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Carboxypeptidase M: a biomarker for the discrimination of well-differentiated liposarcoma from lipoma

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The discrimination between well-differentiated liposarcomas/atypical lipomatous tumors and lipomas can be diagnostically challenging at the histological level. However, cytogenetic identification of ring and giant rod chromosomes supports the diagnosis of well-differentiated liposarcoma/atypical lipomatous tumor. These abnormal chromosomes are mainly composed of amplified genomic sequences derived from chromosome 12q13-15, and contain several genes, including *MDM2*, *CDK4 (SAS)*, *TSPAN31*, *HMGA2*, and others. *MDM2* is consistently amplified in well-differentiated liposarcomas/atypical lipomatous tumors, and up to 25% in other sarcomas. As part of a large genomic study of lipomatous neoplasms, we initially found *CPM* to be consistently amplified in well-differentiated liposarcomas/atypical lipomatous tumors. To further explore this initial finding, we investigated the copy number status of *MDM2* and *CPM* by fluorescent *in situ* hybridization (FISH) on a series of 138 tumors and 17 normal tissues, including 32 well-differentiated liposarcoma/atypical lipomatous tumors, 63 lipomas, 11 pleomorphic lipomas, 2 lipoblastomas, 30 other tumors and 17 normal fat samples. All 32 well-differentiated liposarcoma/atypical lipomatous tumors showed amplification of *MDM2* and *CPM*, usually >20 copies per cell. The other tumors lacked *MDM2* and/or *CPM* amplification. Chromogenic *in situ* hybridization confirmed the above results on a subset of these tumors ($n=27$). These findings suggest that identification of *CPM* amplification could be used as an alternative diagnostic tool for the diagnosis of well-differentiated liposarcoma/atypical lipomatous tumors.
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Well-differentiated liposarcomas/atypical lipomatous tumors are among the most common sarcomas presenting in older adults.[1–4,8] These tumors often arise in deep tissues as slow growing masses and can occasionally be difficult to distinguish morphologically from lipomas.[1–4,8] Cytogenetically, well-differentiated liposarcomas/atypical lipomatous tumors are characterized by supernumerary ring and large marker chromosomes. These abnormal chromosomes are mainly composed of amplified genomic sequences derived from chromosome bands 12q13-15, and contain several genes. Among them, *MDM2* seems to be the most consistently amplified gene.[1–4] During a high-resolution microarray comparative genomic hybridization experiment on a series of lipomatous neoplasms, we discovered that *CPM*, a gene that encodes carboxypeptidase M, was consistently amplified in liposarcomas but not in different subtypes of lipoma or normal fat.

In this study, we compared *MDM2* and *CPM* on a series of lipomatous tumors to establish whether *CPM* could be used clinically to discriminate well-differentiated liposarcoma/atypical lipomatous tumors from lipomas.

Materials and methods

Tumor Samples

After receiving Institutional Review Board approval, formalin-fixed and paraffin-embedded specimens were identified from the archives of the Department

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Table 1 Results for *MDM2* and *CPM*

Soft tissue tumor	*MDM2*	*CPM*	*Interpretation*
Well-differentiated liposarcoma/atypical lipomatous tumor ($n=17$)	17/17	17/17	Amplification[a]
Lipoma ($n=27$)	0/27	0/27	Negative
Pleomorphic sarcoma ($n=4$)	0/4	0/4	Aneuploidy[b] (4/4)
Pleomorphic lipoma ($n=4$)	0/4	0/4	Aneuploidy[b] (4/4)
Angiomyofibroblastoma ($n=1$)	0/1	0/1	Negative
Myxoid liposarcoma ($n=8$)	0/8	0/8	Aneuploidy (2/8)
Sarcomatoid renal cell carcinoma ($n=2$)	0/2	0/2	Negative
Pleomorphic liposarcoma ($n=1$)	0/1	0/1	Negative

[a]Amplification is defined as a ratio $\geq$3:1 for *MDM2*/CEP12, *CPM*/*CEP12*, or *CPM*/*SYT10* in >10% of 100 cells analyzed.
[b]Aneuploidy is defined as a chromosome number that are not an exact multiple of the haploid set of chromosomes.

of Laboratory Medicine and Pathology at the Mayo Clinic, Rochester, MN, USA. Hematoxylin and eosin (H&E)-stained sections were reviewed in all cases for diagnostic confirmation. The verified diagnostic categories included well-differentiated liposarcoma/atypical lipomatous tumors ($n=32$), ordinary lipomas ($n=63$), myxoid liposarcomas ($n=7$), undifferentiated pleomorphic sarcomas ($n=15$), pleomorphic lipomas ($n=11$), sarcomatoic renal cell carcinomas ($n=2$), angiomyofibroblastoma ($n=2$), low-grade myxofibrosarcoma ($n=2$), lipoblastoma ($n=2$), hibernoma ($n=1$), nodular fasciitis ($n=1$), and normal fat samples ($n=17$) (Tables 1 and 2).

