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Fluorescence *in situ* hybridization for *MDM2* gene amplification as a diagnostic tool in lipomatous neoplasms

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Well-differentiated liposarcoma/atypical lipomatous tumor and dedifferentiated liposarcoma can be difficult to distinguish from benign lipomatous neoplasms and other high-grade sarcomas, respectively. Cytogenetics in these tumors has identified ring and giant chromosomes composed of 12q13-15 amplicons including the *MDM2* gene. Identifying *MDM2* amplification by fluorescence *in situ* hybridization may prove an adjunctive tool in the diagnosis of lipomatous neoplasms. Dual color fluorescence *in situ* hybridization employing a laboratory-developed BAC label probe cocktail specific for *MDM2* (12q15) and a probe for the centromeric region of chromosome 12 (Abbott Molecular, DesPlaines, IL) was performed on formalin-fixed and paraffin-embedded tissue including whole sections from atypical lipomatous tumors ($n=13$), dedifferentiated liposarcomas ($n=14$), benign lipomatous tumors ($n=30$), and pleomorphic sarcoma, not otherwise specified ($n=10$), and a tissue microarray containing a variety of high-grade sarcomas ($n=63$). An *MDM2*/chromosome 12 ratio ≥ 2.0 was considered amplified, <2.0 nonamplified, and cases displaying >2 signals of both probes and an *MDM2* ratio <2.0 polysomic for chromosome 12. Of the well-differentiated and dedifferentiated liposarcomas, 100% showed amplification of *MDM2*. Chromosome 12 polysomy was noted in 89% of spindle cell/pleomorphic lipomas, while all angiolipomas and lipomas were nonamplified and eusomic. *MDM2* amplification was observed in 40% of pleomorphic sarcomas and a small subset of high-grade sarcomas (3/63). *MDM2*/chromosome 12 fluorescence *in situ* hybridization is a sensitive and specific tool (both 100%) in evaluating low-grade lipomatous neoplasms. The specificity decreases in high-grade sarcomas, as *MDM2* amplification was observed in a small portion of pleomorphic sarcomas and high-grade sarcomas other than dedifferentiated liposarcomas. Importantly, none of the benign lipomatous lesions were *MDM2* amplified and even cells in areas of well-differentiated liposarcomas with minimal cytologic atypia were amplified, making the probe a valuable tool in the diagnosis of even limited biopsy samples of well-differentiated lipomatous neoplasms.
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Well-differentiated liposarcoma/atypical lipomatous tumor and dedifferentiated liposarcoma are among the most common malignant soft tissue tumors presenting in older adults.[1–3] These tumors typically arise in the deep soft tissue of the proximal lower extremities as slow growing masses or in the retroperitoneum with symptoms related to the presence of an abdominal mass.[4,5] Well-differentiated liposarcomas/atypical lipomatous tumors are often difficult to distinguish morphologically from benign lipomatous tumors, while dedifferentiated liposarcomas may be challenging to distinguish from other high-grade sarcomas, especially on needle biopsy specimens that lack areas of well-differentiated liposarcomas.

Well-differentiated liposarcoma/atypical lipomatous tumors and dedifferentiated liposarcomas have been shown by cytogenetics to harbor ring and giant marker chromosomes consisting of amplicons of the 12q13-15 region, resulting in amplification of several genes, including most notably *MDM2*. PCR, comparative genomic hybridization (CGH), and fluorescence *in situ* hybridization (FISH) can also

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be used to demonstrate amplification in this chromosomal region.[6–10] *MDM2* (murine double minute) gene is an oncogene whose expression plays important roles in controlling the cell cycle and tumorigenesis by degradation of TP53.[11] With the increased use of small needle core biopsy and fine needle aspiration (FNA) in which only limited tissue is available for histologic evaluation, increased utilization of molecular studies to identify the characteristic molecular aberrations of well-differentiated liposarcoma/atypical lipomatous tumors and dedifferentiated liposarcomas may prove useful in clinical practice.

