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MDM2 and CDK4 immunohistochemistry is a valuable tool in the differential diagnosis of low-grade osteosarcomas and other primary fibro-osseous lesions of the bone

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Low-grade osteosarcoma is a rare malignancy that may be subdivided into two main subgroups on the basis of location in relation to the bone cortex, that is, parosteal osteosarcoma and low-grade central osteosarcoma. Their histological appearance is quite similar and characterized by spindle cell stroma with low-to-moderate cellularity and well-differentiated anastomosing bone trabeculae. Low-grade osteosarcomas have a simple genetic profile with supernumerary ring chromosomes comprising amplification of chromosome 12q13–15, including the *cyclin-dependent kinase 4* (*CDK4*) and *murine double-minute type 2* (*MDM2*) gene region. Low-grade osteosarcoma can be confused with fibrous and fibro-osseous lesions such as fibromatosis and fibrous dysplasia on radiological and histological findings. We investigated MDM2-CDK4 immunohistochemical expression in a series of 72 low-grade osteosarcomas and 107 fibrous or fibro-osseous lesions of the bone or paraosseous soft tissue. The *MDM2-CDK4* amplification status of low-grade osteosarcoma was also evaluated by comparative genomic hybridization array in 18 cases, and the *MDM2* amplification status was evaluated by fluorescence *in situ* hybridization or quantitative real-time polymerase chain reaction in 31 cases of benign fibrous and fibro-osseous lesions. MDM2-CDK4 immunostaining and *MDM2* amplification by fluorescence *in situ* hybridization or quantitative real-time polymerase chain reaction were investigated in a control group of 23 cases of primary high-grade bone sarcoma, including 20 conventional high-grade osteosarcomas, two pleomorphic spindle cell sarcomas/malignant fibrous histiocytomas and one leiomyosarcoma. The results showed that MDM2 and/or CDK4 immunoreactivity was present in 89% of low-grade osteosarcoma specimens. All benign fibrous and fibro-osseous lesions and the tumors of the control group were negative for MDM2 and CDK4. These results were consistent with the *MDM2* and *CDK4* amplification results. In conclusion, immunohistochemical expression of MDM2 and CDK4 is specific and provides sensitive markers for the diagnosis of low-grade osteosarcomas, helping to differentiate them from benign fibrous and fibro-osseous lesions, particularly in cases with atypical radio-clinical presentation and/or limited biopsy samples.
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Low-grade osteosarcoma is a rare malignancy that represents about 5–7% of all osteosarcomas and is typically divided into two main subgroups on the basis of location in relation to the bone cortex, that is, parosteal osteosarcoma and low-grade central osteosarcoma. Parosteal osteosarcoma is the most frequent form of low-grade osteosarcoma and represents 4–5% of all osteosarcomas. It is the most common type of osteosarcoma of the bone surface. It occurs mostly in young adults during the third decade of life, with a slight female predominance.[1] Most cases (about 70%) develop on the lower posterior femoral shaft, although some occur on other long bones.[2] Parosteal osteosarcoma of the flat bones is exceptional. Parosteal osteosarcoma is a slow growing tumor revealed by the appearance of a palpable mass, pain or swelling. Radiographic studies show a heavily mineralized mass attached to the cortex with a broad base, and the tumor has a tendency to wrap around the involved bone.[1] Low-grade central osteosarcoma accounts for 1–2% of all osteosarcomas. Men and women are equally affected and the peak incidence occurs in the second and third decades of life. Approximately 80% of these tumors are located in the long bones, with a distinct predilection for the distal femur and proximal tibia.[1,3] The radiographic appearance of low-grade central osteosarcoma is highly variable at presentation, but the majority of these tumors will show some degree of cortical disruption, with or without extension to soft tissue.[1] Nevertheless, a significant number of these lesions may initially present a misleading radiographic appearance, suggestive of a benign lesion. Parosteal osteosarcoma and low-grade central osteosarcoma share the same histological appearance.[4] They are characterized by a spindle cell stroma with low-to-moderate cellularity and fairly mature bone trabeculae. The stromal cells resemble fibroblasts or myofibroblasts, are minimally cytologically atypical and have low mitotic activity. Areas composed of stroma can be prominent, with rare ossification clusters, and may mimic fibromatosis or a desmoplastic fibroma. More than 50% of parosteal osteosarcomas have a significant cartilaginous tumor component.[1] Eighteen percent of low-grade central osteosarcomas also have cartilage differentiation, seen as small scattered foci of atypical cartilage.[3] The clinical behavior of both parosteal osteosarcoma and low-grade central osteosarcoma is favorable and indistinguishable, with identical rates of local recurrence (7%) and 5-year survival above 80%.[2,5,6] The prognosis is mainly conditional on the risk of local recurrence after inadequate resection and dedifferentiation.[6–9] Unless the tumor is dedifferentiated, it does not metastasize.[2] About 15–29% of low-grade osteosarcomas will show dedifferentiation.[1,3,7,10] Dedifferentiated low-grade osteosarcomas are defined by the presence of areas of undifferentiated spindle cells and/or pleomorphic sarcoma (ie, fibrosarcoma or malignant fibrous histiocytoma) or high-grade osteosarcoma.[3,7,10,11] Wide surgical excision is recommended for primary lesions, and adjuvant chemotherapy is reserved for dedifferentiated lesions.[5]

Several studies have documented difficulties in the diagnosis of low-grade osteosarcoma.[2,3,8,9,12] Inability to diagnose the lesion correctly often leads to inadequate initial operative procedures. In two reviews of the literature, low-grade osteosarcomas were initially misdiagnosed as benign tumors in 32–60% of cases.[13,14] In the Mankin series, the rate of misdiagnosis reached 50% in specialist centers.[14] The differential diagnosis may include diverse entities such as fibrous dysplasia, non-ossifying fibroma, myositis ossificans, fracture callus, ossifying hematoma, osteochondroma, desmoplastic fibroma, osteoma and giant bone island.[2–4,6,12,15] Little information on low-grade osteosarcoma genetics is available in the literature. These tumors seem to share similarities in changes in cytogenetics and molecular genetics involving ring or giant marker chromosomes, which contain amplification of the 12q13–15 region, including *murine double-minute type 2* (*MDM2*) and *cyclin-dependent kinase 4* (*CDK4*).[16–21]

The purpose of this study was to determine the value of MDM2 and CDK4 immunostaining for the differential diagnosis of low-grade osteosarcomas and benign fibrous or fibro-osseous lesions. We then investigated the immunohistochemical status of 72 low-grade osteosarcomas and 107 fibrous or fibro-osseous lesions of the bone or paraosseous soft tissue. We also analyzed *MDM2* and *CDK4* amplification levels in 18 cases of low-grade osteosarcoma by comparative genomic hybridization array (aCGH) and *MDM2* amplification of 31 fibrous/fibro-osseous lesions by real-time quantitative polymerase chain reaction (Q-PCR) ($n=16$) or fluorescence *in situ* hybridization (FISH) ($n=15$), depending on the material available. A control group of 23 cases of primary high-grade bone sarcoma, including 20 conventional high-grade osteosarcomas, two pleomorphic spindle cell sarcomas/malignant fibrous histiocytomas and one leiomyosarcoma, were evaluated for both MDM2 and CDK4 by immunohistochemistry and for *MDM2* by molecular study (FISH or Q-PCR).