Table 2 Results for *CPM* only (independent set)

Soft tissue tumor	*CPM*	*Interpretation*
Well-differentiated liposarcoma/atypical lipomatous tumor ($n=15$)	15/15	Amplification[a]
Lipoma ($n=36$)	0/36	Negative
Pleomorphic sarcoma ($n=11$)	0/11	Aneuploidy[b] (11/11)
Pleomorphic lipoma ($n=7$)	0/7	Aneuploidy[b] (7/7)
Angiomyofibroblastoma ($n=1$)	0/1	Negative
Myxoid liposarcoma ($n=1$)	0/1	Negative
Hibernoma ($n=1$)	0/1	Negative
Nodular fasciitis ($n=1$)	0/1	Negative
Low-grade myxofibrosarcoma ($n=2$)	0/2	Negative

[a]Amplification is defined as a ratio $\geq$3:1 for *CPM*/CEP12 or *CPM*/*SYT10* in >10% of 100 cells analyzed.
[b]Aneuploidy is defined as a chromosome number that are not an exact multiple of the haploid set of chromosomes.

FISH

All cases were evaluated with laboratory-developed fluorescent *in situ* hybridization (FISH) probe sets. Bacterial artificial chromosome (BAC) clones spanning the *CPM* and *MDM2* loci, 12q13-15, were obtained from the Children's Hospital Oakland Research Institute (Oakland, CA, USA). BAC clones for *CPM* are RP11-717F7, RP11-426B12, RP11-630N19, RP11-1104N20, and RP11-1036O8. BAC clones for *MDM2* are RP11-61F20, RP11-816C9, RP11-185H13, RP11-450G15. BAC clones spanned the *CPM* and *MDM2* genes and are 729 kb and 765 kb, respectively. The chromosome 12 reference probes for FISH and chromogenic *in situ* hybridization (CISH) were CEP12 (Abbott Laboratory, North Chicago, IL, USA) and the chromosome 12 pericentromeric gene *SYT10* (RP11-8P13, RP11-267D19, RP11-855O3, RP11-88P4, RP11-102G23, RP11-51O12 and RP11-1143D16), respectively. All of the identities for the BAC clones were individually confirmed by PCR and by hybridization on metaphase preparations from the peripheral blood of five normal individuals. Analytical sensitivity and specificity of each probe were calculated and their performance on paraffin-embedded tissue was verified on numerous normal tissue types, including the skeletal muscle, adipose tissue, and others. Amplification was defined as *CPM*/CEP12 and/or *MDM2*/CEP12 or *CPM*/*SYT10* ratio $\geq$3. Normal structures that could be readily identified under the 4′, 6-diamidino-2-phenylindole (DAPI) staining on thin sections, such as the blood vessels and epidermis, were used as internal controls for the cases analyzed. Normal signal patterns were established by scoring 100 cells from each normal tissue.

DNA isolation was carried out using the Qiagen Plasmid Maxi kit (Qiagen, Valencia, CA, USA). DNA was labeled using a nick translation kit (Abbott Laboratory). Interphase molecular cytogenetic studies were carried out using 4-μm paraffin-embedded thin sections that were deparaffinized twice in xylene (15 min pretreatment), dehydrated once in 100% ethanol (5 min), and treated with 10 mmol/l citric acid (10 min, in a humidified microwave). Tissue sections were incubated in warm (37°C) sodium chloride–sodium citrate buffer (2 × SSC) for 5 min. Protein was digested with Digest-All 3 (Invitrogen Corporation, Carlsbad, CA, USA). After a brief wash in phosphate-buffered saline (1 × PBS), slides were sequentially dehydrated in ethanol (70, 80, and 100%) and air dried at room temperature. Tissue sections were denatured at 85°C for 5 min, and BAC probe hybridization was performed overnight in a humidified chamber at 37°C. Tissue sections were washed in 0.1% Nonidet P-40 (NP40)