A dual-color *MDM2* FISH probe was utilized, which permits recognition of *MDM2* gene (12q13) amplification in formalin-fixed, paraffin-embedded tissues (FFPET). This probe, coupled with a centromeric reference probe for chromosome 12 (CEP12), could provide an important ancillary diagnostic tool in cases where fresh tissue is either not available for conventional cytogenetic analysis and/or PCR-based studies, or where performing such studies is impractical. Here, we report our experience with this novel probe set to distinguish well-differentiated liposarcoma/atypical lipomatous tumors and dedifferentiated liposarcomas from their morphologic mimics.

Materials and methods

After receiving Institutional Review Board approval, formalin-fixed and paraffin-embedded resection specimens and excisional biopsies were identified from the archives of the Department of Anatomic Pathology at the Cleveland Clinic. Hematoxylin and eosin-stained sections were independently reviewed by two soft tissue pathologists (JRG and MS). The consensus-verified diagnostic categories included well-differentiated liposarcoma/atypical lipomatous tumors ($n=13$), low-grade dedifferentiated liposarcomas ($n=7$), high-grade dedifferentiated liposarcomas ($n=7$), spindle cell/pleomorphic lipoma ($n=9$), angiolipoma ($n=10$), lipoma ($n=10$), lipoblastoma ($n=1$), pleomorphic sarcoma, not otherwise specified ($n=10$), myxoid liposarcoma ($n=5$), extra-skeletal myxoid chondrosarcoma ($n=10$), myxofibrosarcoma ($n=1$), clear cell sarcoma ($n=4$), low-grade fibromyxoid sarcoma ($n=10$), solitary fibrous tumor/hemangiopericytoma ($n=10$), malignant melanoma ($n=1$), epithelioid sarcoma ($n=1$), neuroblastoma ($n=1$), Ewing's sarcoma/primitive neuroectodermal tumor ($n=3$), desmoplastic small round cell tumor ($n=1$), alveolar rhabdomyosarcoma ($n=3$), gastrointestinal stromal tumor ($n=4$), leiomyosarcoma ($n=1$), synovial sarcoma ($n=4$), and malignant peripheral nerve sheath tumor ($n=4$). Whole tissue sections of the lipomatous tumors were used, as the hypocellularity seen in many of these tumors may preclude representative evaluation in tissue microarray format. The remaining soft tissue neoplasms, except the cases of pleomorphic sarcoma, not otherwise specified, were represented on tissue microarray sections consisting of duplicate 1.5 mm diameter tissue cores, as described previously.[12] Whole tissue sections of the pleomorphic sarcoma, not otherwise specified were utilized.

All cases were evaluated with a laboratory-developed BAC probe set. BAC DNAs were purchased from Roswell Park Cancer Institute, Buffalo, NY. BAC DNAs used for this study were RP11-775J10 and RP11-450G15 both containing genomic sequence for the *MDM2* gene. The probes were generated by nick translation of the BAC DNAs using a kit purchased from Vysis Inc. In a typical labeling reaction, 1 μg of DNA was nick translated in a 50 μl reaction containing 10 μM spectrum green dUTP, 10 μM dTTP, and 20 μM dNTP mix (dATP, dCTP, and dGTP) in 1X nick translation buffer provided with 5 μl of enzyme in 8 h reaction at 15°C. Enzyme was inactivated at 70°C for 10 min and reactions precipitated along with human placental DNA (Sigma). Precipitates were centrifuged in cold for 30 min and resuspended in 20 μl of water. Probes were validated against controls such as normal human tonsil and known *MDM2* amplified samples before staining the cohort of sample slides.

The FISH assay was performed according to established laboratory protocol, as described previously.[13] Blinded to the histologic diagnosis and using an Olympus IX-50 microscope (Olympus, Tokyo, Japan), cases were scored by counting a minimum of 40 nuclei per case under oil immersion at $\times 100$ magnification with a DAPI/green/red triple band pass filter. Only nuclei with at least two CEP12 signals were evaluated to minimize nuclear truncation artifact that occurs in paraffin-embedded 4-μm sections. Overlapping tumor nuclei were also excluded from evaluation to decrease false-positive scoring. The average number of *MDM2* and CEP12 signals was then determined and a *MDM2*/CEP12 ratio was calculated for each case. A ratio ≥ 2.0 was considered amplified for the *MDM2* gene, while a ratio <2.0 was considered nonamplified. A ratio of <2.0 with >2 signals of both probes was considered polysomic for CEP12.

