

Lead article

Dedifferentiation of a well-differentiated liposarcoma to a highly malignant metastatic osteosarcoma: amplification of 12q14 at all stages and gain of 1q22–q24 associated with metastases

Anne Forus^{a,*}, Marcelo L. Larramendy^{b,c}, Leonardo A. Meza-Zepeda^a, Bodil Bjerkehagen^d, Linda H. Godager^a, Anine B. Dahlberg^a, Gunnar Saeter^e, Sakari Knuutila^b, Ola Myklebost^a

^aDepartment of Tumor Biology, The Norwegian Radium Hospital, 0301 Oslo, Norway

^bDepartment of Medical Genetics, Haartman Institute, University of Helsinki, Helsinki, Finland

^cLaboratorio de Citogenética y Cátedra de Citología, Facultad de Ciencias Naturales, Universidad Nacional de La Plata, La Plata, Argentina

^dDepartment of Pathology, The Norwegian Radium Hospital, 0310 Oslo, Norway

^eDepartment of Clinical Oncology, The Norwegian Radium Hospital, Oslo, Norway

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Abstract

Well-differentiated liposarcomas (WDLPS), especially those located in the retroperitoneum, may occasionally undergo dedifferentiation. Although this process is associated with a more aggressive clinical course, dedifferentiated liposarcomas rarely produces metastases. The case reported here is rather uncommon: A retroperitoneal WDLPS gave lung metastases that were diagnosed as highly malignant osteosarcomas. We used comparative genomic hybridization (CGH), fluorescence in situ hybridization (FISH), and Southern blot analyses to characterize the copy number changes and genetic aberrations occurring at different stages of the disease. In the primary tumor, the only detectable aberration was amplification of 12q13–q14, which was present only in a fraction of the cells and revealed by FISH analysis. High-level amplification of 12q13–q14, involving *CDK4*, *MDM2*, and *HMGIC*, was seen both in the relapse and the metastases. The second most common change, gain or high-level amplification of 1q22–q24, was detectable by CGH only in the osteogenic metastases, as was loss of the distal 2q. FISH analyses revealed considerable heterogeneity in the samples, and the percentage of cells showing aberrations was significantly higher in the metastatic samples. In particular, increased copy numbers of 789f2, a marker for 1q21 amplification in sarcomas, was observed in more than 65% of the cells in the metastatic samples, but in less than 10% of the cells from the recurrent samples. These observations could indicate that 1q amplification, in particular, may be indicative of a more malignant phenotype and ability of metastasis in WDLPS, as has also been suggested by others. © 2001 Elsevier Science, Inc. All rights reserved.

1. Introduction

Well-differentiated liposarcomas (WDLPS) are tumors of low malignancy grade. When located in subcutis they are usually cured by local surgery and rarely recur. However, WDLPS in deep soft tissues, such as retroperitoneum, abdomen or muscle of the extremities have a tendency to recur, but rarely develop metastases [1]. Still, the long-term prog-

nosis of retroperitoneal tumors could be poor because of relentlessly recurring, locally aggressive disease [2].

WDLPS may undergo dedifferentiation [3]. A dedifferentiated liposarcoma is a tumor that contains components of WDLPS (or atypical lipoma) and cellular, mitotically active non-lipogenic sarcoma [4,5]. In more than two-thirds of the cases, the poorly differentiated component of the tumor resembles malignant fibrous histiocytoma (MFH). Less frequently, poorly differentiated fibrosarcoma-, leiomyosarcoma-, or rhabdomyosarcoma-like areas [2,6], as well as focally osteosarcomatous differentiations, heterotopic ossifications or whorls and bone-formation have been described

* Corresponding author. Tel. +47-22-93-54-11; fax: +47-22-52-24-21.
E-mail address: anne.forus@labmed.uio.no (A. Forus).

[2,7,8]. Dedifferentiation of WDLPS is encountered most often in tumors of the retroperitoneum, and in most cases, there is a time period of 7–11 years from the first diagnosis and dedifferentiation [3].

Several reports have suggested associations between dedifferentiation and altered expression of specific proteins. Dei Tos et al. [9] report overexpression of *MDM2* as the most frequently observed abnormality in dedifferentiated liposarcomas, but it is not known whether this was associated with amplification. In another series of WDLPS, all cases with giant marker chromosomes showed consistent amplification of *MDM2*, and in most cases also (over)expression of the protein [10]. In these tumors, poorly differentiated regions were not reported, suggesting that *MDM2* overexpression probably is not causally linked to dedifferentiation.

Interestingly, a recent study reported expression of *p53* only in the dedifferentiated areas of WDLPS with meningothelial-like whorls, whereas the differentiated areas were negative [2]. Similar observations have also been reported for a series of dedifferentiated chondrosarcomas where overexpression of *p53* was consistently observed in the dedifferentiated component [11]. Abnormal *p53* expression has been found also in low-grade areas of such tumors [9], thus, *p53* expression is not a reliable marker for dedifferentiation.

We have studied in detail a rather peculiar case of WDLPS dedifferentiation: The primary tumor was diagnosed as a WDLPS, the relapse was a WDLPS showing areas with ectopic bone formation and areas with bizarre-looking cells, and the tumor gave rise to metastases that were diagnosed as highly malignant osteosarcomas. Comparative genomic hybridization (CGH), fluorescence in situ hybridization (FISH), Southern and Northern blotting analyses were used to determine whether the presence of specific chromosomal and genetic aberrations could be correlated with different stages of the disease.

2. Materials and methods

2.1. Case report

A 26-year-old woman noticed an abdominal mass in 1987. Clinical and radiographic investigation showed a retroperitoneal mass localized between the right kidney and adrenal gland. The mass was resected (P). Pathological examination revealed a tumor measuring 15 cm in maximum diameter, and a diagnosis of WDLPS with myxoid areas was made. Postoperative radiation therapy (50 Gy) was given. Five years later, in 1992, at routine follow-up, radiographic examination showed lesions suspected to be metastases in the lung, and at the same time, a local recurrence with multiple abdominal foci was found, and was confirmed by needle biopsy. Microscopically, the biopsy of the recurrent tumor showed WDLPS with myxoid areas and benign ossification (1-LS, -MYX, and -OS, respectively). Preoperative chemotherapy was given with etoposide and ifosfamide. The patient underwent resection of the abdominal tu-

mor (1992) and two resections for lung metastasis: from the left lung early in April 1993 (samples 2a, b, c) and a from the right lung (samples 3a, b) a few weeks later. The various samples a, b, and c were selected on the basis of macroscopic appearance of the biopsies.