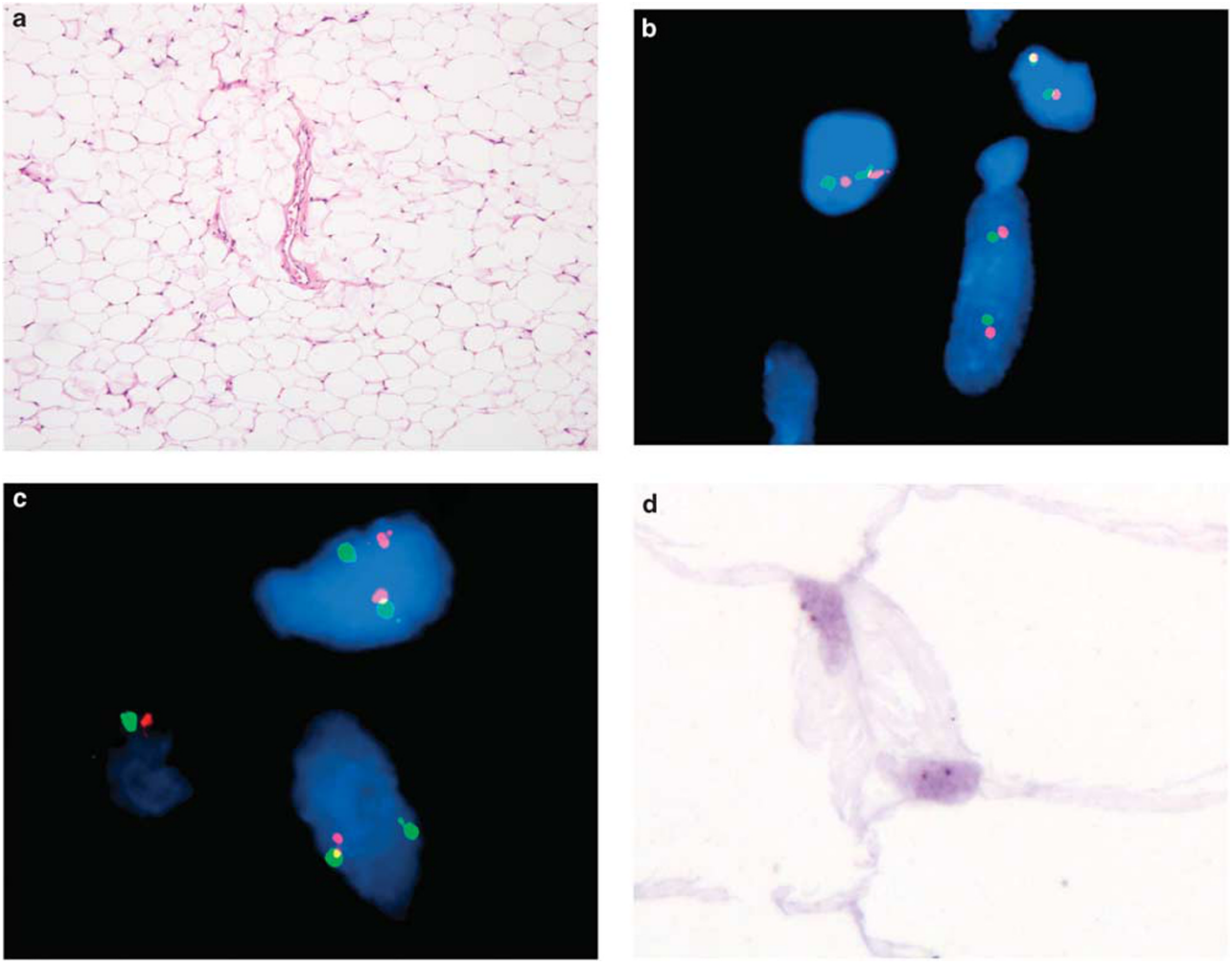

Figure 1 Lipoma. (**a**) Histological features of a representative case. (**b**) and (**c**) FISH images of this case with *MDM2* and *CPM* probes, respectively. Orange signals represent either *MDM2* or *CPM*. Green signals show chromosome 12 centromeres. (**d**) Normal CISH pattern for *CPM* in the same case. Brown signals represent normal copies of *CPM*.

in 2 × SSC at 76°C for 2 min and then washed in the same solution at room temperature for 1 min. Slides were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) with 1.5 μg/ml DAPI medium (Vector Laboratories). Tumor samples were considered positive for amplification if a ratio ≥3:1 *CPM*/CEP12 and/or *MDM2*/CEP12 was found in >10% of 100 cells. Tumors were evaluated and scored by three independent investigators (MEJ, ARS, and CWR).