Results

All cases analyzed by FISH had detectable and evaluable nuclear signals. The data presented in Table 1 depict the results for each tumor category. Of the well-differentiated liposarcomas/atypical lipomatous tumors studied, 100% (13/13) showed amplification of *MDM2* (mean: >16 signal gains/nucleus; *MDM2*/CEP12 ratio: 7.2). A total of 14 of 14 (100%) dedifferentiated liposarcomas showed amplification of *MDM2* (mean: >18 signal gains/nucleus; *MDM2*/CEP12 ratio: 8.2). Both the well-differentiated and dedifferentiated areas showed a

Table 1 *MDM2* gene amplification by FISH in a variety of soft tissue neoplasms

Soft tissue tumor	MDM2 *amplification (mean signal gains per cell)*	*CEP12 amplification (mean signal gains per cell)*	*Ratio* MDM2/ *CEP12*	*Interpretation*
Lipoma ($n=10$)	0/10 (1.8)	0/10 (2.0)	0.9	Absent
Angiolipoma ($n=10$)	0/10 (1.9)	0/10 (2.1)	0.9	Absent
Spindle cell/pleomorphic lipoma ($n=9$)	8/9 (3.4)	8/9 (3.6)	0.9	Polysomic
Lipoblastoma ($n=1$)	0/1 (2.1)	0/1 (2.3)	0.9	Absent
Well-differentiated liposarcoma/atypical lipomatous tumor ($n=13$)	13/13 (16.0)	0/13 (2.3)	7.2	Present
Dedifferentiated liposarcoma ($n=14$)	14/14 (18.2)	0/14 (2.3)	8.2	Present
Pleomorphic sarcoma, not otherwise specified ($n=10$)	10/10 (5.1)	10/10 (3.3)	1.6	40% present
Myxoid malignant fibrous histiocytoma/ myxofibrosarcoma ($n=1$)	0/1 (2.0)	1/1 (4.1)	0.5	Absent
Extra-skeletal myxoid chondrosarcoma ($n=10$)	0/10 (2.0)	0/10 (2.0)	1.0	Absent
Clear cell sarcoma ($n=4$)	0/4 (2.0)	0/4 (2.0)	1.0	Absent
Low-grade fibromyxoid sarcoma ($n=10$)	0/10 (2.0)	0/10 (2.0)	1.0	Absent
Myxoid liposarcoma ($n=5$)	1/5 (2.9)	0/5 (2.1)	1.4	20% present 80% absent
Solitary fibrous tumor/hemangiopericytoma ($n=10$)	0/9 (2.0)	0/9 (2.0)	1.0	Absent
Epithelioid sarcoma ($n=1$)	1/1 (20.0)	0/1 (2.0)	10.0	Present
Malignant melanoma ($n=1$)	0/1 (2.0)	0/1 (2.0)	1.0	Absent
Neuroblastoma ($n=1$)	0/1 (2.0)	0/1 (2.0)	1.0	Absent
Ewing's sarcoma ($n=3$)	0/3 (2.0)	0/3 (2.0)	1.0	Absent
Desmoplastic small round cell tumor ($n=1$)	0/1 (2.0)	0/1 (2.0)	1.0	Absent
Alveolar rhabdomyosarcoma ($n=3$)	0/3 (2.0)	0/3 (2.0)	1.0	Absent
Gastrointestinal stromal tumor ($n=4$)	0/4 (2.00)	0/4 (2.00)	1.0	Absent
Leiomyosarcoma ($n=1$)	0/1 (2.0)	0/1 (2.0)	1.0	Absent
Synovial sarcoma ($n=4$)	0/4 (2.0)	0/4 (2.0)	1.0	Absent
Malignant peripheral nerve sheath tumor ($n=4$)	1/4 (6.5)	0/4 (2.0)	3.3	25% present 75% absent

similar degree of *MDM2* amplification (Figure 1). In well-differentiated liposarcomas/atypical lipomatous tumors, *MDM2* amplification was identified in both the cytologically atypical and nonatypical cells (Figure 2). CEP12 polysomy was noted in 89% (8/9) spindle cell/pleomorphic lipomas (*MDM2*/CEP12 ratio: 0.97) (Figure 3). All angiolipomas, lipomas, and the lipoblastoma were not amplified (*MDM2*/CEP12 ratio: 1) (Figure 3).