Histological analysis of the abdominal recurrence showed a mixed liposarcoma. There were areas of WDLPS of sclerosing and lipoma-like type, and areas of myxoid differentiation. This part did not show any histological evidence of chemotherapy response. Other parts of the tumor revealed areas of hyalinized tissue corresponding to ectopic ossification, and parts with rather bizarre cells not seen in the needle biopsy. The latter cells were thought to show changes induced by treatment.

The lung metastases displayed highly malignant osteosarcoma with osteoblastic differentiation. Only minor areas revealed histological evidence of response to preoperative chemotherapy. The patient did not have any signs or symptoms of a primary bone tumor. Scintigraphic examination (bone scan) showed high soft tissue uptake in the lung metastases consistent with metastases from an osteogenic sarcoma. There were no signs of a primary bone tumor in the skeleton (not shown).

In 1994, the patient was admitted to the hospital because of abdominal pain, symptoms of sepsis and gastrointestinal bleeding. Anaerobe bacteria were observed in the blood cultures. The patient died, and postmortem examination showed no residual tumor, but evidence of disseminated intra-vascular coagulation with hemorrhage and septicemia was found.

2.2. Histology

Paraffin and frozen sections were stained with hematoxylin and eosin according to standard procedures, and the tumor cell percentage as well as the histological subtype was evaluated by a specialist in pathology (BB). From the primary lesion, 11 blocks were examined, from the recurrent 20, and from the lung metastases, 12 and 5 blocks, respectively.

2.3. Tumor specimens

Tumor specimens were obtained from the patient during different stages of the disease (Table 1). From the primary tumor, only paraffin embedded tissue was available. Fresh tissue was obtained directly after surgery from the recurrent sample and two different metastases to the lungs. These samples were frozen in liquid nitrogen and stored at -80°C .

2.4. Xenograft samples

Tissue from several regions of the two lung metastases were transplanted to immunodeficient mice and established as xenografts. Sections made from the xenografts were examined by the pathologist (BB), and the histological subtype was confirmed. The xenograft lines, 2bx and 3bx, respectively, have been passaged in nude mice 13 and 14 times since they were established.

Table 1
Tumor material

Sample	% Tumor cells	Location	Stage	Sarcoma subtype
P	85–100%	Retroperitoneum	Prim	Well-differentiated liposarcoma
1-LS	100%	Kidney/colon	Rec	Well-differentiated liposarcoma
1-LSp	90%	Kidney/colon	Rec	Well-differentiated liposarcoma
1-MYX	NA	Kidney/colon	Rec	NA
1-OS	100%	Kidney/colon	Rec	Hyalinized tissue and bizarre cells
2a	100%	Lung (left side)	Met	Osteosarcoma
2b	100%	Lung (left side)	Met	Osteosarcoma
2c	95–100%	Lung (left side)	Met	Osteosarcoma
3a	75–80%	Lung (right side)	Met	Osteosarcoma
3b	95%	Lung (right side)	Met	Osteosarcoma
Xenografts				
2bx	100%	Subcutaneous		Osteosarcoma
3bx	100%	Subcutaneous		Osteosarcoma

2.5. DNA extraction

DNA was extracted from paraffin embedded tissue from the primary tumor (P) and a part of the recurrent tumor (1-LSp), and from frozen material from the different parts of the recurrent tumor (1-LS, 1-OS and 1-MYX), metastases in the left and right lungs (2a, 2b, 2c, 3a, and 3b), and xenograft samples established from the metastases (2bx and 3bx) as described earlier [12,13].

2.6. Comparative genomic hybridization (CGH) analysis

CGH was performed using direct fluorochrome-conjugated DNAs for all samples essentially as previously described [14]. Tumor DNA was labeled with fluorescein isothiocyanate (FITC)-dUTP (DuPont, Boston, MA, USA), and reference DNA was labeled with Texas Red-dUTP (DuPont), and the hybridization mixture consisted of 400 ng of labeled tumor DNA, 400 ng of labeled female reference genomic DNA, and 10 µg of human Cot-1 DNA (GIBCO BRL).

Digital image analysis was done largely as described previously [14], using the ISIS digital image analysis system (MetaSystems Hard & Software, Altlußheim, Germany). Three-color images were acquired for 8–12 metaphases per sample. Chromosomes not suitable for CGH analysis were excluded, and chromosomal regions were interpreted as over-represented when the corresponding ratio exceeded 1.17 (gains) or 1.5 (high-level amplifications), and as under-represented (loss) when the ratio was lower than 0.85. A negative (peripheral blood DNA from normal controls) and a positive (tumor DNA with known copy number changes) control were included and run simultaneously with the test samples (tumors). All results were confirmed using a 99% confidence interval.

2.7. Fluorescent in situ hybridization (FISH) on interphase nuclei

Preparation of interphase nuclei was done as described previously [15].

2.7.1. Preparation of probes

YAC and cosmid DNA was labeled with biotin-14-dATP or digoxigenin-11-dUTP (Boehringer-Mannheim, Germany) by nick translation. For each hybridization, 200–500 ng of labeled DNA was prehybridized with 50- to 100-fold excess of human Cot-1 DNA and, in the case of YAC DNA, 2–5 µg of yeast DNA.