CISH

Using the same DNA extracted for FISH probes (*CPM*, *MDM2*, and *SYT10*), DNA was labeled using the DIG DNA-labeling mix (Roche Diagnostics, Mannheim, Germany). In brief, 5 μl of 0.2 μg/ml DNA and 2.5 × random primers were mixed and placed on a thermal cycler at 99°C for 5 min, followed by placing on ice for 1 min. In all, 5 μl 10 × DIG-dNTP (Roche Diagnostics), 19 μl water, and 1 μl Klenow fragment (Roche Diagnostics) were added, gently mixed, and placed on a thermal cycler overnight at 37°C. DNA was then purified as per the manufacturer's instructions using a microspin s-200HR column (GE Healthcare, Fairfield CT, USA) and precipitated by the addition of 1 μl of 20 μg/ml glycogen (Invitrogen Corporation) and 33 × 7.5 M ammonium acetate (Sigma-Aldrich, St Louis, MO, USA) and 2 × 100% ethanol. Samples were precipitated overnight in an ethanol bath at −80°C. Labeled probe was centrifuged at 14 000 r.p.m. for 15 min at 4°C and pellets were resuspended in 100 μl LSI/WCP hybridization buffer (Abbott Laboratory). CISH slides were prepared following the FISH processing procedure until the addition of probe. CISH probe (5 μl) and 2 μl COT-1 (Invitrogen Corporation) were added to the slides and hybridized at 80°C for 5 min, and then held at 37°C overnight. On the following day, coverslips were removed and slides were washed in 76°C 0.5 × SSC for 2 min,