In pleomorphic sarcoma, not otherwise specified, *MDM2* amplification was identified in 40% of (4/10) cases (*MDM2*/CEP12 ratio: 3.3, 2.3, 6.3, and 2.1), while 100% (10/10) were polysomic for CEP12 (average *MDM2*/CEP12 ratio: 1.6). Of the remainder of the neoplasms studied, extra-skeletal myxoid chondrosarcoma ($n=10$), myxofibrosarcoma ($n=1$), clear cell sarcomas ($n=4$), low-grade fibromyxoid sarcomas ($n=10$), solitary fibrous tumors/hemangiopericytomas ($n=10$), malignant melanoma ($n=1$), neuroblastoma ($n=1$), Ewing's sarcomas/ primitive neuroectodermal tumors ($n=3$), desmoplastic small round cell tumor ($n=1$), alveolar rhabdomyosarcomas ($n=3$), gastrointestinal stromal tumors ($n=4$), leiomyosarcoma ($n=1$), and synovial sarcomas ($n=4$) displayed no evidence of *MDM2* amplification or chromosome 12 polysomy. However, a small subset of these soft tissue neoplasms (5% or 3/63) did have evidence of *MDM2* amplification, including one epithelioid sarcoma ($n=1$), one malignant peripheral nerve sheath tumor ($n=4$), and one myxoid liposarcoma ($n=5$, diagnosis confirmed by *DDIT3* break-apart FISH).

Discussion

Well-differentiated liposarcoma/atypical lipomatous tumor is the most common type of liposarcoma, but can be difficult to distinguish from benign lipomatous tumors. Lipoblasts may not be present in well-differentiated liposarcoma/atypical lipomatous tumor, and alternatively, even benign lipomatous tumors such as spindle cell lipomas may have scattered lipoblasts. Enlarged hyperchromatic nuclei, the histologic feature most reliable for a diagnosis of well-differentiated liposarcoma/ atypical lipomatous tumor, may be widely scattered and not even present in a small biopsy specimen. In addition, atypical nuclei are characteristically found in spindle cell/pleomorphic lipomas, a tumor that is difficult to distinguish from well-differentiated liposarcoma/atypical lipomatous tumor based solely on histological features. In our experience, one of the more common diagnostic dilemmas that arise in our consultation practice is the distinction of

