technical issue is not likely. Tumor heterogeneity is a possible culprit. If this tumor was genetically heterogeneous for *MDM2* copy number, with a mixture of amplified and unamplified nuclei, selection of different target areas for CISH and FISH analysis

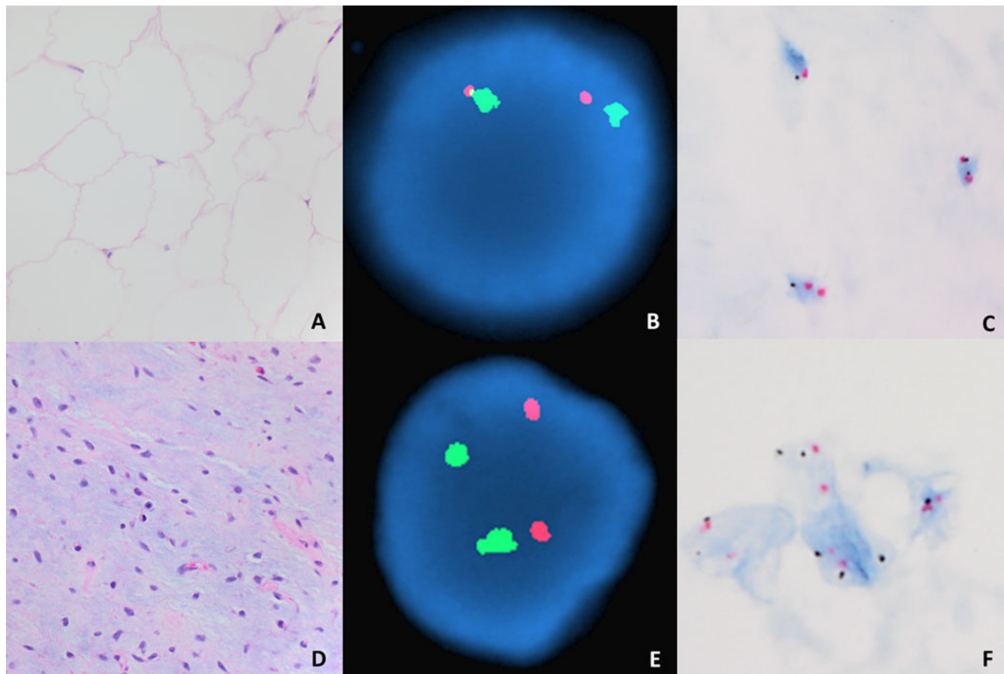

Fig. 1. Lipoma (A) and myxoid liposarcoma (D) with negative MDM2 amplification as demonstrated by FISH (B, E) and CISH (C, F), respectively. A and D, hematoxylin, and eosin, 40×. C and F, CISH, 100×.