2.7.2. In situ hybridization

Pretreatment and denaturation of the slides was done as described previously [15], or as follows (Drs. E. Schröck and T.Ried, Cold Spring Harbor course “Advanced Molecular Cytogenetics” 1998). Briefly, slides were treated with pepsin (4 mg/ml) for 10 min at 37°C and washed in 1× PBS, then with 1% formaldehyde in 1× PBS/MgCl₂ for 10 min at room temperature, followed by washes in 1× PBS. Denaturation (1.5–2 min 74°C), hybridization and washes were according to standard procedures. For detection of green fluorescence we used fluorescein isothiocyanate (FITC)-conjugated sheep anti-digoxigenin (Boehringer Mannheim, Germany) followed by FITC- or ALEXA 488-labeled donkey anti-sheep (Molecular Probes, Leiden, The Netherlands), for detection of red fluorescence we used avidin-conjugated Cy3 (Amersham Life Science, Little Chalfont, UK).

2.7.3. Evaluation of results

Hybridized slides were examined visually using a Zeiss Axioplan microscope equipped with appropriate single bypass filter for excitation of DAPI, and double bypass filters for DAPI/FITC and DAPI/Rhodamine (Cy3), and a triple bypass filter for excitation of DAPI/FITC/Rhodamine(Cy3). For each probe, the number of hybridization signals was counted in at least 150 nuclei per slide.

2.8. FISH to paraffin embedded tissue sections and nuclei

2.8.1. Preparation of sections from formalin-fixed tissues

Paraffin blocks were sectioned at 5 µm and transferred to polylysine-coated slides that were baked at 65°C overnight.

2.8.2. Nucleus extraction from formalin-fixed tissue sections

For isolation of nuclei we used sections of 40 μm treated according to the protocol established by D. Gisselson (Lund, Sweden, personal communication) with minor modifications. The following steps were done in Eppendorf tubes: Paraffin was removed (3×10 min in xylene), washed in 100% methanol and dehydrated (100, 85, and 70% ethanol). Thereafter, the sections were treated with proteinase K (5 mg/ml in 50 mM Tris pH 7.5/10 mM EDTA/5 mM NaCl/1% Triton X-100) for 1–2 h at 37°C, disaggregated by pipetting, and the suspension was centrifuged and treated with protease-K for another 30 min at 37°C with shaking. The tissue was fixed by adding 1 ml of fixative (3:1 methanol:acetic acid). After centrifugation, the pellet was resuspended in 1 ml of fixative, left at room temperature for 30 min, and centrifuged again. Fixative was removed down to 100 μl , the pellet was resuspended, and drops of 10 μl were added onto silanized slides (Oncor, Gaithersburg, MD, USA). The slides were dried by incubation at 60°C overnight.

2.8.3. Pretreatment of slides

Tissue sections: pretreatment of sections was done according to a procedure described by A. Hopman and F. Raemakers (personal communication, “In situ hybridisation” course at the University of Brabant, April 1998). Briefly, paraffin was removed in xylene (3×10 min, room temperature) followed by washes in 100% methanol. To avoid unspecific binding of streptavidin-HRP when using TSA detection (see Section 2.8.4.), slides were immersed in 1% H_2O_2 in 100% methanol for 30 min, washed in 100% methanol and air-dried. Thereafter, slides were immersed in 1M NaSCN for 10 min at 80°C, rinsed with distilled water, and treated with pepsin (4 mg/ml) in 0.2 M HCl for 30–60 min, rinsed in water and dehydrated in ethanol (70, 90, 96, and 100%).

Interphase nuclei: Slides were baked overnight at 65°C and treated with 1% formaldehyde in PBS for 10 min at room temperature. After washes in PBS, slides were treated with 4 mg/ml pepsin in 0.01 M HCl for 20 min at 37°C, washed in PBS, dehydrated, and dried. A large fraction of the nuclei were lost during this procedure.

2.8.4. Preparation of probes, in situ hybridization and washes

Probe-labeling, pretreatment, and hybridization was performed as described in Section 2.8.3. Slides were denatured for 2–5 min at 70°C. Posthybridization washes and detection of digoxigenin-labeling was done as described previously, using green fluorescence.

2.8.5. Detection of biotin labeling by tyramide signal amplification

After incubation in 0.1 M Tris-HCl pH 7.5/0.15 M NaCl/0.5% blocking reagent for 30 min at room temperature, hybridization signals were detected by deposition of biotinylated tyramides [16,17] according to the instructions of the kit supplier (NEN, Life Science Products, Boston, MA, USA). The antibodies used were avidin-Cy3 (Amersham),

FITC-labeled streptavidin (NEN) and biotinylated anti-avidin D (Vector Laboratories).

2.9. Southern blot analyses

Preparation of filter blots and hybridization was performed as described previously [12]. For detection of amplification in 12q13–q14, copy numbers of *MDM2*, *HMGIC*, and *CDK4* were compared to the copy number of *APOB*. For detection of 9p deletions, a probe for p15^{INK4B} (*CDKN2B*) was compared to the copy number of *APOB*. Southern blots were sequentially hybridized to probes from each locus, and to the control probe (*APOB*) [18]. Quantitation of signal intensity was done by two-dimensional densitometry on a Molecular Dynamics laser densitometer. The net signals from specific bands were corrected for unequal sample loading by calibration relative to the signal obtained with the *APOB* control probe. Average signals from at least three different blots were used to quantitate the amplification levels in the tumor.

The signals were compared to signals from normal control samples (leukocytes). Signals with an intensity less than 50% of the signal from the normal samples were scored as deletions.

2.10. Northern blot analyses

Total cellular RNA was isolated from frozen tissues, electrophoresed and blotted onto membranes essentially as described previously [12]. Filter blots were hybridized with probes for alkaline phosphatase (*ALP*) and lipoprotein lipase (*LPL*), and with an 18S rRNA oligonucleotide probe for loading control. Hybridization conditions were as described previously.

2.11. Probes

2.11.1. Southern analyses

cDNA probes for *MDM2* [19] (Drs. D. L. George and B. Vogelstein), *CDK4* cDNA was the clone pSS-CDK4-5 [20] (Dr. P. S. Meltzer), the *HMGIC* probe covered the 5' part of the gene including the complete protein coding region [21] (Dr. W. Van de Ven), cDNA probe for *CDKN2B* [22] (Dr. Beach). Calibration probe was *APOB* clone pB27 [18] (Dr. Breslow).