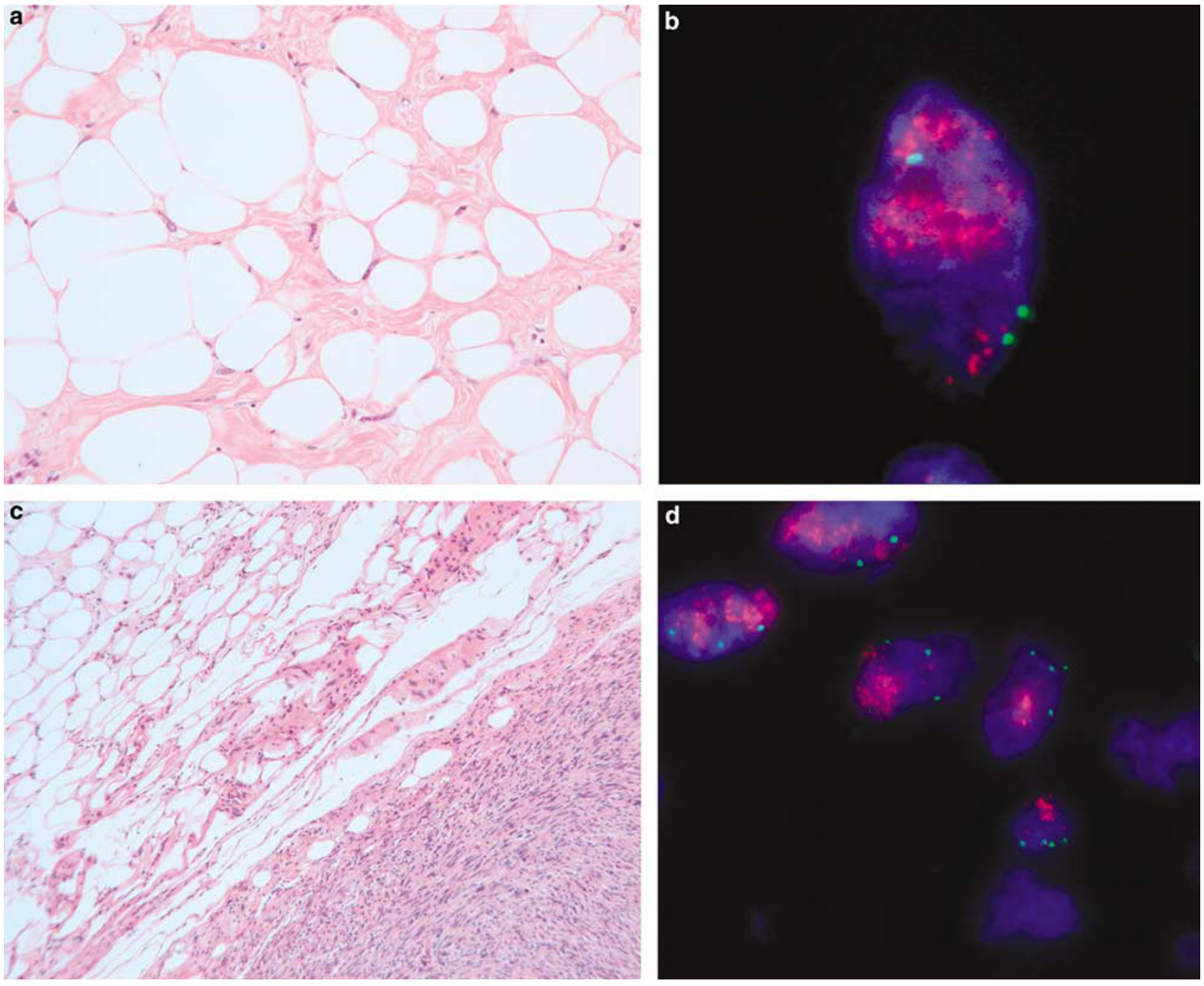

Figure 1 (**a**) Well-differentiated liposarcoma/atypical lipomatous tumor, adipocytes with cytologic atypia cells. (**b**) *MDM2* gene amplification in a well-differentiated liposarcoma/atypical lipomatous tumor (many *MDM2*-red signals and only three CEP12-green signals). (**c**) Dedifferentiated liposarcoma, a well-differentiated liposarcoma/atypical lipomatous tumor component adjacent to a high-grade sarcomatous component. (**d**) And a positive *MDM2*/CEP12 FISH assay in a dedifferentiated liposarcoma similar to the fluorescent photomicrograph in panel b.

well-differentiated liposarcomas/atypical lipomatous tumors from benign lipomatous tumors. However, with the identification of characteristic ring and giant chromosomes largely comprised of 12q13-15 amplicons in well-differentiated liposarcomas/atypical lipomatous tumors, ancillary diagnostic tools can now be utilized to distinguish benign lipomatous tumors from well-differentiated liposarcomas/atypical lipomatous tumors.