Materials and methods

Tumor Specimens

Institutional ethical guidelines were followed for this retrospective study. All cases (including low-grade osteosarcomas, fibrous and fibro-osseous bone lesions and the control group of high-grade sarcomas) came from the database of the French Bone Pathology Group (Cochin Hospital, Paris; Trousseau Hospital, Tours; Timone Hospital, Marseille; Rangueil Hospital, Toulouse; Bergonié Institute, Bordeaux; Pontchaillou Hospital, Rennes) and from the University Institute of Pathology, Lausanne,

Switzerland. They were reviewed by three pathologists (MBNB, FD and GdP). Formalin-fixed and paraffin-embedded tissues were available from all tumors, and snap-frozen material (stored at −80°C) was available in 32 cases (18 low-grade osteosarcomas, 11 fibro-osseous lesions and three high-grade sarcomas). Bony specimens were decalcified according to a standardized protocol with 6.5% nitric acid or rapid bone decalcifier (hydrochloric acid-based product) rotating with a longer period of fixation in buffered formalin. We finally collected 72 cases (31 male, 41 female) of low-grade osteosarcoma/dedifferentiated low-grade osteosarcoma divided into 54 primary low-grade osteosarcomas and 18 dedifferentiated low-grade osteosarcomas, including 64 parosteal osteosarcomas and eight low-grade central osteosarcomas. All samples originated from biopsy, except for six samples of low-grade osteosarcoma/dedifferentiated low-grade osteosarcoma, which originated from surgical resection specimens. Mean age at presentation was 33.1 years (range 10–78 years). Long bones were the site of involvement for 64 patients, and the knee area (distal femur, 45 cases and proximal tibia, four cases) was the main site. The clinical symptoms were nonspecific and the diagnosis was confirmed by histological analysis correlated with radiographic features in most cases. The initial histological diagnosis in two cases with atypical radio-clinical presentation included desmoplastic fibroma and osteofibrous dysplasia. One case was initially misdiagnosed as myositis ossificans. The histological features of low-grade osteosarcoma included well-delineated long bone trabeculae separated by a spindled fibrous stroma (Figure 1). The stroma had to be devoid of cytological abnormalities to be considered low grade. Ten low-grade osteosarcomas had a particular growth pattern, seven lesions simulating the appearance of fibrous dysplasia (irregular bone trabeculae resembling 'Chinese characters' in a spindle cell stroma) (Figure 2), two presenting as desmoid-type fibromatosis (prominent spindle cell proliferation with heavy collagenization and only scattered bone trabeculae) (Figure 3) and one as osteofibrous dysplasia. In 18 patients, there were areas of dedifferentiation at presentation (synchronous, $n=17$) (Figure 4a) or at the time of recurrence (metachronous, $n=1$). These dedifferentiated low-grade osteosarcomas included 12 high-grade osteosarcomas and four malignant fibrous histiocytomas (Figure 4b), and two were unspecified. The majority of dedifferentiated low-grade osteosarcomas dedifferentiated into high-grade osteosarcomas that were osteoblastic subtype, but two cases were classified as chondroblastic subtype.

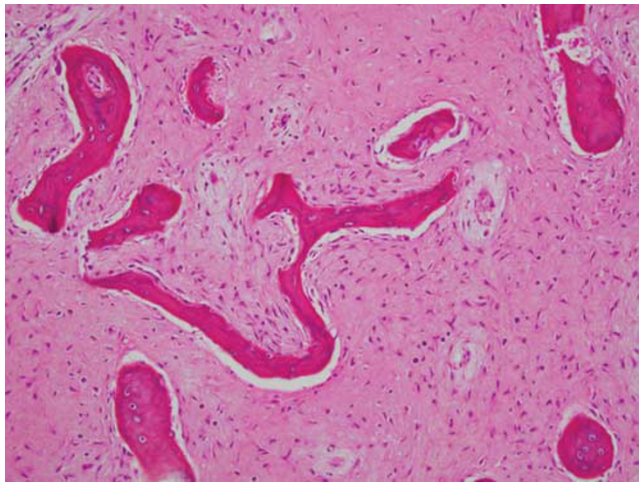

Figure 2 Fibrous dysplasia-like area in a low-grade osteosarcoma with characteristic irregularly-shaped bony trabeculae (hematoxylin–eosin–safran (HES) × 200).

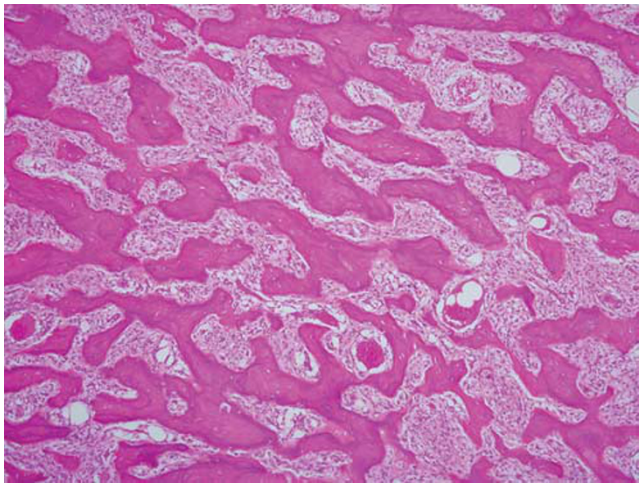

Figure 1 Low power magnification showing the typical, well-differentiated osseous trabeculae and spindle cell stroma seen in low-grade osteosarcoma (hematoxylin and eosin staining, original magnification, hematoxylin–eosin–safran (HES) × 40).

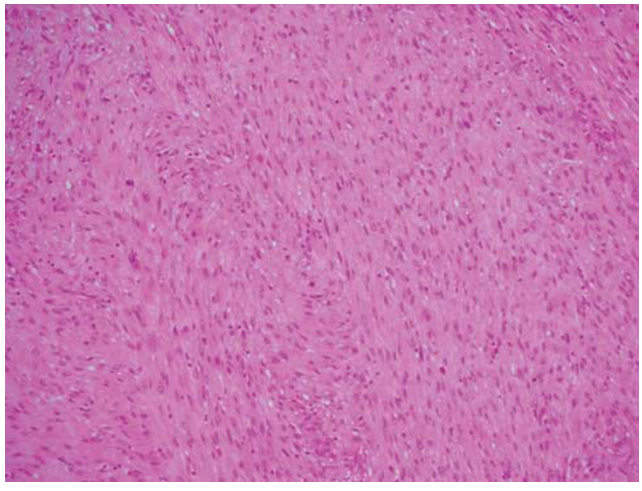

Figure 3 Desmoid-like area in a low-grade central osteosarcoma: microscopically, it was a neoplasm composed of a fibrous stroma containing fibroblast-like cells showing no pleomorphism (hematoxylin–eosin–safran (HES) × 100).