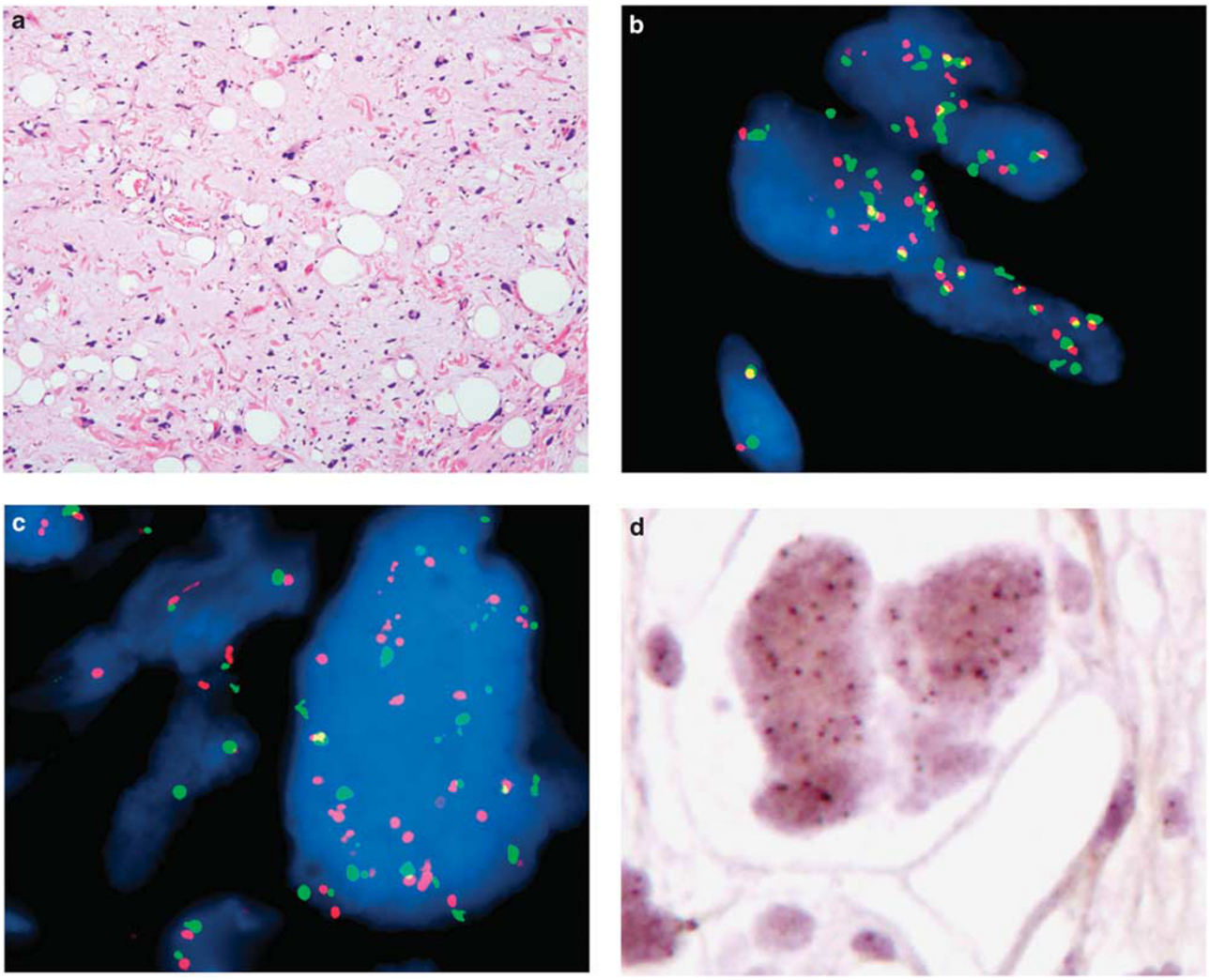

Figure 2 Pleomorphic lipoma. (**a**) Histological features of a representative case. (**b**) and (**c**) FISH images of this case with *MDM2* and *CPM* probes, respectively. Orange signals represent either *MDM2* or *CPM*. Green signals show chromosome 12 centromeres. (**d**) CISH image of a *CPM* probe hybridized to a pleomorphic lipoma. Scattered brown signals represent multiple copies of *CPM* without amplification (clustering). These images show FISH and CISH signatures consistent with numerical abnormalities of chromosome 12 but no amplification of either *MDM2* or *CPM* (*CPM/SYT10* and *MDM2*/CEP12 ratios = 1).

followed by a wash in 1 × PBS for 2 min. Slides were visualized by using the Spot-Light CISH Polymer detection kit (Zymed Laboratories, South San Francisco, CA, USA). Briefly, slides were incubated in 3% hydrogen peroxide for 5 min followed by three washes in PBS for 2 min each. Slides were blocked using in CAS-Block (Invitrogen Corporation) for 10 min. Reagent B (mouse-anti DIG) was added and incubated for 60 min followed by three washes in 1 × PBS, followed by the addition of 3′3-diaminobenzidine (DAB), incubated for 5 min, and washed in PBS. Following the addition of DAB, the slides were counterstained with hematoxylin, dehydrated in an ethanol series (70, 85, and 100%), immersed in xylene, and coverslipped using histomount. Tumor samples were considered positive for amplification if a ratio ≥3:1 *CPM/SYT10* or *MDM2/SYT10* was found in >10% of 100 cells analyzed. Tumors were evaluated and scored by two independent investigators (MEJ and AMO).

Comparative Genomic Hybridization

This study is part of a major genomic analysis of a large series of lipomatous neoplasms, which is currently in progress. Briefly, *CPM* was discovered using the Human CNV370-Quad BeadChip (Illumina, San Diego, CA, USA), which contains over 370 000 markers. Analysis was by multiple copy number variation (CNV) detection tools, including cnvPartition (Illumina), CGHAnalyzer (open source software from University of Pennsylvania), and Segmentation algorithm in Partek Genomic Suit (Partek Incorporated, St Louis, MO, USA).