Immunohistochemistry (IHC) is often the first ancillary tool performed in current day diagnostic pathology to differentiate tumors resembling one another morphologically. The 12q13-15 amplicons characteristic of well-differentiated liposarcomas/atypical lipomatous tumors and dedifferentiated liposarcomas have been shown to include both *MDM2* and *CDK4* genes. Therefore, one might expect their corresponding proteins to be overexpressed in these lesions as a result of their amplification, thereby facilitating this evaluation. To evaluate this possibility, Binh *et al*[14] analyzed a large number of soft tissue tumors by IHC using antibodies to MDM2 and CDK4. In their hands, the sensitivity and specificity of MDM2 and CDK4 expression in distinguishing well-differentiated liposarcomas/atypical lipomatous tumors, and dedifferentiated liposarcomas from other soft tissue neoplasms was 97 and 92, and 83 and 95%, respectively. In their series, all 44 cases of well-differentiated liposarcomas/atypical lipomatous tumors were MDM2 positive, but 4.2% of benign lipomatous tumors and 18.9% of various sarcomas including malignant peripheral nerve sheath tumor, myxofibrosarcoma, embryonal rhabdomyosarcoma, malignant fibrous histiocytoma, leiomyosarcoma, myxoid liposarcoma, synovial sarcoma, angiosarcoma, dermatofibrosarcoma

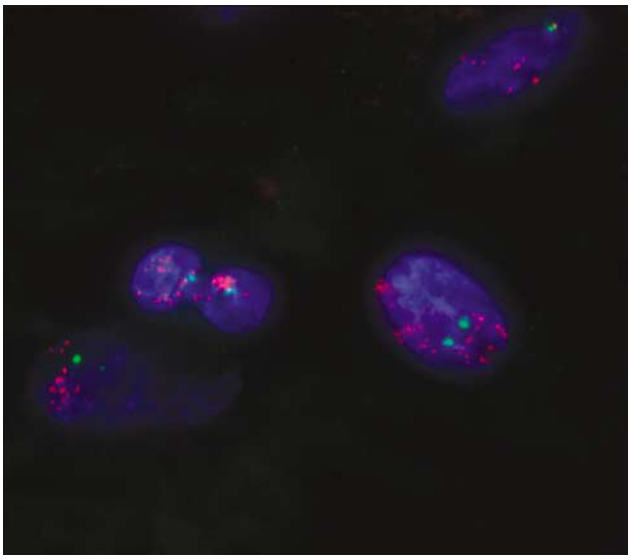

Figure 2 *MDM2* amplification in the cytologically nonatypical cells of a well-differentiated liposarcoma/atypical lipomatous tumor.

protuberans, clear cell sarcoma, Kaposi's sarcoma, epithelioid sarcoma, and desmoplastic small round cell tumor were also immunoreactive. Although there was decreased sensitivity when CDK4 immunostaining was added in combination, the specificity of this staining panel improved, as only 2.3% of benign lipomatous tumors and 3.7% of sarcomas were positive for both MDM2 and CDK4. Similar results have been reported from other laboratories with excellent interobserver concordance.[15] However, as there is typically not an absolute correlation between gene amplification and protein overexpression, detection of the underlying genetic alteration itself might be expected to yield higher sensitivity and specificity when compared to IHC.

Although *MDM2* FISH probes have been used in combination with other molecular techniques in numerous studies to define and demonstrate the 12q13-15 amplicons characteristic of well-differentiated liposarcoma/atypical lipomatous tumor and dedifferentiated liposarcoma, this tool has not been formally evaluated in the published literature as an ancillary test to help differentiate these entities from their morphological mimics.[10,16–19] Jacob *et al*[20] reported using a custom-designed FISH probe for *MDM2* along with a commercially available CEP12 probe. Similar to our data, in their smaller series of lipomatous tumors, all benign lipomatous tumors lacked *MDM2* amplification, while all well-differentiated liposarcoma/atypical lipomatous tumor exhibited amplification.