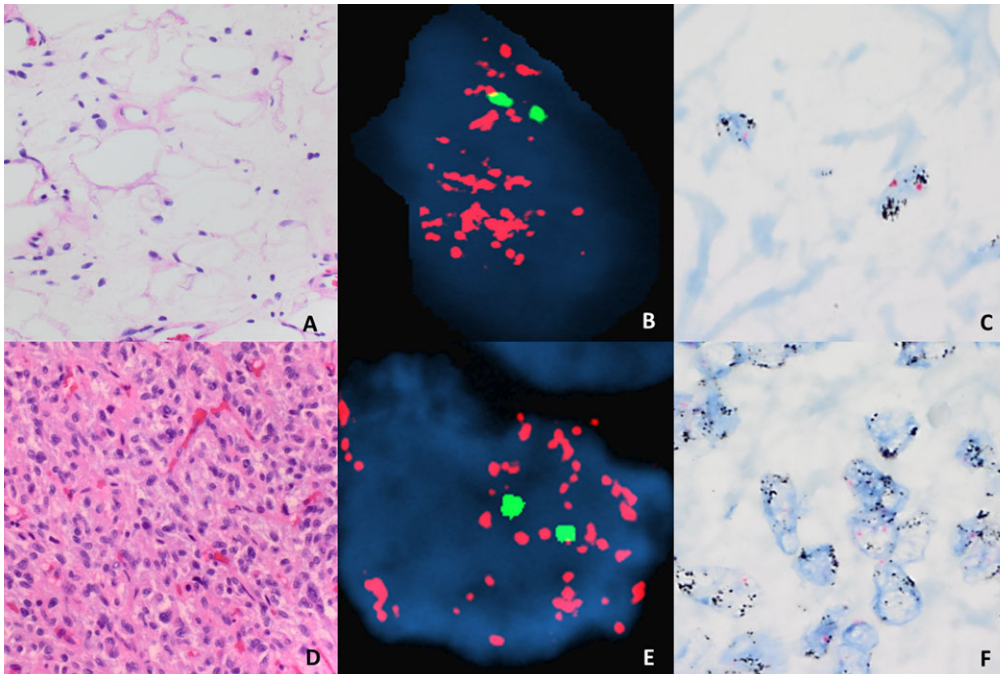

Fig. 2. ALT-WDLPS (A) and DDLPS (D) with positive MDM2 amplification demonstrated by FISH (B, E) and CISH (C, F), respectively. A and D, hematoxylin and eosin, 40×. C and F, CISH, 100x.

would account for these discrepant results. Another case, an ALT-WDLPS, showed amplification by CISH but not by FISH. More specifically, by FISH, 21% of nuclei examined showed *MDM2* amplification, with an overall ratio of 1.45. By CISH, 70% of nuclei examined showed *MDM2* amplification, and determination of an accurate ratio was not possible due to the fact that the majority of these nuclei contained clusters of black signals. Again, tumor heterogeneity could play a role in this case. CISH allows the evaluator to scan for heterogeneity at low power to ensure a localized highly amplified area is not overlooked. An additional finding by FISH in this case is that 50% of cells showed polysomy, ranging from —two to eight chromosome 12 copies. It is unclear whether or not this high polysomy caused apparent increased *MDM2* expression by CISH. Certainly, this warrants further investigation and suggests the need for revised scoring guidelines to take into account the issue of polysomy.

Two cases were not interpretable, even after repeated hybridization by CISH. This compares to Zhang et al., who reported a failure of CISH in two out of 100 cases and attributed this failure to tissue inadequacy (6). In both of our cases, both black and red (control and locus-specific) probes were weak or undetectable, in both the tumor cells and surrounding non-neoplastic stromal cells. Furthermore, the overall staining of cells in these cases appeared uniformly pale, and this pale counterstaining was also evident on the corresponding hematoxylin and eosin stained slides. This observation is suggestive of underfixation of the tissue, but the corresponding FISH in both cases had bright interpretable signals, suggesting that a step unique to the CISH procedure was responsible for the failure with this method. According to Ventana's Interpretation Guide, there are certain steps that can be manipulated to increase signal staining intensity. The cell conditioning times and/or cycle numbers can be increased. The application time of ISH-Protease 3 can be increased, and this extended tissue digestion time is a troubleshooting technique often used with success in FISH. However, at a certain point, extended digestion times begin to distort cell morphology, so there is a delicate balance between increasing signal intensity and sacrificing cell morphology. Lastly, signal staining intensity can be increased with extended incubation time with the Detection Kit reagents (see Materials and Methods), but this comes with a risk of causing nonspecific background staining that may obscure signal interpretation (10). Clearly, there are many variables within the CISH technique that may be adjusted when repeating failed cases, and this should be studied further to increase the overall success rate of CISH.

Determination of *MDM2* gene status in liposarcomas may provide both prognostic and therapeutic significance. Increased *MDM2* expression has been associated with an overall worse clinical prognosis. Not only is there an increased likelihood of distant metastases in *MDM2*-amplified tumors, but there is also a decreased response to therapeutic intervention (14). Specifically, the negative regulation of *p53* by *MDM2* may limit the magnitude of

p53 activation by DNA damaging agents, thereby limiting their therapeutic effectiveness (15). Furthermore, pharmaceuticals that block the interaction between *MDM2* and *p53* are currently in development (16). Therefore, testing of liposarcoma cases for *MDM2* amplification status is both diagnostically helpful and clinically relevant, and CISH proves to be a viable alternative to FISH, with added advantages that make it a more attractive option.

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CONFLICT OF INTEREST

Ventana Medical Systems, Inc. (Tucson, Arizona) provided our institution free-of-charge with the necessary probes and reagents to carry out the CISH portion of the study.

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