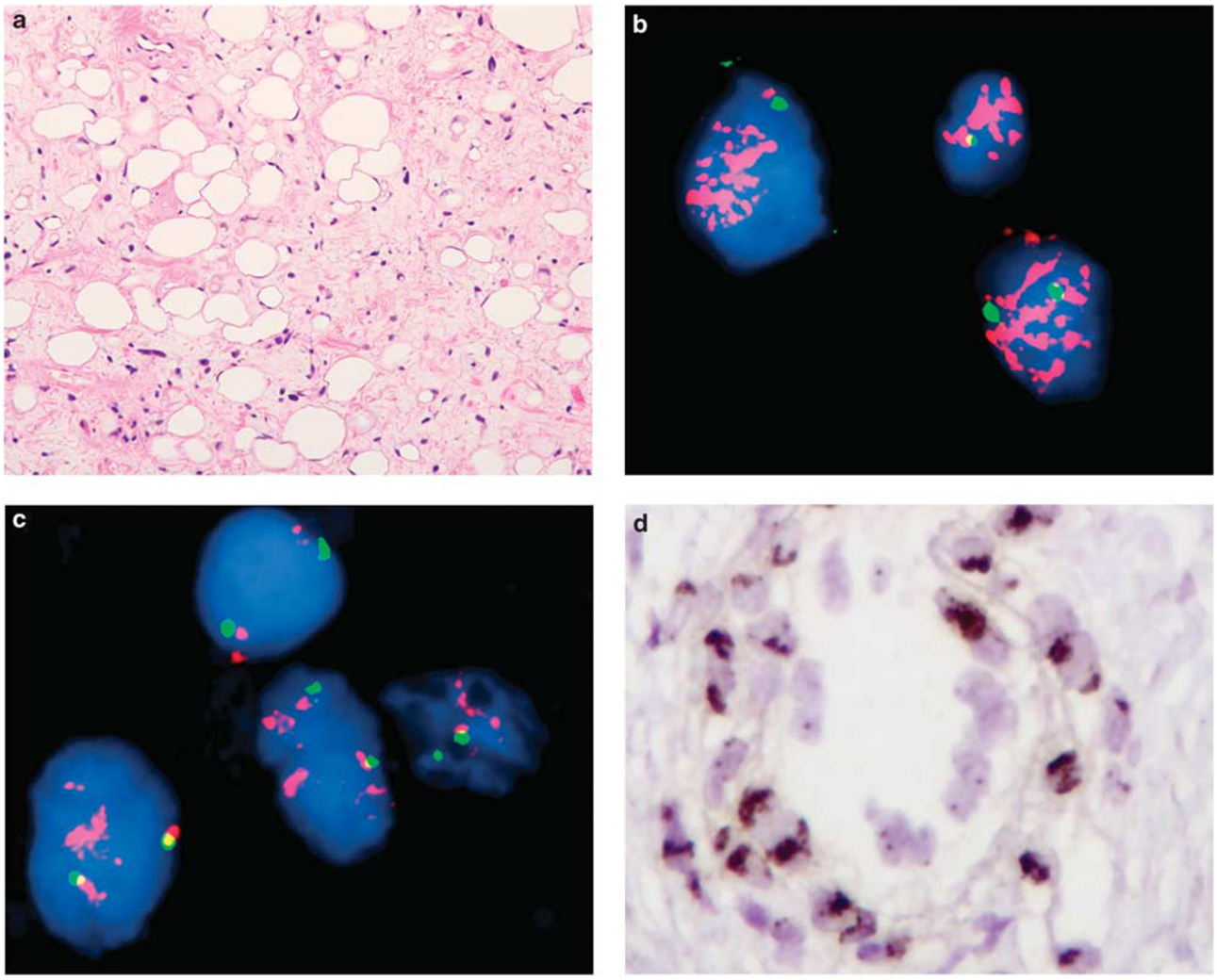

Figure 3 Well-differentiated liposarcoma/atypical lipomatous tumor. (**a**) Histological features of a representative case. (**b**) and (**c**) FISH images of this case show *MDM2* and *CPM* amplification, respectively. Orange signals represent either *MDM2* or *CPM*. Green signals show chromosome 12 centromeres. (**d**) CISH image of *CPM* probe hybridized to this case; clusters of brown signals represent *CPM* amplification. These images show FISH and CISH signatures consistent with the amplification of these genes (*CPM/SYT10* and *MDM2*/CEP12 ratios ≥ 3).

Results

FISH and CISH

All cases were found to have detectable and analyzable signals. The data in Tables 1 and 2 depict the results for each tumor category. All 32 well-differentiated liposarcomas/atypical lipomatous tumors were found to have amplification of *MDM2* and/or *CPM*, usually with >20 copies per cell (Tables 1 and 2, and Figure 3). Lipomas, lipoblastomas, and normal adipose tissue samples showed a normal FISH signal pattern with two copies of *MDM2* and *CPM* (Tables 1 and 2, and Figure 1). Pleomorphic lipomas and pleomorphic liposarcomas showed FISH signal patterns consistent with numerical abnormalities of chromosome 12 but without amplification of either *CPM* or *MDM2* (Figure 2). Two myxoid liposarcomas (29%) exhibited patterns consistent with monosomy 12 or loss of the *CPM/MDM2* loci (Tables 1 and 2). The remaining cases showed normal FISH signal patterns. Using the same FISH scoring criteria, all 27 cases analyzed by CISH (21 lipomas, 4 liposarcomas, and 2 normal fat samples) were found to have detectable and analyzable signals. CISH produced results similar to FISH, resulting in normal patterns for lipomas and normal fat samples (Figure 1), and amplification for liposarcoma (Figure 3). Pleomorphic lipomas and myxoid liposarcomas showed signal patterns consistent with numerical chromosomal abnormalities of chromosome 12 without amplification, similar to what was seen with FISH (Figure 2).