In this study, we have shown the *MDM2*/CEP12 FISH assay to be a sensitive and specific tool for well-differentiated liposarcoma/atypical lipomatous tumor and dedifferentiated liposarcoma, when compared to other lipomatous neoplasms. Thus, this technique provides an even greater sensitivity and specificity than has been previously achieved using immunohistochemical stains. All of the well-differentiated liposarcomas/atypical lipomatous tumors analyzed in this study were lipoma-like or sclerosing subtypes. However, the specificity does decrease when evaluating high-grade sarcomas, as *MDM2* amplification was seen in a subset of high-grade sarcomas other than dedifferentiated liposarcomas. Interestingly, one of our myxoid liposarcomas ($n=5$) showed *MDM2* amplification, while it also showed the evidence of the characteristic t(12;16), as demonstrated by break-apart FISH assays specific for *DDIT3* (12q13 also known as *CHOP*) and *FUS* (16p11). The *MDM2*/CEP12 ratio was low in this neoplasm (3.1). Although we do not have a plausible explanation for the low-level *MDM2* amplification in this neoplasm, loss of p53 in myxoid liposarcoma has been correlated with a worse prognosis, suggesting that *MDM2* amplification could provide an alternate mechanism of affecting the p53 function in such cases.[21] The single case of malignant peripheral nerve sheath tumor with *MDM2* amplification ($n=4$) was from the retroperitoneum and showed divergent rhabdomyosarcomatous differentiation. The S-100 protein stain was only focally positive and definitive areas of well-differentiated liposarcoma/atypical lipomatous tumor, to suggest this could be part of a dedifferentiated liposarcoma, were not identified. Therefore, it is possible that this neoplasm could represent a dedifferentiated liposarcoma in which characteristic areas of well-differentiated liposarcoma/atypical lipomatous tumor were either not sampled or were overrun by the dedifferentiated component. The single example of epithelioid sarcoma in this study showed high-level *MDM2* amplification. Additional evaluation of a larger number of epithelioid sarcomas will be needed to establish the true incidence of *MDM2* amplification in this tumor type.

Pleomorphic sarcoma, not otherwise specified (malignant fibrous histiocytoma, nonmyxoid) tend to have complex karyotypes including extensive polysomy.[22] *MDM2* as well as other proto-oncogenes from the 12q13-15 chromosome region have been reported to be amplified in cases of pleomorphic sarcoma, not otherwise specified.[22] Therefore, it was not a surprise to discover 40% (4/10) of the pleomorphic sarcomas from our study to be amplified for *MDM2* and all (10/10) to be polysomic for CEP12. As the main differential diagnosis for dedifferentiated liposarcoma is pleomorphic sarcoma, not otherwise specified, and both entities display *MDM2* amplification, the described *MDM2* FISH assay is not as useful in this setting for distinguishing between these neoplasms. From a clinical standpoint, differentiation is not essential on needle core biopsy of a deep-seated mass, because both require the same surgical approach. Dedifferentiated liposarcoma and pleomorphic sarcoma, not otherwise specified, can only be differentiated on complete examination of the entire