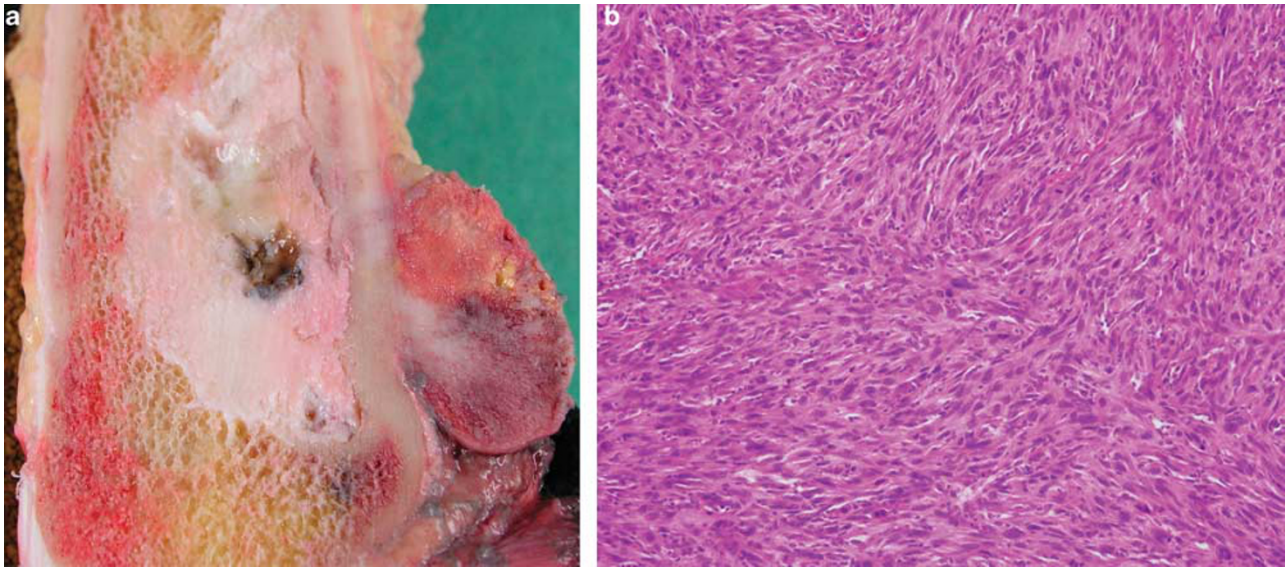

Figure 4 Dedifferentiated parosteal osteosarcoma: a gross specimen viewed coronally (**a**) shows an ossified, hard bone tumor attached to the posterior cortex of the femur. Intramedullary invasion and dedifferentiation areas with a white fibrous tumor component can be seen. High-grade pleiomorphic spindle cell sarcomatous/malignant fibrous histiocytoma dedifferentiated areas were observed (**b**) (hematoxylin–eosin–safran (HES) × 200).

Table 1 *MDM2* and *CDK4* expression on immunohistochemistry in 107 benign fibrous and fibro-osseous bone lesions

Histological diagnosis	*Total number of tumors*	MDM2-*positive cases*	CDK4-*positive cases*
Fibrous dysplasia	44	0	0 1 non-interpretable
Liposclerosing myxofibrous tumor	4	0	0
Non-ossifying fibroma	10	0	0
Myositis ossificans and related lesions	7	0	0
Callus	3	0	0
Fibro-osseous reparative lesion of the rib	3	0	0
Desmoplastic fibroma	4	0	0
Aneurysmal bone cyst (solid areas)	3	0	0
Fibrous and fibro-osseous lesions of jaws	10	0	0
Nora's lesion	6	0	0
Osteofibrous dysplasia	4	0	0
Chronic osteitis	2	0	0
Ossifications (metaplastic or reactive)	2	0	0
Adamantinoma osteofibrous dysplasia-like	1	0	0
Paget's disease	3	0	0
Desmoid tumor	1	0	0

Abbreviations: CDK4, cyclin-dependent kinase 4; MDM2, murine double-minute type 2.

In addition, we included 107 benign fibrous or fibro-osseous lesions of the bone or paraosseous soft tissue (Table 1): 44 cases of fibrous dysplasia, 11 with secondary aneurysmal bone cysts, two with cartilaginous differentiation and two with psammomatoid variants, four cases of liposclerosing myxofibrous tumor, one osteofibrous dysplasia-like adamantinoma, four desmoplastic fibromas, 10 non-ossifying fibromas, three solid variants of aneurysmal bone cysts, six Nora's lesions, four osteofibrous dysplasia, three fracture callus, two chronic osteitis, three fibro-osseous reparative lesions of the rib, three Paget's disease, one ectopic metaplastic bony tissue, one reactive periosteal osteogenesis, one desmoid-type fibromatosis of soft tissue with bone destruction, 10 maxillo-facial fibro-osseous lesions (six ossifying fibroma, two fibrous dysplasia, one osseous dysplasia and one solitary myofibroma), six myositis ossificans and one florid reactive periostitis. Furthermore, 20 conventional primary high-grade osteosarcomas, two pleomorphic spindle cell sarcomas/malignant fibrous histiocytomas of the bone and one intra-osseous leiomyosarcoma were investigated as a control group.

Immunohistochemistry

For MDM2 and CDK4 immunostaining, 4-μm-thick paraffin sections were cut and mounted on glass slides (Superfrost + ®, Menzel Glazer). Preparations were dried for 1 h at 58°C, then overnight at 37°C, and sections were then deparaffinized with toluene and rehydrated with ethanol. Preparations were pretreated with EDTA (MS-Unmasker-Diapath, Microstain division, Martinengo, Italy), and a heat-based antigen retrieval method was used before incubation. Endogenous peroxidase was blocked using 3% H_2O_2 solution for 5 min. The primary antibodies used were: MDM2 (Zymed Laboratories, South San Francisco, CA, USA; clone IF2) dilution

1:25, and CDK4 (Biosource International, Camarillo, CA, USA; clone DCS-31) dilution 1:50. Sections were incubated with the primary antibodies for 1 h at 22°C, followed by staining with the Envision kit (Dako, Carpinteria, CA, USA). Sections were then revealed in a diaminobenzidine solution for 7 min and stained with hematoxylin for 16 s. This analysis was used for the three subgroups (low-grade osteosarcomas, fibrous and fibro-osseous lesions and control group of high-grade bone sarcomas). For dedifferentiated low-grade osteosarcomas, the samples were obtained from well-differentiated and dedifferentiated components. Immunostaining was performed on whole-tissue sections and slides were evaluated by three independent pathologists (MBNB, FD and GdP). Discordant cases were re-evaluated collegially. The stained MDM2-CDK4 slides were viewed at low magnification to identify areas potentially containing the greatest number of positive cells. Ten high power fields ($\times 400$ magnification) were then counted in these areas. A tumor was considered as MDM2 or CDK4 positive when at least one cell nucleus was stained per high power field. The percentage of positive tumor cell nuclei was evaluated approximately by visual scanning of the slides at medium power. Four classes of immunostaining positivity were defined: ≤ 10, 11–25, 26–50 and >50%. The negative control was provided by the molecular results (CGH, Q-PCR and FISH analysis) when material was available and the technology feasible.