Discussion

Well-differentiated liposarcomas/atypical lipomatous tumors with minimal or no cytological atypia

can be difficult to distinguish from lipomas and fibrolipomas.[1,3,5–9] However, the identification of ring and giant rod chromosomes by cytogenetic analysis supports the diagnosis of well-differentiated liposarcomas/atypical lipomatous tumor. These abnormal chromosomes are mainly composed of amplified genomic sequences derived from chromosome bands 12q13-15 and contain several genes, including *MDM2*, *CDK4 (SAS)*, *TSPAN31*, *HMGA2*, among others.[8–21] *MDM2* seems to be amplified in almost all cases of well-differentiated liposarcoma/atypical lipomatous tumors, especially when molecular cytogenetic analysis is carried out.[1,9] In addition, *MDM2* amplification has been described in up to 25% of other sarcomas.[1–3]

As a part of large genomic study of lipomatous neoplasms, we found *CPM* to be consistently amplified well-differentiated liposarcomas/atypical lipomatous tumors in a CNV screening array CGH.[22] Our experiments showed co-amplification of *MDM2* and *CPM* in all well-differentiated liposarcoma/atypical lipomatous tumors evaluated in an initial test set. An independent group of well-differentiated liposarcoma/atypical lipomatous tumors was then tested only for *CPM*. Amplification of this gene was observed in all instances. All other tumors were negative for *CPM* amplification. These results correlate with previous studies that showed consistent amplification of *MDM2* in well-differentiated liposarcoma/atypical lipomatous tumors, but not in lipomas.[1,9,22] FISH results were also corroborated by a CISH assay in 27 of these tumors. Both FISH and CISH are closely related techniques resulting in the ability to enumerate chromosome and/or gene status. FISH requires a fluorescent microscope, trained technologists, and the signals of slides fade over time. In the other hand, CISH signals can be read out by any pathologist in a bright field microscope, and the findings can be directly correlated with specific morphological details of the tumor. However, CISH signals may be more difficult to be visualized and individually enumerated, especially if one is dealing with low levels of gene amplification. The final decision on which technique to use will depend on the comfort level and familiarity of the performing laboratory with each one of them.

CPM spans ~113 kb of genomic DNA, contains 11 exons, and encodes for a membrane-bound zinc-dependent protease that cleaves C-terminal basic residues from peptides and proteins. *CPM* is located 11 kb downstream from *MDM2* and the encoded protein has been implicated in many functions, such as adipose tissue differentiation, osteogenesis, inflammation, and coagulation.[23] *CPM* is expressed at high levels in many tissues and cell types, including the pulmonary membranes, placenta, and kidney.[23] Gene expression profiling has shown *CPM* transcriptional upregulation in response to specific external stimuli, which may be used as an indicator of body's response to infection or disease.[23] Recently, *CPM* has been found to be upregulated in soft tissue sarcomas, such as synovial sarcoma, gastrointestinal stromal tumors, and dedifferentiated/pleomorphic liposarcomas.[23] However, whether *CPM* has an important oncogenic role in those tumors or in liposarcomas is still unknown.

In summary, we have found that *CPM* is consistently amplified in well-differentiated liposarcoma/atypical lipomatous tumors but not in ordinary or pleomorphic lipomas. These results offer another avenue for the investigation of the biology of well-differentiated liposarcomas and suggest that *CPM* could be used as an alternative and novel diagnostic tool for these tumors.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