2.11.2. Northern analyses

Clone pSV2Aalp (ATCC clone 59635) for *ALP* [23] and cDNA clone lpl (ATCC number 59635) for *LPL* [24]. An oligonucleotide complementary to nucleotides 287 to 305 in human 18S rRNA for RNA loading control.

2.11.3. Centromere probes

Centromeric regions of chromosome 1 and 12 were revealed by biotin or digoxigenin-labeled human chromosome 1 α -satellite (D1Z5) and chromosome 12 α -satellite (D12Z3, Oncor, Inc., Gaithersburg, MD, USA), and digoxigenin-labeled human satellite III specific for the pericentric region of chromosome 1 (pUC1.77, Boehringer Mannheim).

2.11.4. Yeast artificial chromosome (YAC) and cosmid clones

We used YAC 789f2 from the CEPH mega-YAC library to detect gains in the 1q21–q23 region [25], covering the region from *FLG* to D1S3620. For detection of 12q13–q14 amplification we used a cosmid for *CDK4* (CDK4cos 10-3, Dr. T. Look) [26].

3. Results

3.1. Histology

Microscopic examination of the primary tumor showed a WDLPS of a sclerosing and lipoma-like type (Figs. 1a and b). Some myxoid areas could be seen. The recurrent tumor, like the primary, was a WDLPS, but parts with benign-looking heterotopic ossification (Figs. 1e and f) and areas with bizarre looking cells, thought to represent chemotherapy induced changes, could be seen (Fig. 1g). No clear osteosarcoma component was seen. The metastases in the lung, which were detected at the same time as the abdominal recurrence, were highly malignant osteosarcomas (Figs. 1j and k). No liposarcoma components could be recognized in these tumors. In accordance with these findings, the tumor was diagnosed as a dedifferentiated liposarcoma.

3.2. Comparative genomic hybridization (CGH)

CGH did not detect any changes in the primary, paraffin-embedded tumor (P), but several recurrent chromosomal aberrations were seen in all the other tumors (Table 2).

Gain of 12q material was seen in all samples except the primary tumor (P) and the recurrent 1-LSp, and was, therefore, the most frequently observed alteration. The specimen 1-OS from the recurrent tumor had a local high-level amplification of the 12q21–q22 region. This high-level amplification was detected in all samples from the metastasis in the left lung, which was diagnosed as an osteosarcoma (2a, b, and c), and in addition, the region 12q14–q24.2 was amplified at lower levels. The metastasis in the right lung, part 3b,

showed exactly the same pattern, but sample 3a had a gain of 12q13–qter and not the high-level amplification of 12q21–q22.

Gain of 1q material, affecting various parts of 1q21–q32 and with a minimal overlapping region at 1q23, was the second most common aberration. This gain was observed only in the samples from the lung metastases, of which 2b and 3b showed high-level amplification narrowed down to 1q21–q31.

The two metastatic tumors also showed loss of 2q material; most of the long arm was lost, but a region from the centromere and down to q14 or q21/22 was retained. In the metastasis from the left lung, this aberration was observed in all different parts of the tumor, but only in the 3b sample from the metastasis in the right lung. In the recurrent tumor (1-OS and 1-MYX) and a part of the metastasis in the left lung (2a) loss of 9p-material was observed, but this was not seen in the other metastases, nor in any of the xenografts from samples 2b and 3b established in nude mice (2bx and 3bx, respectively).

The xenograft 2bx had fewer aberrations than the corresponding lung metastasis (2b), but the high-level amplification of 12q14–q24.2 and gain of 1q material were retained. In contrast, the xenograft 3bx had a CGH profile very similar to the other lung metastasis (3b), including the gain of 1q21–q31, but the high-level amplification of 1q22–q24 was lost.

In general, the number and complexity of aberrations increased as the tumor progressed towards a more malignant phenotype. Furthermore, copy number changes varied for the different parts of the same tumor, most notably in the metastasis to the right lung where one part (3a) showed gain of 12q13–qter alone whereas the second, 3b, had this gain as well as numerous other aberrations.

3.3. FISH analyses

The two most common aberrations, gain or amplification of regions within 1q21–q31 and 12q14, were additionally analyzed by FISH. Copy numbers of centromere 1 and 12 as

Table 2
DNA sequence copy number changes revealed by CGH

Sample	Copy number changes
Patient samples	
P	No changes
1-LS	NA
1-LSp	No changes
1-OS	–9p21–pter, +12q13–q24.2(12q21–q22)
1-MYX	+4q, –9p21–pter, +12q14–q24.2, –13cen–q14
2a	+1q23–q31, –2q22–qter, –9p, +12q (12q14–q24.2), +17p12–pter
2b	+1cen21–q32(1q21–q31), –2q14.2–qter, 6q16–qter, +12q(12q14–q24.2), +14, –15cen–q15, +17q(17q23–q24)
2c	+1q21–q32, –2q22–qter, –11q14–q24, +12q(12q14–q24.2), –13
3a	+12q13–qter
3b	1q21–q31(1q22–q24), –2q14.2–qter, +6p, –6q, –11q24–qter, +12q(12q14–q24.1), –13q21–qter, +17, –18, +20, +22
Xenografts	
2bx	+1q24–q25, +12q(12q14–12q24.2)
3bx	+1q21–q32, –2q21–qter, –6q21–qter, +12q(12q13–q24.2), +17q22–qter, –18, +20

Abbreviation: NA, no DNA available for analysis.

Gains are marked with +, losses with – and high-level amplifications are marked in bold.