Molecular Analysis

CGH analysis

Eighteen low-grade osteosarcomas for which frozen tissue from the tumor was available were studied using aCGH developed at INSERM U830, Institut Curie, Paris, France. DNA was extracted from frozen samples using a standard phenol–chloroform procedure. After digestion with *Dpn*II (Ozyme, Saint Quentin en Yvelines, France) and column purification (Qiaquick PCR purification kit; Qiagen, Courtaboeuf, France), tumor DNA for each sample was labeled by the random priming method (Bioprime DNA labeling system; Invitrogen, Cergy-Pontoise, France) incorporating dCTP-cyanine-5 (Perkin-Elmer, Wellesley, MA, USA). Using the same procedure, we labeled control DNA incorporating dCTP-cyanine-3 (Perkin-Elmer, Wellesley, MA, USA). After ethanol co-precipitation with 210 μg of Human Cot-1 DNA (Invitrogen, Cergy-Pontoise, France), resuspension in hybridization buffer (50% formamide), denaturation at 95°C for 10 min and prehybridization at 37°C for 90 min, probes were cohybridized on aCGH. The aCGH slide was previously preblocked with a buffer containing 2.6 mg succinic anhydride/118 ml *N*-methyl-2-pyrrolidinone/32 ml sodium tetraborate decahydrate, pH 8.0 (Sigma-Aldrich, Lyon, France). The aCGH developed in the laboratory contains 3342 sequence-validated BACs. After washing, arrays were scanned using a Genepix 4000B scanner (Axon, Union City, CA, USA). Image analysis was performed with the Genepix 5.1 software (Axon) and ratios of Cy5/Cy3 signals were determined after normalization with the MANOR algorithm (Neuvial2006 BMC bioinformatics).

FISH analysis

FISH was performed on 15 benign fibro-osseous lesions (11 cases of fibrous dysplasia, one desmoplastic fibroma, two osteofibrous dysplasia and one florid reactive periostitis) and 20 cases of conventional high-grade osteosarcoma. Five-μm-thick sections of the formalin-fixed, paraffin-embedded decalcified tissues on silanized slides were deparaffinized for 3×10 min in xylene, washed in 100% ethanol, air-dried, incubated in $2 \times$ SSC (sodium saline citrate) at 72°C for 40 min, incubated in a proteinase K solution (500 μg/ml in $2 \times$ SSC; Roche, Meylan, France) at 45°C for 5–80 min, washed in $2 \times$ SSC for 2×3 min at room temperature and stored in 70% ethanol at 4°C. For each slide, 200 ng of the biotin-labeled BAC clone RP11-775J10 containing the *MDM2* gene was hybridized according to standard procedures. A minimum of 100 nuclei were visualized per slide. The number of fluorescent signals was evaluated for each nucleus analyzed. If at least one or two bright fluorescent spots per nucleus could not be seen on at least 80% of cells, the result was considered to be uninterpretable (ie, failure of hybridization owing to inappropriate deproteinization conditions). Amplification was defined as more than five fluorescent signals per cell. For each series of experiments, a control slide from a case of well-differentiated liposarcoma known to be positive for *MDM2* amplification was also examined.

Q-PCR

Q-PCR was performed on 16 other benign fibro-osseous lesions (10 cases of fibrous dysplasia, one desmoid-type fibromatosis, one non-ossifying fibroma, one Nora's lesion, one fracture callus, one solitary myofibroma and one myositis ossificans) and three high-grade bone sarcomas (control group). The presence of a tumor on the frozen specimens used for Q-PCR was investigated by obtaining an HES-stained frozen section. DNA was prepared from frozen tissue samples using the QiAmp DNA mini Kit from Qiagen. Q-PCR was performed using a LightCycler 480 (Roche) to analyze the amplification status of *MDM2*. Genomic amplification was performed with a starting amount of DNA (50 ng) using the LightCycler 480 Sybr Green I Master kit (Roche). Primer sequences for albumin (used as reference gene) and *MDM2* were: MDM2 forward, 5′-CCGGAT GATCGCAGGTG-3′; MDM2 reverse, 5′-AAAAGCTG AGTCAACCTGCCC-3′; Albumin forward, 5′-TGAA ACATACGTTCCCAAAGAGTTT-3′; Albumin reverse,

5′-CTCTCCTTCTCAGAAAGTGTGCATAT-3′. PCR was carried out as follows: after an initial 5 min preincubation step at 95°C, 45 amplification cycles were run, each consisting of 10 s at 95°C, 10 s at 60°C and 10 s at 72°C. The relative amount of gene was compared with the reference gene (Albumin) and calculated with LightCycler Relative Quantification software (Roche).

Statistical Analysis

The specificity and sensitivity for MDM2 and CDK4 immunostaining were evaluated in low-grade osteosarcomas with or without the dedifferentiation subgroup and compared with that of the fibrous and fibro-osseous lesions subgroup. We hypothesized that low-grade osteosarcoma with or without dedifferentiation would show amplification or immunostaining different from that of other bone lesions studied. MDM2 and CDK4 sensitivity was calculated as the ratio of positively immunostained low-grade osteosarcomas to the total number of low-grade osteosarcomas examined. MDM2 and CDK4 specificity was calculated as the ratio of unstained non-low-grade osteosarcoma lesions to the total number of non-low-grade osteosarcoma lesions.

The Youden index is the sum of sensitivity and specificity −1.0. It expresses the overall power of the test, the best index being the closest to 1.0. We could not study the correlation between *MDM2* amplification detected by FISH, CGH or Q-PCR analysis and MDM2-CDK4 immunostaining because of the non-matching of the series.

Results

Immunohistochemistry Analysis

Histological slides from 72 patients with low-grade osteosarcoma/dedifferentiated low-grade osteosarcoma were reviewed. MDM2 and CDK4 staining is summarized in Table 2 according to histology. Only nuclear staining was considered to be a positive result. Immunohistochemistry analysis was interpretable in all low-grade osteosarcomas. MDM2 and/or CDK4 were expressed in 89% of low-grade osteosarcomas (64/72) (sensitivity). Each antibody stained 82% of cases (59/72) and 75% of low-grade osteosarcomas were stained with both antibodies. Nine of ten low-grade osteosarcomas with a particular growth pattern simulating benign fibrous or fibro-osseous lesions showed MDM2 and/or CDK4 immunostaining. The percentages of positive tumor cell nuclei, according to the four classes of immunostaining positivity (≤10, 11–25, 26–50, >50%), are set out separately for MDM2 and CDK4 and for low-grade osteosarcomas and dedifferentiated low-grade osteosarcomas in Tables 3 and 4, respectively. The percentage of tumor cell MDM2-stained nuclei did not exceed 10% in any

Table 3 Results of immunohistochemical positivity of *MDM2* and *CDK4* in low-grade osteosarcomas/dedifferentiated low-grade osteosarcomas ($n=72$)

Percentage of nuclear staining	MDM2	CDK4
≤10	59 (100%)	17 (29%)
11–25	0	42 (71%)
26–50	0	0
>50	0	0
Total (positive low-grade osteosarcomas)	59/72 (82%)	59/72 (82%)

Abbreviations: CDK4, cyclin-dependent kinase 4; MDM2, murine double-minute type 2.

Table 4 Results of immunohistochemical positivity of *MDM2* and *CDK4* in dedifferentiated areas of dedifferentiated low-grade osteosarcomas ($n=18$)

Percentage of nuclear staining	MDM2	CDK4
≤10	6 (46 %)	1 (6%)
11–25	7 (54 %)	3 (19 %)
26–50	0	8 (50 %)
>50	0	4 (25 %)
Total (positive dedifferentiated low-grade osteosarcomas)	13/18 (72%)	16/18 (88%)

Abbreviations: CDK4, cyclin-dependent kinase 4; MDM2, murine double-minute type 2.