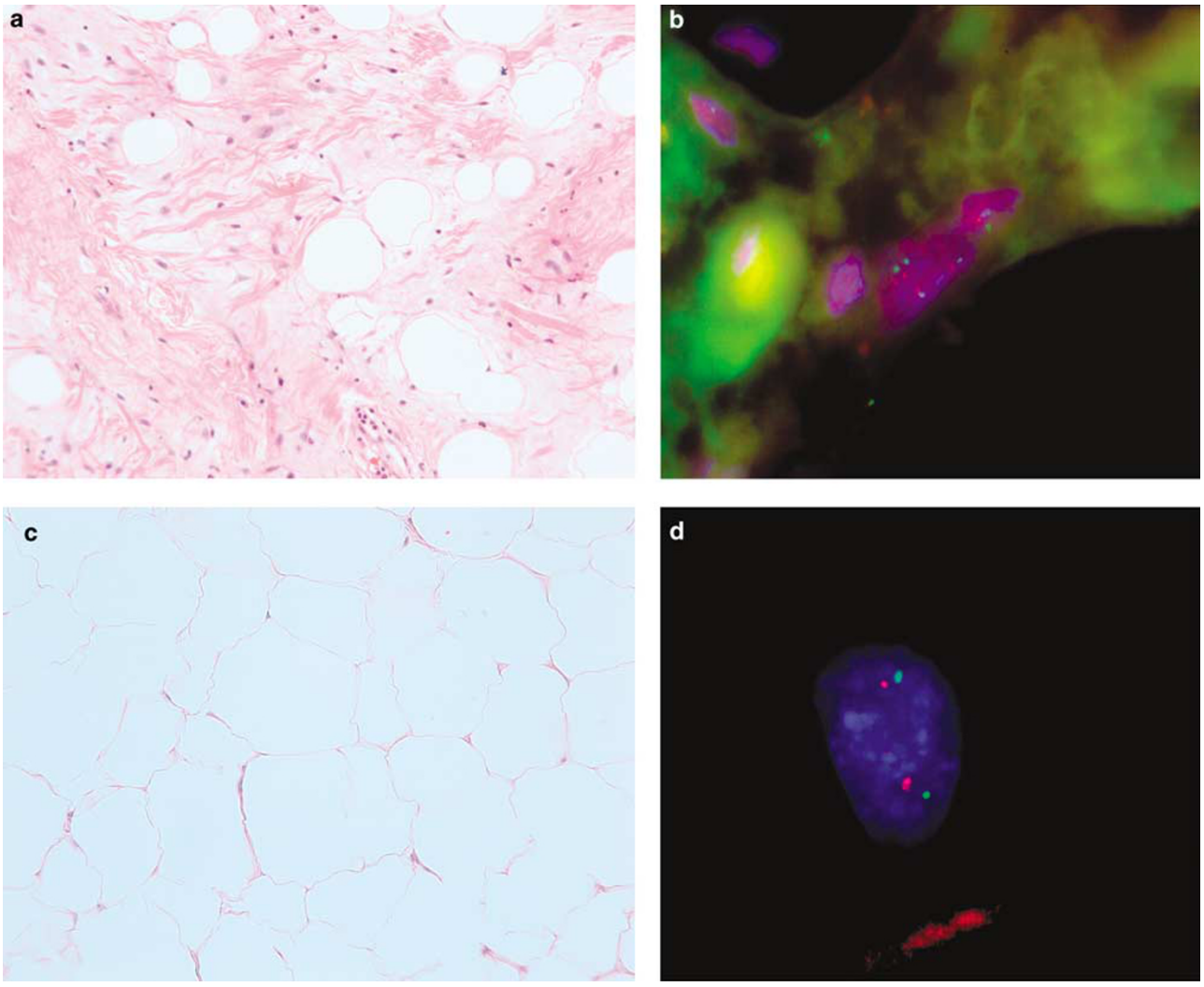

Figure 3 (**a**) Spindle cell/pleomorphic lipoma, adipocytes with atypical nuclei admixed with 'ropey' collagen. (**b**) Polysomic *MDM2*/CEP12 FISH assay in a spindle cell/pleomorphic lipoma (same number of *MDM2*-red signals as CEP12-green signals). (**c**) Lipoma, adipocytes lacking atypia. (**d**) Lipoma with a negative *MDM2*/CEP12 FISH assay (two *MDM2*-red signals and two CEP12-green signals).

neoplasm by identifying a well-differentiated liposarcoma component in dedifferentiated liposarcomas. Further more, the high-grade sarcoma component of a dedifferentiated liposarcoma may overgrow the well-differentiated liposarcoma component becoming indistinguishable from a pleomorphic sarcoma, not otherwise specified. This phenomenon along with not sampling the well-differentiated liposarcoma component are plausible explanations for the *MDM2* amplification detected in a percentage of our classified pleomorphic sarcoma, not otherwise specified cases. In fact, recent studies suggest that most pleomorphic sarcoma, not otherwise specified cases, otherwise known as malignant fibrous histiocytomas and their variants that arise in the retroperitoneum are actually dedifferentiated liposarcomas based on histological review, immunoprofile, and genomic profile.[23,24]

In conclusion, the identification of *MDM2* amplification by FISH is a useful ancillary tool in the diagnosis of well-differentiated liposarcomas/atypical lipomatous tumors, as none of the benign lesions commonly considered in its histologic differential diagnosis were found to harbor *MDM2* amplification. Moreover, the nonatypical cells in well-differentiated liposarcomas/atypical lipomatous tumors showed *MDM2* amplification, making the probe a valuable diagnostic tool even in small specimens where cytologic atypia is not identified but where well-differentiated liposarcoma/atypical lipomatous tumor remains a strong diagnostic consideration. While this study included only well-characterized tumor samples to illustrate the probe's performance, we predict that the *MDM2* FISH assay will become a valuable tool in the evaluation of difficult lipomatous lesions.

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