Table 3
FISH analyses using probes from the most frequently altered regions

Patient samples	Stage/Histology	Cen 1	Cen 12	YAC789f2	CDK4 ^a
P	P/WDLPS	1s: 21% 2s: 73% 3–6s: 6%	1s: 25% 2s: 71% 3–6s: 4%	1s: 7% 2s: 77% 3–6s: 16%	1s: 7% 2s: 62% 3–9s: 16% ≥10s: 15%
1-LSp	Rec/WDLPS	1s: 14% 2s: 82% 3–9s: 4%	1s: 11% 2s: 76% 3–9s: 13%	1s: 12% 2s: 82% 3–9s: 6%	2s: 57% 4s: 15% ≥10s: 28%
Patient samples	Stage/Histology	Cen1 and YAC789f2		Cen 12 and CDK4	
1-LS	Rec/WDLPS	2 cen and 2s: 96% 2 cen and 3–9s: 1% 4 cen and 4s: 3%		Not analyzed	
1-OS	Rec/WDLPS, hyalinized tissue and atypical cells	2 cen and 2s: 91% 2 cen and 3–9s: 8% 4 cen and 4s: 1%		2 cen and 2s: 58% 2 cen and 3–9s: 4% 2 cen and ≥10s: 38%	
2a	Met/OS	2 cen and 2s: 19% 3–9s of each: 43% 3–9 cen and ≥10s: 38%		2 cen and 2s: 7% 3–9 cen and ≥10s: 93%	
2b	Met/OS	2 cen and 2s: 11% 3–9s of each: 73% ≥10s of each: 16%		2 cen and 2s: 6% 3–9 cen and ≥10s: 94%	
2c	Met/OS	2 cen and 2s: 7% 2 cen and 3–9s: 5% 3–9s of each: 44% 3–9 cen and ≥10s: 38% ≥10s of each: 6%		2 cen and 2s: 3% 3–9 cen and ≥10s: 97%	
3a	Met/OS	2 cen and 2s: 80% 2 cen and 3–9s: 11% 3–9s of each: 4% 3–9 cen and ≥10s: 5%		2 cen and 2s: 72% 2 cen and 3–9s: 4% 2 cen and ≥10s: 24%	
3b	Met/OS	2 cen and 2s: 18% 2 cen and 3–9s: 9% 3–9s of each: 62% ≥10s of each: 11%		2 cen and 2s: 1% 2–4 cen and ≥10s: 99%	
Xenografts	Stage/Histology	Cen 1 and YAC789f2		Cen 12 + CDK4	
2bx	X/OS	2 cen and 2s: 14% 2 cen and 3–9s: 17% 3–9s of each: 48% 3–9 cen and ≥10s: 12% ≥10s of each: 9%		2 cen and 2s: 4% 2–4 cen and ≥10s: 96%	
3bx	X/OS	2 cen and 2s: 12% 2 cen and 3–9s: 17% 3–9s of each: 60% 3–9 cen and ≥10s: 11%		2 cen and 2s: 2% 2–4 cen and ≥10s: 98%	

FISH analysis on paraffin sections and interphase nuclei isolated from paraffin embedded primary tumor (P) and recurrent 1-LSp, and on interphase nuclei from frozen tissue of recurrent (Rec), metastatic (Met), and xenograft (X) samples. For the frozen tissue, the centromere probes were hybridized together with YAC or cosmid from the same chromosome, and the signals were counted in the same nuclei, thus the number before “and” refers to the centromere whereas the number after is the count for YAC789f2/CDK4, respectively. For the primary tumor (P), the probes for cen 1 and cen 12 were hybridized to separate paraffin sections. A relatively large fraction of the nuclei showed only one signal, and this is probably a result of nuclei being cut during the preparation of the sections. YAC789f2 and the CDK4 cosmid were hybridized to interphase nuclei isolated from paraffin embedded tissue. The most frequent signal pattern is shown in boldface.

Cen = centromeres, s = signals.

^aCDK4 signals were counted in nuclei on four different preparations to get sufficient material for analysis.

well as two genomic probes representing sequences known to be highly amplified were analyzed and the results presented in Fig. 1 and Table 3.

3.3.1. Centromere 1

The samples from the primary tumor (P) had normal copies of centromere 12. Interestingly, several nuclei with high-

level amplification of *CDK4* were observed (Fig. 1c), although the majority showed normal copy numbers of this gene. In the recurrent tumor, the sample 1-OS showed two centromere 12 signals, but here copy numbers of *CDK4* were even more heterogeneous (Fig. 1h). While approximately 60% of the nuclei gave two signals, the remaining 40% had amplification. All parts of the metastasis in the left

lung, samples 2a, 2b and 2c, had increased numbers of centromere 12 and high-level amplification of *CDK4* in most of the nuclei (Fig. 1l). This was also the case for the 3b sample from the metastasis in the right lung, and the xenografts. Again, samples 3a and 3b had extremely different patterns. In 3a, only a small fraction of the nuclei showed amplification of *CDK4*, and aneusomy of chromosome 12 was never observed. In 3b, most of the nuclei showed amplification of *CDK4*, and about 25% of the nuclei had more than two centromeres.

3.3.2. Centromere 1 (*cen1*) and 1q21 marker

YAC 789f2, which is so far the best marker for the 1q21-amplification [15] was used to detect this amplicon. The samples from the primary tumor (P) had normal copy numbers of YAC789f2 and the centromere in a majority of the nuclei (Fig. 1d). A relatively large fraction of the nuclei showed only one centromere signal, probably because the nuclei have been cut during the preparation of the sections. In the recurrent sample, the majority of the nuclei showed normal copy numbers of both probes (Fig. 1i). In the samples from the metastases in the left lung (2a, b, c) we observed aneusomy of chromosome 1, and the most common pattern was gain of both the centromere and YAC789f2 (Fig. 1m). Samples 2a and 2c had a relatively large fraction of nuclei with increased copy numbers of centromere 1 and high-level amplification of YAC789f2, but this pattern was less striking for 2b, although CGH revealed high-level amplification of 1q21–q23 in this sample. Sample 3b, from the metastasis in the right lung, had a signal pattern similar to 2b, whereas in 3a, almost all the nuclei analyzed showed normal copy numbers of both probes. The CGH analyses confirmed this results, indicating that (at least) two very different tumor cell populations are present in this sample. In the two xenografts (2bx and 3bx), increased copy numbers of both *cen1* and YAC789f2 was most frequent, as was the case in the corresponding tumor samples, 2b and 3b. All the metastatic samples, apart from 3a, showed gain or amplification of 1q material in more than 65% of the cells analyzed, whereas the recurrent tumor showed such aberrations in less than 10% of the cells. This could suggest that cells with 1q aberrations are selected for during the metastatic process.