Table 2 *MDM2* and *CDK4* expression on immunohistochemistry in parosteal and low-grade central osteosarcomas (low-grade osteosarcomas) and dedifferentiated low-grade osteosarcomas

Histological diagnosis	*Total number of tumors*	MDM2-*positive cases*	CDK4-*positive cases*	MDM2- *and* CDK4-*positive cases*	MDM2- *and/or* CDK4-*positive cases*
Low-grade parosteal osteosarcoma	48	40 (83%)	37 (77%)	35 (73%)	42 (87%)
Low-grade central osteosarcoma	6	6 (100%)	6 (100%)	6 (100%)	6 (100%)
Dedifferentiated parosteal and low-grade central osteosarcomas	18	13 (72%)	16 (88%)	13 (72%)	16 (88%)
Total					
Low-grade osteosarcoma/dedifferentiated low-grade osteosarcoma	72	59 (82%)	59 (82%)	54 (75%)	64 (89%)

Abbreviations: CDK4, cyclin-dependent kinase 4; MDM2, murine double-minute type 2.

well-differentiated area of low-grade osteosarcomas (Figure 5a) and they were relatively evenly distributed across any given lesion (Table 3), whereas the proportion of MDM2-stained nuclei was higher in dedifferentiated areas (between 11 and 25% for more than 50% of cases) (Table 4). There were more CDK4- than MDM2-stained nuclei in most cases (between 11 and 25% for 71% of low-grade osteosarcomas studied) (Figure 5b) (Table 3). CDK4 staining was also more diffuse in dedifferentiated low-grade osteosarcomas, with more than 25% of stained nuclei in 75% of cases (Table 4). Finally, the percentages of both MDM2- (Figure 6a) and CDK4- (Figure 6b) stained nuclei were higher in dedifferentiated areas.

Negative immunohistochemistry staining was observed in all cases (100%) in the subgroup of benign fibrous and fibro-osseous bone or soft-tissue lesions (specificity). Only one result was not interpretable for CDK4 (fibrous dysplasia). MDM2 and CDK4 were not expressed in the control group (primary high-grade osteosarcomas and high-grade bone sarcomas). MDM2 and CDK4 specificity was 100% in low-grade osteosarcomas. The Youden index (0.89 for MDM2 and/or CDK4 expression) confirmed the power of the test.

CGH Analysis

CGH analysis was successfully performed in 18 cases of low-grade osteosarcoma, for which frozen tissues were available. All cases showed an amplification of chromosome 12q13–15 as recurrent genomic imbalance. BACs containing *MDM2* and *CDK4* genes were amplified (Figure 7). All samples showed amplification of *MDM2* and *CDK4*, whereas 2/18 of these low-grade osteosarcomas

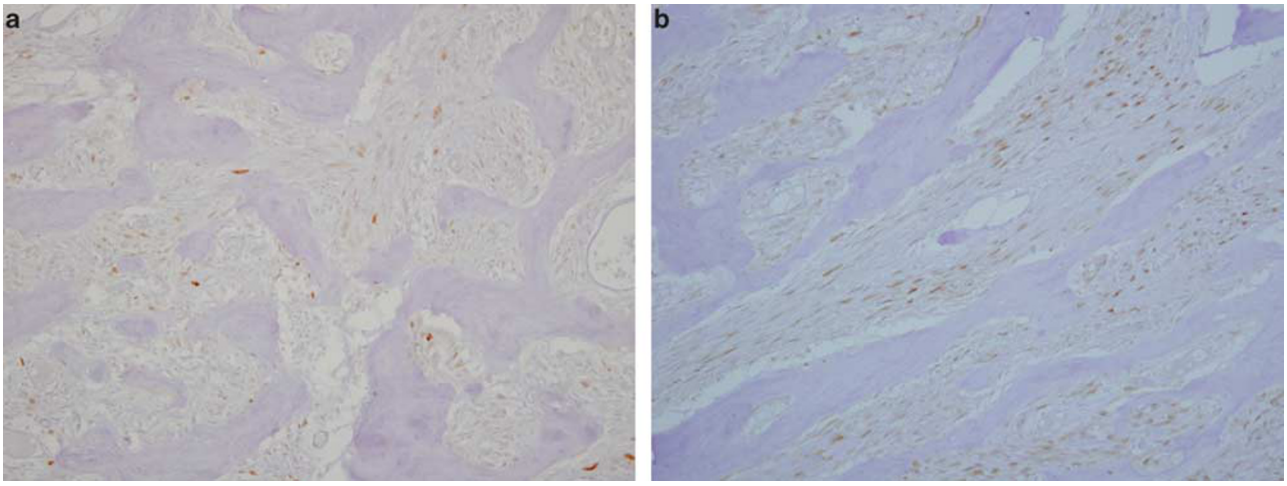

Figure 5 Murine double-minute type 2 (MDM2) and cyclin-dependent kinase 4 (CDK4) immunostaining in a case of low-grade osteosarcoma. The spindle cells are focally positive for MDM2 (**a**) and more diffusely stained with CDK4 (**b**) (× 200).

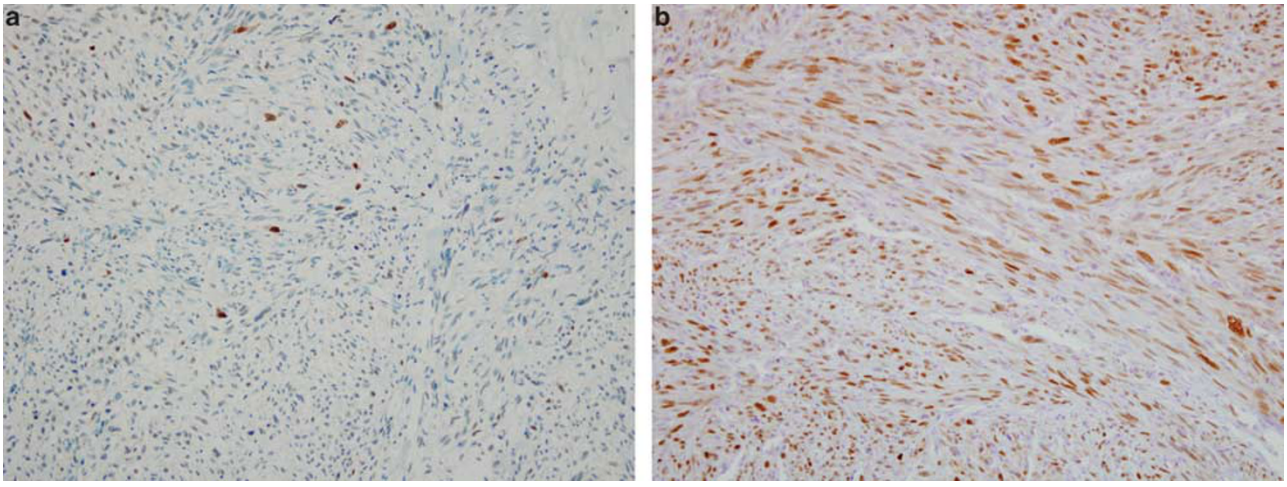

Figure 6 Murine double-minute type 2 (MDM2) and cyclin-dependent kinase 4 (CDK4) immunostaining in a case of dedifferentiated low-grade osteosarcoma (high-grade pleiomorphic spindle cell sarcomatous/malignant fibrous histiocytoma dedifferentiated areas). MDM2 staining (**a**) was more focal than CDK4 staining (**b**). The percentages of both MDM2- and CDK4-stained nuclei were higher than in well-differentiated areas (× 200).