References

1 Jacob EK, Erickson-Johnson MR, Wang X, *et al.* Assessment of MDM2 amplification using fluorescence *in situ* hybridization of paraffin embedded tissues discriminates atypical lipomatous tumors from lipomas. Mod Pathol 2006;19(13A):45.
2 Sirvent N, Forus A, Lescaut W, *et al.* Characterization of centromere alterations in liposarcomas. Genes Chromosomes Cancer 2000;29:117–129.
3 Mef Nilbert AR, Felix M, Paul SM, *et al.* Characterization of the 12q13-15 amplicon in soft tissue tumors. Cancer Genet Cytogenet 1995;83:32–36.
4 Nilsson M, Meza-Zepeda LA, Mertens F, *et al.* Amplification of chromosome 1 sequences in lipomatous tumors and other sarcomas. Int J Cancer 2004; 109:363–369.
5 Skubitz K, Cheng E, Clohisy D, *et al.* Differential gene expression in liposarcoma, lipoma, and adipose tissue. Cancer Invest 2005;23:105–118.
6 Pilotti S, Della Torre G, Lavarino C, *et al.* Distinct mdm2/p53 expression patterns in liposarcoma subgroups: implications for different pathogenetic mechanisms. J Pathol 1997;181:14–24.
7 Weiss SW. Enzinger and Weiss's Soft Tissue Tumors 4th edn. Mosby: St Louis, MO, 2001.
8 Weaver J, Downs-Kelly E, Goldblum JR, *et al.* Fluorescence *in situ* hybridization for MDM2 gene amplification as a diagnostic tool in lipomatous neoplasms. Mod Pathol 2008;21:943–949.
9 Trahan S, Erickson-Johnson MR, Rodriguez F, *et al.* Formation of the 12q14-q15 amplicon precedes the development of a well-differentiated liposarcoma arising from a nonchondroid pulmonary hamartoma. Am J Surg Pathol 2006;30:1326–1329.

10 Fletcher C, Unni K, Mertens F, (eds) WHO Classification of Tumours; Pathology and Genetics: Tumours of Soft Tissue and Bone. IARC Press: Lyon, France, 2002.
11 Laurino L, Furlanetto A, Orvieto E, *et al.* Well-differentiated liposarcoma (atypical lipomatous tumors). Semin Diagn Pathol 2001;18:258–262.
12 Pedeutour F, Forus A, Coindre J, *et al.* Structure of the supernumerary ring and giant rod chromosomes in adipose tissue tumors. Genes Chromosomes Cancer 1999;24:30–41.
13 Gisselsson D, Hoglund M, Mertens F, *et al.* The structure and dynamics of ring chromosomes in human neoplastic and non-neoplastic cells. Hum Genet 1999; 104:315–325.
14 Borden E, Baker L, Bell R, *et al.* Soft tissue sarcomas of adults: state of the translational science. Clin Cancer Res 2003;9:1941–1956.
15 Berner J, Forus A, Elkahloun A, *et al.* Separate amplified regions encompassing CDK4 and MDM2 in human sarcomas. Genes Chromosomes Cancer 1996;17:254–259.
16 Ida CM WX, Erickson-Johnson MR, Wenger DE, *et al.* Primary retroperitoneal lipoma: a soft tissue pathology heresy?: report of a case with classic histologic, cytogenetics, and molecular genetic features. Am J Surg Pathol 2008;32:951–954.
17 Gustafson P, Akerman M, Alvegard T, *et al.* Prognostic information in soft tissue sarcoma using tumour size, vascular invasion and microscopic tumour necrosis-the SIN-system. Eur J Cancer 2003;39:1568–1576.
18 Nielsen T, West R, Linn S, *et al.* Molecular characterization of soft tissue tumours: a gene expression study. Lancet 2002;359:1301–1307.
19 Golub T, Slonim D, Tamayo P, *et al.* Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. Science 1999;286:531–537.
20 Helman L, Meltzer P. Mechanisms of sarcoma development. Nat Rev Cancer 2003;3:685–694.
21 Fanburg-Smith JC, Miettinen M. Liposarcoma with meningothelial-like whorls: a study of 17 cases of a distinctive histological pattern associated with dedifferentiated liposarcoma. Histopathology 1998;33: 414–424.
22 Johnson MR, Seys AR, Roth CW, *et al.* Assessment of carboxypeptidase M (CPM) amplification: a surrogate marker for MDM2 amplification in the discrimination of well-differentiated liposarcomas from lipomas. ASHG Meeting Booklet. Phildelphia. Abstract 1190. 2008, p.246.
23 Deiteren K, Hendriks D, Scharpé S, *et al.* Carboxypeptidase M: multiple alliances and unknown partners. Clinica Chimica Acta 2009;399:24–39.