In general, the FISH analyses revealed considerable heterogeneity in copy numbers among nuclei from the same tu-

mor, and there was also some variation between the different parts (a, b, and c). This was most striking for the metastasis in the right lung (3a and b), where 3a presented mostly normal copy numbers of all probes whereas the other, 3b, showed gains and amplifications. Furthermore, the increased numbers of hybridization signals from both centromere 1 and 12 in the metastatic samples could indicate that these tumors were aneuploid.

3.4. Southern analyses

We analyzed the 12q13–q14 amplicon in more detail using probes for *CDK4*, *MDM2*, and *HMGIC* (12q14–q15), and we used *CDKN2B* (p15^{INK4B}) to examine whether a deletion of 9p could be observed also in samples where CGH analyses showed an apparently normal copy number of this region. The results are shown in Fig. 2.

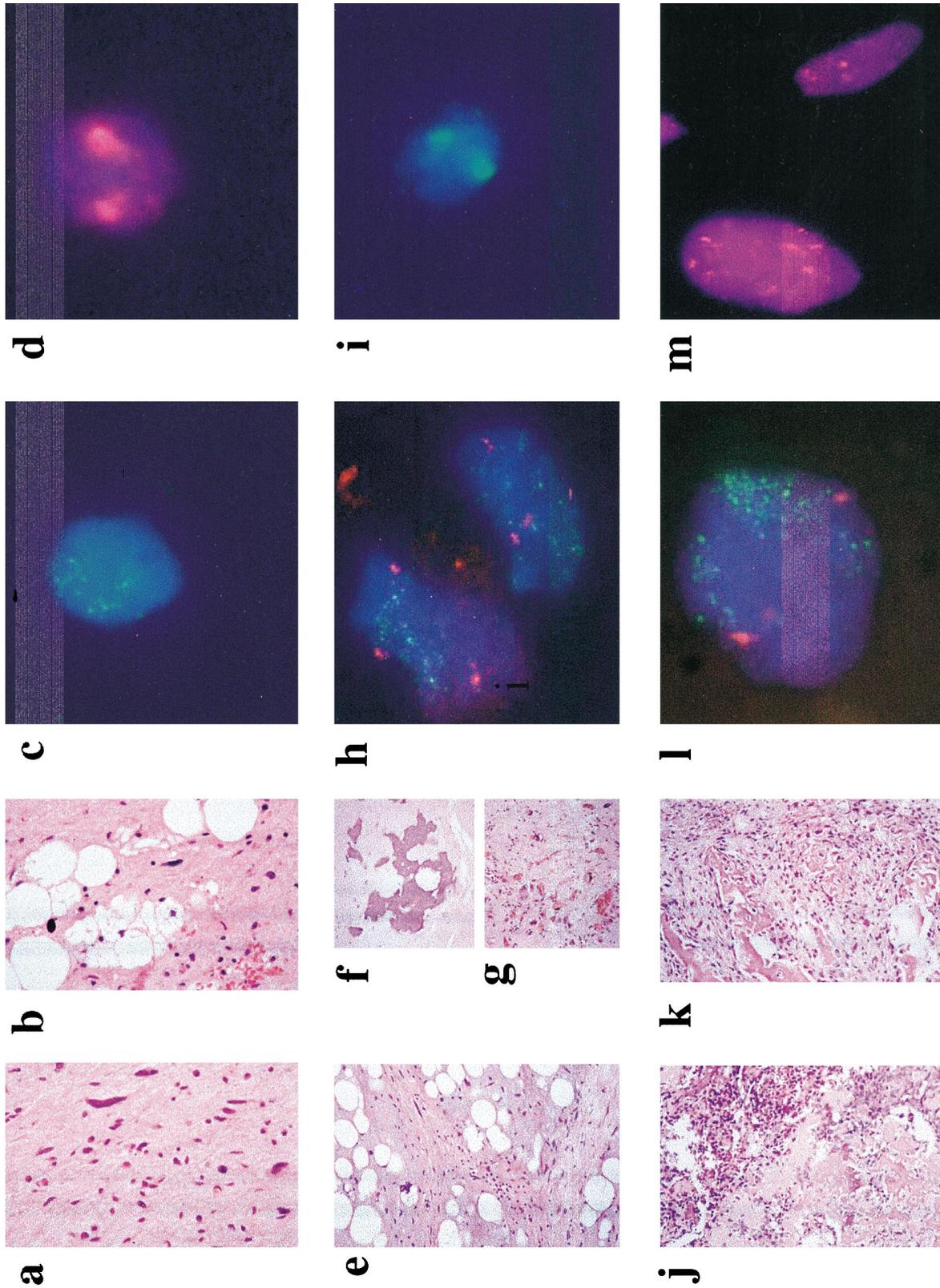
We found amplification of all three genes from 12q13–q14 in all samples checked, but the amplification levels varied (Fig. 2). The recurrent samples generally showed lower amplification levels than the metastases, but again, sample 3a from the metastasis in the right lung was an exception. In this part of the tumor, all three probes showed low or moderate amplification (2- to 4-fold or 5- to 9-fold increases), which is in keeping with CGH and FISH results.

We detected deletions of *CDKN2B* in sample 2a from the metastasis in the right lung. This sample showed deletion of the whole 9p arm by CGH (Fig. 2). Also in 2bx, this deletion was seen, although CGH analyses showed normal copy numbers of 9p. In contrast, *CDKN2B* was normal in the two other samples, 1-OS and 1-MYX, where CGH detected deletion of 9p21–pter. These deletions were smaller, and might not include this gene.