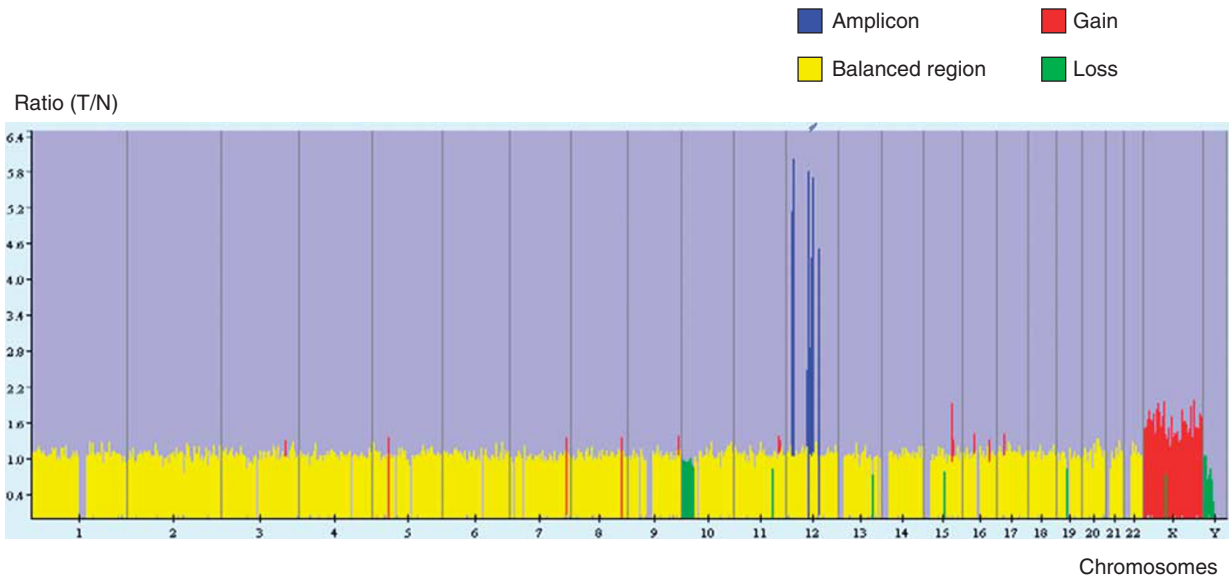

Figure 7 Comparative genomic hybridization array (aCGH) profile of case 42 showing chromosome 12q13–15 amplification containing *cyclin-dependent kinase 4* (*CDK4*) and *murine double-minute type 2* (*MDM2*) genes. Tumoral *versus* normal DNA ratio for each bacterial artificial chromosome (BAC) clone is plotted according to genome order (hg18 assembly). Balanced regions are represented in yellow, the regions gained are in red, the regions lost in green and amplicons in blue.

Table 5 CGH results of 18 low-grade osteosarcomas and comparison with *MDM2* and *CDK4* immunohistochemical status

Case no.	*Histological diagnosis*	*Dedifferentiation*	*CGH results—amplification 12q15*	*Immunohistochemistry*	
				MDM2 *expression*	CDK4 *expression*
28	Low-grade central osteosarcoma	–	+	+	+
29	Low-grade central osteosarcoma	–	+	+	+
30	Parosteal osteosarcoma	+ (osteosarcoma)	+	+	+
33	Parosteal osteosarcoma	–	+	+	+
37	Parosteal osteosarcoma	+ (osteosarcoma)	+	–	+
38	Parosteal osteosarcoma	–	+	–	+
42	Parosteal osteosarcoma	–	+	–	–
43	Parosteal osteosarcoma	–	+	–	–
44	Parosteal osteosarcoma	–	+	+	+
45	Parosteal osteosarcoma	–	+	+	–
49	Low-grade central osteosarcoma	–	+	+	+
52	Parosteal osteosarcoma	–	+	+	+
56	Parosteal osteosarcoma	+ (osteosarcoma)	+	+	+
57	Low-grade central osteosarcoma	–	+	+	+
62	Low-grade central osteosarcoma	–	+	+	+
66	Parosteal osteosarcoma	–	+	+	+
68	Parosteal osteosarcoma	+ (osteosarcoma)	+	+	+
69	Parosteal osteosarcoma	–	+	+	+

Abbreviations: CDK4, cyclin-dependent kinase 4; CGH, comparative genomic hybridization; MDM2, murine double-minute type 2.

were MDM2 and CDK4 immunohistochemistry staining negative (Table 5).

FISH Analysis

Of the benign fibro-osseous lesions analyzed ($n=15$), only two cases were interpretable, and they did not show amplification of *MDM2* consistent with the immunohistochemistry results. FISH results were interpretable in all cases of conventional high-grade osteosarcoma ($n=20$), none of them showed amplification of *MDM2*, and this was consistent with the immunohistochemistry results (Table 6).

Q-PCR Analysis

None of the fibrous or fibro-osseous lesions ($n=16$) or the tumors of the control group tested ($n=3$) showed amplification of *MDM2*, which was

Table 6 FISH results and comparison with MDM2 and CDK4 immunohistochemical status

Histological diagnosis	*Total number of tumors*	*FISH results*—MDM2 *amplification*			*Immunohistochemistry*	
		Amplification	*No amplification*	*Not interpretable*	MDM2 *expression*	CDK4 *expression*
Fibrous and fibro-osseous bone lesions	15	0	2	13	0	0
Conventional osteosarcomas	20	0	0	0	0	0

Abbreviations: CDK4, cyclin-dependent kinase 4; FISH, fluorescence *in situ* hybridization; MDM2, murine double-minute type 2.

Table 7 PCR results and comparison with MDM2 and CDK4 immunohistochemical status

Histological diagnosis	*Total number of tumors*	*PCR results*—MDM2 *amplification*	*Immunohistochemistry*	
			MDM2 *expression*	CDK4 *expression*
Fibrous and fibro-osseous bone lesions	16	0	0	0
High-grade primary bone sarcomas (two pleomorphic spindle cell sarcoma/malignant fibrous histiocytoma and one leiomyosarcoma)	3	0	0	0

Abbreviations: CDK4, cyclin-dependent kinase 4; MDM2, murine double-minute type 2; PCR, polymerase chain reaction.

also consistent with the immunohistochemistry results (Table 7).

Discussion

Low-grade osteosarcomas are well-differentiated tumors, which are locally aggressive, but with limited potential for distant spread. However, the prognosis may be worsened by the occurrence of dedifferentiation that signifies progression towards a higher-grade osteosarcoma or heterologous sarcoma with metastatic potential. This dedifferentiation may occur either at presentation (synchronous type) or at the time of recurrence (metachronous type) without any difference in outcome between the two groups.[7] In our study, 16/17 dedifferentiated low-grade osteosarcomas were synchronous tumors.