3.5. Northern analyses

RNA was available from part 1-OS of the recurrent tumor, from two parts of the metastasis in the left lung (2a and 2b), and from the xenograft established from metastasis in the right lung (3bx). The metastatic samples and the xenograft were classified as high-grade osteosarcomas, and sample 1-OS showed hyalinized tissue and heterotopic ossification. All these samples expressed high levels of alkaline phosphatase (*ALP*), indicative of bone-forming tissue (Fig. 3), whereas the unrelated liposarcoma samples on the same blot showed no expression at all, or low levels in a few

Fig. 1. Representative histology and FISH-analyses of the primary, recurrent, and metastatic tumors. Histology: Stained paraffin-embedded tissue was evaluated by microscopy ($\times 20$ and $\times 40$ magnification). FISH analyses: Interphase nuclei isolated from paraffin-embedded or frozen tissue was hybridized with probes for centromere 12 and/or a cosmid for *CDK4* (12q13–q14), or with YAC789f2 (1q21) as described. (a and b): Histological slides from the abdominal primary tumor show well-differentiated liposarcoma. No osteosarcoma component was seen. (c and d): Interphase nuclei isolated from paraffin tissue hybridized with *CDK4* (c, green) and YAC789f2 (d, red). *CDK4* is amplified whereas the YAC show normal copy numbers. (e, f, and g): Histological slides from the recurrent abdominal tumor show liposarcoma with ossification and areas of bizarre looking cells (g) thought to be chemotherapy induced. (h and i): Interphase nuclei from 1-OS hybridized with *CDK4* (green) and *cen12* (red, h), and YAC789f2 (red, i). *CDK4* is amplified whereas the YAC show normal copy numbers. (j and k): Histological slides from the metastasis in the left lung show a highly malignant osteosarcoma. (l and m): Interphase nuclei from the metastasis in the left lung hybridized with *CDK4* (green) and *cen12* (red, l) and YAC789f2 (red, m). *CDK4* is highly amplified whereas the YAC show variable copy numbers.



cases (Fig. 3 and results not shown). We also checked the expression of samples lipoprotein lipase (*LPL*), which is expressed in muscle, heart and adipose tissue, but not in bone [24]. No expression was found in the samples from me-

tastases (2a, 2b, and 3ax), and also the sample 1-OS from the recurrent tumor did not express this gene. *LPL* expression was high in the other unrelated liposarcomas present on the blot (Fig. 3), as expected [27].

Patient Sample	CDK4	HMGIC	MDM2	CDKN2B
1-MYX				N
1-LS				N
1-OS				N
2-A				Deletion
2-B				N
2-C				N
3-A				N
3-B				N
2-Bx				Deletion
3-Ax				N

Normal	2-4	5-9	≥10	Deletion
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Fig. 2. Summary of Southern blot analyses of the 12q13–q15 and 9p21 regions in recurrent, metastatic, and xenograft samples. Probes for *CDK4*, *HMGIC*, and *MDM2* (12q13–q14) as well as *CDKN2B* (9p21) were sequentially hybridized to filter blots. Gene copy numbers relative to a reference probe was determined densitometrically, and divided into five categories as indicated. Measurements were based on average signal intensities from at least three different blots. Gene dosage values of 0.5 or lower were scored as deletions.

4. Discussion

One of the most extensive reports on dedifferentiated liposarcomas to date is the one by Henricks et al. [7] from 1997 where 155 cases were analyzed in order to define how the extent and degree of dedifferentiation affected the clinical outcome. These investigators found that the majority of dedifferentiated liposarcomas were de novo cases, and only a minority developed from pre-existing WDLPS similar to the one described here. There was no direct correlation between the amount of dedifferentiated zones and the clinical

behavior. Rather, the location seemed to be the most important prognostic factor since retroperitoneal tumors had significantly worse survival than those located in other sites [7]. This has also been suggested by others [1,6], and seems to be a general feature also for ordinary WDLPS in this region [2].

The case presented here was first diagnosed as a WDLPS of the retroperitoneum. It was not possible to perform karyotyping, so it is not known whether the tumor had the giant rods or ring chromosomes which are so typical for WDLPS. The recurrent tumor, which was excised from the same location 5 years later, had regions of ectopic ossification and bizarre cells thought to be induced by chemotherapy, and the metastases to the left and the right lungs were highly malignant osteosarcomas.

Upon scintigraphic examination of the patient, no tumor cells could be detected in the bone. It is, therefore, unlikely that the multiple metastases seen in the lungs did not originate from the WDLPS, and instead had developed from an occult, undetectable osteogenic tumor. Moreover, the frequency of 12q13–q14 amplification, including *MDM2* or *CDK4*, in sarcomas has been reported to be about 15% [28], and it is rather unlikely that two entirely independent and rare tumors should arise at the same time and both have the high-level amplification of 12q13–q14 (*CDK4*) observed here. Additionally, an osteogenic cell population expressing high levels of *ALP*, was present in the recurrent tumor. Although sampling was satisfying, one cannot rule out that a small component of osteosarcoma differentiated tumor cells was overlooked in the primary or recurrent tumor. The parts with benign looking osteogenic metaplasia and bizarre cells were difficult to evaluate. One cannot rule out that this part contained small osteosarcoma foci with chemotherapy induced morphologic changes.

There are reports in the literature of malignant mesenchymas, which are sarcomas that exhibit more than two

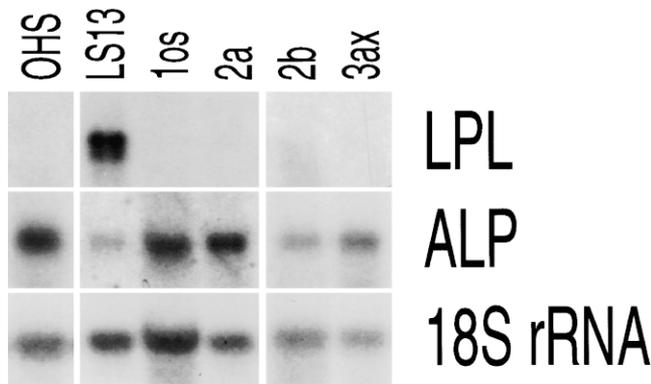


Fig. 3. Northern blot analyses of lipoprotein lipase (*LPL*) and alkaline phosphatase (*ALP*) in samples from the recurrent and metastatic tumors. Probes for *LPL*, *ALP* and 18srRNA were sequentially hybridized to filter blots with the recurrent 1-OS, two different parts of the left lung metastasis (2a and 2b) and the xenograft from the right lung metastasis (3ax), as well as some unrelated liposarcoma and osteosarcoma samples. Lipoprotein lipase (*LPL*) was only expressed in the liposarcoma samples (LS13, and results not shown). *ALP* showed high expression levels only in the osteogenic samples (1-OS, 2a, 2b, 3ax and the osteosarcoma cell line OHS and results not shown). Very low *ALP* expression could be detected in some liposarcoma samples.

types of specialized differentiation [5], that could be WDLPS and osteosarcoma. Some of these may resemble the case presented here, e.g., Geurts van Kessel et al. [29] have reported that such tumors may have ring chromosomes as well as amplification of 12q13–q14.

Looking at the chromosomal and genetic aberrations occurring at the different stages, we found that the first, low grade tumor had no aberrations detectable by CGH, but FISH-analyses detected *CDK4* amplification in some nuclei. All the other samples had chromosomal and genetic changes detectable by all the methods used. The number of aberrations increased as the tumor became more aggressive, in keeping with the general assumption that accumulation of genetic aberrations is necessary for progression and metastasis.

Already in the primary WDLPS (P), and more profound in the recurrent tumor (1-OS), a high-level amplification of regions from chromosome 12, including *CDK4* (also *MDM2* and *HMGIC* in the recurrent tumor), was observed. All but one metastatic sample (3a) showed high-level amplification of 12q-material by CGH, FISH and molecular analysis, as well as extra copies of the centromere.

Many studies of amplification in WDLPS report consistent amplification of *MDM2* [10,30–33], which probably acts through inactivation of *p53* [19]. Various other genes in the 12q13–q14 region may be included, in particular *CDK4* and *HMGIC* [10,30–33]. In other sarcoma subtypes, such as the more aggressive osteosarcomas, *CDK4* is most consistently amplified, alone or together with *MDM2* [34–36]. Therefore, there seems to be two different kinds of 12q13–q14 amplicons in sarcomas: one associated with low-grade or borderline tumors such as WDLPS, where *MDM2* is always involved, and one associated with more aggressive tumors, always including *CDK4* [34,37].

Loss of 9p-material was detected (by CGH) in the recurrent tumor and a part of the metastasis in the left lung (2a) but not in any of the other metastatic samples or the xenografts. This aberration, frequently affecting the putative tumor suppressor proteins *p16/p19* and/or *p15* is common in several cancers, including sarcomas, but seems to be uncommon in liposarcomas [35]. In this series of tumors, Southern analyses showed that the *CDKN2B* gene was affected in the sample with deletion of the whole arm (2a) and in the xenograft 2bx, but not in the recurrent tumors with deletions of a smaller part of 9p, nor in any other part of the metastatic tumors. The variable deletion pattern of 9p that was observed here probably reflect changes in the cellular composition of these tumors during the process. It also seems likely that other genes than *CDKN2B* may be the selective target for these deletions.

Notably, gain of chromosome 1 material was not detectable by CGH in the primary and recurrent samples, and only detected in few cells (<10%) by FISH. The metastatic samples and the xenografts from those had high-level amplifications of 1q22–q24, and high copy numbers of the marker 789f2. Previously, high-level amplifications of 1q21–q22

have been reported as a particularly frequent aberration in several different kinds of sarcomas [15,38–41] and have been detected both in primary and metastatic tumors. In the case presented here, amplification within 1q21–q31 is a late event, and it seems to be associated with metastasis and transformation to a highly malignant tumor type. Similar observations have been made by others: Tarkkanen et al. [42] reported that osteosarcoma patients with tumors that had gains of 1q21 showed a tendency toward short overall survival. In addition, in other tumor types, i.e., renal clear-cell carcinomas, gain of 1q21–q22 is particularly frequent in metastases [43].

It is also interesting that loss of the distal 2q was observed only in the osteogenic lung metastases. This aberration is not among the most commonly reported, and although observed in some cases, has not previously been associated specifically with osteosarcomas or metastatic disease [42,44].

The heterogeneity within each of the tumors, as shown by variable gains and amplification patterns of cen 1 and the 1q21 marker, is conserved in the xenografts. This could indicate that the various subpopulations of tumor cells will grow at similar rates during and after the establishment of the xenograft, and that there is no selection for further amplification of 1q material during this process. Alternatively, tumor cells with different kinds of aberrations may be selected for. A third, and perhaps more likely explanation, is that there is some dynamic equilibrium, resulting in uneven distribution of amplified segments during cell division. There is little heterogeneity in the 12q amplifications compared to the gain or amplifications of 1q21. The presence of 12q amplicons on stable extra-chromosomal segments, such as giant rods or marker chromosomes, that would be expected to be more strongly selected [45], and 1 q amplicons, e.g., on double minutes, could explain these differences.

Already in the primary tumor a percentage of the cells had amplification of *CDK4*. Thus, the amplification of 12q13–q14, also including *HMGIC* and *MDM2* at least in the recurrent and metastatic samples, was not induced by the post-operative radiation or chemotherapy given after excision of that sample. Nevertheless, we cannot rule out that other aberrations have been induced by therapy.

There is still no good explanation as to why well-differentiated tumors of low malignancy may change into aggressive, poorly differentiated tumors. Random oncogenic activation may abrogate the almost complete differentiation of a WDLPS, and give rise to a dedifferentiated tumor. In this case, dedifferentiation have produced osteogenic cells that have completely taken over during metastasis, as confirmed by the presence of osteoid cells and expression of alkaline phosphatase (*ALP*), and the absence of adipocytic cells expressing lipoprotein lipase (*LPL*). How this process is regulated is unknown, but one may speculate that the amplification of 1q21/q22–q24 may be involved, and possibly also loss of 2q material, as it correlates with malignant osteogenic differentiation as well as metastasis.

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