Low-grade central osteosarcoma and parosteal osteosarcoma share similar histological[12] and ultrastructural features.[22] They may arise simultaneously.[23] Low-grade osteosarcomas are rare tumors characterized clinically by slow growth. They appear histologically as quiescent tumors with low cellularity. Atypia and mitoses are rare or absent. Cases of low-grade osteosarcoma are therefore often misdiagnosed as benign lesions.[13,14] An experienced team can diagnose parosteal osteosarcoma on typical radiological features, that is, a heavily mineralized mass attached to the cortex of the distal posterior femur. In some cases, primary surgical resection without previous biopsy can then be proposed. Nevertheless, a significant number of parosteal osteosarcomas may develop at other locations and most low-grade central osteosarcomas have a misleading radiographic appearance at initial presentation. A low-grade osteosarcoma may both radiologically and histologically mimic a group of fibrous or fibro-osseous benign lesions involving the medullary canal or the bone surface. The main differential diagnoses for low-grade central osteosarcoma are, in practice, fibrous dysplasia[3,6,11,12,24] and desmoplastic fibroma.[3,12] The key factor differentiating low-grade central osteosarcoma from fibrous dysplasia is therefore the infiltrative growth pattern of the tumor, with permeation of the bone marrow and encasement of the pre-existing trabecular bone.[3,4] However, this feature is rarely observed on a biopsy specimen. Usually any bone formation rules out desmoplastic fibroma and supports the diagnosis of low-grade central osteosarcoma.[3] In everyday practice, differentiating both tumors can be difficult on a biopsy specimen: bone trabeculae can be sparse in some cases of low-grade osteosarcoma and reactive bone may sometimes be observed at the periphery of a desmoplastic fibroma.[12] The infiltrative tumoral pattern, cortical disruption and soft-tissue extension cannot totally provide a differential diagnosis, but are more suggestive of the low-grade malignancy of a low-grade central osteosarcoma.[12,25] The presence of a cloud-like tumor matrix pattern on radiological findings, reflecting the presence of intra-tumoral bone formation, is also a good argument for low-grade central osteosarcoma. Another pitfall when diagnosing low-grade central osteosarcoma is the liposclerosing myxofibrous tumor, a lesion possibly linked to fibrous dysplasia and characteristically

located in the proximal femur, at the base of the femoral neck.[26] It can also be (more rarely) confused with Paget's disease of the bone[15] or with a giant bone island. The latter lesion is exclusively made of cortical haversian bone without any cell proliferation within the haversian canals.[27]

Several jawbone lesions may present a fibro-osseous pattern with overlapping histological features, often making their diagnosis difficult. This group of lesions includes fibrous dysplasia such as those in the extra-gnathic skeleton and also more specific entities such as ossifying/cementifying fibroma and periapical cemental dysplasia. Low-grade osteosarcoma should be included in the differential diagnosis of these lesions. The osseous component of fibrous dysplasia of the jawbone may show a different pattern, with long and anastomosing bone trabeculae mimicking the tumor osteogenesis of low-grade osteosarcoma. Moreover, atypical microscopic features have been described in some cases of fibro-osseous lesions of the jawbone.[28]

Parosteal osteosarcoma should be differentiated from osteochondromas, benign paraosteal and periosteal bone lesions and periosteal processes, and from reactive soft-tissue processes adhering secondarily to the bone surface. These lesions include Nora's lesions, fracture callus, myositis ossificans, soft-tissue fibromatosis, reactive periostitis and ossifying fibromyxoid tumor.[9,29–31] The cartilaginous component of parosteal osteosarcoma may be significant and, in approximately 25% of cases, is located on the surface of the tumor as a cartilaginous cap mimicking osteochondroma.[2] Lin *et al*[31] reported six cases of osteochondroma-like parosteal osteosarcoma initially misdiagnosed as benign lesions on pathology evaluation. Parosteal osteosarcoma can be confused with Nora's lesions mainly in cases involving the long bones or in cases with unusual radiographic features.[29,32] In later phases of reactive soft-tissue and periosteal processes such as myositis ossificans or reactive periostitis, bone trabeculae appear more mature and these lesions may be confused with low-grade osteosarcoma on a biopsy sample from which the zonal architecture may be missing.[4] Rare cases of paraosteal osteomas involving long bones may also simulate parosteal osteosarcoma. However, they histologically consist of dense sclerotic lamellar bone with haversian systems, without any spindle cell proliferation.[33] Finally, protuberans fibrous dysplasia is a rare variant of fibrous dysplasia mimicking surface lesions of the bone. It is a benign fibro-osseous exophytic mass with heterogeneous sclerosis located eccentrically in the intra-medullary cavity of an adjacent bone.[34] Without detailed radiographic imaging and meticulous histological examination, this benign lesion is likely to be misdiagnosed, especially in parosteal osteosarcoma.

Significant sampling of low-grade osteosarcoma is very important because it may be difficult or impossible to distinguish it histologically from such benign bone lesions on limited samples from a core biopsy or even more so from needle aspiration.[12] An open surgical biopsy is often necessary to obtain a large tumor sample, but even this may be sometimes insufficient. Muramatsu *et al*[24] reported a case for which the initial microscopic diagnosis was fibrous dysplasia on open biopsy, and Bertoni *et al*[12] reported a case for which the original histological diagnosis was Paget's disease and/or fibrous dysplasia. The diagnosis of low-grade osteosarcoma was made a few years later in the recurrence in the bone and soft tissue and at the time of amputation with areas of high-grade osteosarcoma (dedifferentiation). Low-grade osteosarcoma characterized by a deceptively benign-looking histological appearance is an important diagnostic pitfall for pathologists that may often lead to underdiagnosis of malignancy. Finding new, easily performed diagnostic markers is thus a challenge for management of low-grade osteosarcoma.

Several markers (ie, actin, osteonectin, osteocalcin, c-fos and c-jun oncoproteins and ezrin) were shown to be of no value in distinguishing low-grade osteosarcoma from benign fibro-osseous lesions.[18,35–40] Okada *et al*[41] studied eight cases of low-grade central osteosarcomas retrospectively and showed that proliferative cell activity evaluated by AgNOR and MIB-1 immunohistochemical staining was significantly higher in cases of low-grade central osteosarcoma than in fibrous dysplasia and might be helpful in differentiating low-grade central osteosarcoma from fibrous dysplasia. Pollandt *et al*[42] showed that Gsalpha gene mutations were a constant finding in cases of monostotic fibrous dysplasia. Nevertheless, they also reported a Gsalpha gene mutation in one of five cases of fibrous dysplasia-like low-grade central osteosarcoma studied. Such mutations appear to be not totally specific of fibrous dysplasia.

In contrast to the complex karyotypes previously reported in conventional osteosarcoma,[43] low-grade osteosarcoma is characterized by a simple karyotype with supernumerary ring chromosomes[19,44–46] and over-representation of 12q sequences on CGH.[16,17,21] Ring chromosomes are composed of 12q13–15 clusters containing *MDM2* and *CDK4* and are due to telomeric deletions.[16,47] Similar cytogenetic abnormalities have previously been described in other low-grade malignancy mesenchymal lesions such as well-differentiated liposarcoma.[48] Overexpression and gene amplification of *MDM2* and *CDK4* located on chromosome 12q13–15 have been reported to occur in various human sarcomas as the pathway of tumorigenesis or tumor progression.[49,50] As a result, two major growth regulation pathways may be inhibited. *MDM2* may downregulate the p53-mediated growth control and *CDK4* may affect retinoblastoma tumor suppressor protein (pRb)-mediated events.[51] These genes have been reported to be related to amplification of the 12q13–15 region, but they are located in two discontinuous regions,

which are closely linked and frequently co-amplified.[48] Amplification of *CDK4* and *MDM2* can lead to deregulation of the cell cycle and may be an important step in tumor progression. Although some studies have reported 12q amplification in conventional high-grade osteosarcomas, with a frequency ranging from 0 to 27%,[19,50,52–59] the majority have indicated that amplification of *MDM2* and *CDK4* is a typical feature of parosteal osteosarcoma and low-grade central osteosarcoma.[16,17,19,21,47,52,60,61] One study showed a high level of *CDK4* and *MDM2* amplification in a series of nine osteosarcomas of the jaw, including five intermediate and high-grade osteosarcomas.[51] However, gnathic osteosarcomas have particular clinical and biological profiles that differ from conventional high-grade osteosarcomas of long bones and that require further specific investigation. In our series, we did not study low-grade osteosarcomas of the jaw. However, the negativity of CDK4 and MDM2 immunostaining of 10 fibrous and fibro-osseous maxillary lesions shows that MDM2 and CDK4 can help to differentiate these benign lesions from osteosarcomas. Low-grade osteosarcoma studies analyzing MDM2 expression by immunohistochemistry have reported a frequency range of 22–70 and 66–92% for CDK4.[20,21,59–61] This wide range can be explained by technical differences, such as formalin fixation, decalcification of tissues, antibody dilution and antigen retrieval method. The biomolecular study of *MDM2* amplification in low-grade osteosarcomas, including aCGH, FISH and PCR, has revealed a frequency range of 19–100 and 12–80% for *CDK4*.[19,47,52,59,60] The highest amplification frequencies were obtained with Q-PCR[52] on frozen samples and with FISH and Southern blotting[19,47] on unspecified material. DNA extraction from paraffin blocks can explain the disappointing results with Q-PCR in two studies.[59,60]

In our study, overexpression of MDM2 and/or CDK4 was observed by immunohistochemistry in 89% of cases of low-grade osteosarcoma (sensitivity) with a specificity of 100%, with or without dedifferentiation. All low-grade central osteosarcomas showed expression of MDM2 and/or CDK4. The results obtained from our eight patients are promising and will need further investigation. These results were consistent with *MDM2* amplification findings provided by aCGH-tested low-grade osteosarcomas ($n=18$). Neither the fibrous/fibro-osseous lesions tested by Q-PCR ($n=16$) or FISH ($n=15$) nor the tumors of the control group tested by FISH or Q-PCR showed *MDM2* amplification. Moreover, investigation of *MDM2* amplification by aCGH provided a correct diagnosis in two cases of low-grade osteosarcoma whose immunohistochemical study was negative (Table 3). Consequently, although molecular study often needs to be executed on frozen samples and is not easily available in a routine setting, this technique is required in certain cases to improve overall diagnostic sensitivity.

As in our study, a recent analysis tested MDM2 and CDK4 immunostaining in low-grade osteosarcomas (23 cases) and in benign fibro-osseous lesions (40 cases).[61] Their results confirmed the very good sensitivity of the test (100%) and its specificity (97.5%). However, the number of cases studied was lower and the *MDM2* and *CDK4* amplification status was not investigated. This would have been particularly interesting in the immunostaining-positive Nora's lesion in this study.

Our results confirm that 12q13–15 genes are involved in tumor progression in low-grade osteosarcoma. Concurrent amplification and overexpression of these genes might help to diagnose low-grade osteosarcomas, particularly when they mimic benign fibro-osseous lesions. In the 10 cases of low-grade osteosarcoma with a histologically unusual growth pattern simulating benign fibro-osseous lesions, nine were positive on MDM2 and/or CDK4 immunostaining. The case of MDM2-CDK4-negative immunostaining (fibrous dysplasia-like low-grade osteosarcoma) showed *MDM2* amplification on CGH analysis.

Dedifferentiated areas in dedifferentiated low-grade osteosarcomas and other primary high-grade bone sarcomas such as conventional high-grade osteosarcomas can share histological features even if they have a different oncogenesis. In our study, amplification/overexpression of *MDM2* and *CDK4* was clearly observed in differentiated and dedifferentiated low-grade osteosarcoma components, in contrast to the control group of high-grade conventional osteosarcomas ($n=20$) and other rare subtypes of intra-osseous primary sarcomas ($n=3$). Although Wold *et al*[10] showed that the prognosis for patients with dedifferentiated parosteal osteosarcoma appears to be similar to that of patients with high-grade centromedullary osteosarcoma, some recent studies have suggested that the prognosis of dedifferentiated low-grade osteosarcomas may be better than that of primary conventional centromedullary or surface high-grade osteosarcomas.[5,7] This potential difference in malignant behavior may be explained by the different changes in oncogenesis in these two kinds of tumor. In summary, proving overexpression of MDM2 and CDK4 in dedifferentiated low-grade osteosarcomas may allow pathologists to distinguish dedifferentiated low-grade osteosarcomas from conventional high-grade osteosarcomas, although the clinical outcome remains unclear.[7] However, the difference would be important with the discovery of targeted molecular therapy.[62]

Despite standardized protocols, the formalin fixation of tissues and decalcification by acid-based products may result in the failure of FISH by hydrolysis of DNA and in the loss of sensitivity of immunohistochemical analysis. For example, of six cases of low-grade osteosarcoma with both biopsy and resection material available (data not shown), the IHC results were similar for both specimens in

our series, except for one case. Positive tumor cell immunostaining was observed for both MDM2 and CDK4 on biopsy sample, thus confirming the diagnosis. However, this immunohistochemical positivity was not found in the surgical resection specimen, whereas there was *MDM2* amplification according to molecular studies on both specimens. This can be explained by the longer decalcification period required for the surgical specimen. Moreover, the failure of FISH for 13/15 fibro-osseous lesions analyzed can also be explained by the acid decalcification. Using chelating agents such as EDTA for decalcification might circumvent this problem. Decalcification in EDTA has little or no effect on tissues other than on the bone mineral matrix itself. Nevertheless, the application of EDTA as a decalcifying agent in a routine setting is hampered by the long period required for incubation.[63] New methods such as decalcification in EDTA using a microwave oven and ultrasonic decalcification[64] are reported to considerably reduce the time of decalcification and preserve antigenic sites, DNA and mRNA.[63]

In conclusion, overexpression and amplification of MDM2 and CDK4 are confirmed in low-grade osteosarcomas (parosteal osteosarcomas, low-grade central osteosarcomas and dedifferentiated low-grade osteosarcomas). The evaluation of MDM2 and CDK4 overexpression by immunohistochemistry appears to be a valuable diagnostic tool to distinguish low-grade osteosarcomas from look-alike benign fibrous and fibro-osseous lesions with quite good sensitivity. These markers are particularly interesting when there are limited samples from a core biopsy or when the initial presentation is unrecognized. MDM2 and CDK4 are also markers distinguishing between dedifferentiated low-grade osteosarcomas and conventional high-grade osteosarcomas. Improving the sensitivity of these markers by the use of chelating agents such as EDTA should be tested in further studies. In some cases, the use of molecular analysis such as CGH or Q-PCR on frozen samples is necessary to detect *MDM2* and/or *CDK4* gene amplification.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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