ORIGINAL ARTICLE

Frequent expression of smooth muscle markers in malignant fibrous histiocytoma of bone

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Background/Aims: Malignant fibrous histiocytoma (MFH) of bone, a relatively rare primary malignant bone tumour, is a distinct clinicopathological entity as opposed to MFH derived from soft tissue. Although the true histogenesis of this condition is still controversial, a considerable number of cases of MFH in soft tissue show positive immunohistochemical reactivity for muscle markers such as desmin, common muscle actin (HHF35), and α smooth muscle actin (SMA), suggesting that MFH cells are myofibroblastic in nature.

Methods: This study investigated immunoreactivity for several different muscle markers in 19 cases of MFH of bone together with reverse transcription polymerase chain reaction (RT-PCR) analysis on frozen tissue samples that were available in four cases, and compared the data with those found in 11 cases of osteosarcoma and 11 cases of soft tissue MFH treated over the same period.

Results: Immunohistochemistry revealed that MFH of bone showed relatively frequent expression of smooth muscle markers, including calponin (nine cases), α-SMA (nine cases), and SM22α (18 cases), and this was confirmed by RT-PCR analysis. However, only one, two, and three cases of MFH of bone showed positive staining for desmin, MyoD1, and HHF35, respectively. Similarly, 11 osteosarcoma cases were relatively frequently positive for α-SMA (five cases), calponin (four cases), and SM22α (seven cases), and less frequently positive for desmin (one case), MyoD1 (none), and HHF35 (none). In contrast, very few MFH of soft tissue cases (n = 11) showed positive reactivity for all of these muscle markers. It has recently been reported that human bone marrow stromal cells also express various kinds of smooth muscle markers including α-SMA and calponin.

Conclusions: These results suggested that MFH of bone may derive from mesenchymal stromal cells in bone marrow and has a more myofibroblastic differentiation than soft tissue MFH.

Malignant fibrous histiocytoma (MFH) of bone is a relatively rare, malignant bone tumour that comprises less than 2% of all primary malignant bone tumours.[1] This distinct entity was first described by Feldman and Norman[2] in 1972, and by Mirra and colleagues[3] in 1974 as a counterpart of MFH derived from soft tissue. Most cases of MFH of bone were previously classified as pleomorphic or poorly differentiated fibrosarcomas, pleomorphic reticulum cell sarcomas, spindle cell and giant cell sarcomas, or osteolytic/fibroblastic osteosarcomas.[4 5] Although the true histogenesis of this disease is still controversial, MFH of bone is now a widely accepted clinicopathological entity.[1 5–11] It is microscopically characterised by an admixture of fibroblastic or myoblastic spindle cells with a storiform or cartwheel pattern and histiocyte-like pleomorphic cells accompanied by a varying number of giant cells and inflammatory cell infiltrations. It can also include varying amounts of stromal collagen and myxoid matrix. Because the histological appearance of MFH of bone can vary greatly, it is often difficult to differentiate it from other malignant bone tumours, such as osteosarcoma, fibrosarcoma, leiomyosarcoma, dedifferentiated chondrosarcoma, and even malignant lymphoma.

> "Although the true histogenesis of this disease is still controversial, malignant fibrous histiocytoma of bone is now a widely accepted clinicopathological entity"

No specific immunohistochemical markers have been recognised for MFH of soft tissue, and a considerable number of cases of MFH show positive immunoreactivity for muscle markers such as desmin, common muscle actin (HHF35), and α smooth muscle actin (α-SMA),[12] suggesting a "myoid" or myofibroblastic differentiation of MFH cells. However, the immunohistochemical study of MFH of bone is very limited because of its relative rarity compared with MFH of soft tissue.[8 13 14] In our present study, we have looked at immunoreactivity for various muscle markers and undertaken reverse transcription polymerase chain reaction (RT-PCR) analysis in 19 cases of MFH of bone. We further discuss the importance of their expression in MFH of bone.

MATERIALS AND METHODS

Patients

Between 1979 and 1997, 29 patients with MFH of bone were treated at Osaka University Medical Hospital, Osaka Medical Center for Cancer and Cardiovascular Diseases, and Osaka National Hospital. Of these 29 patients, we selected the 19 consecutive patients whose paraffin wax embedded, non-calcified biopsy and/or surgically resected tumour specimens were available for immunohistochemical staining. There were 12 male and seven female patients, with ages ranging from 15 to 77 years (mean, 39.9). The locations of the primary tumours were: 11 cases in the femur, two cases in the ilium, two cases in the fibula, and one case each in the scapula, humerus, tibia, and lumbar spine. All cases were histologically reviewed and confirmed as MFH of bone by two of the authors (TU and

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Table 1 Antibodies used in our immunohistochemical study

Antibody/antigen pretreatment	Clone	Dilution	Source	Mirowave
Calponin	hCP	1/4000	Sigma, St Louis, Missouri, USA	+
α-SMA	1A4	1/500	Sigma	−
Desmin	D33	1/200	Dako, Carpinteria, California, USA	+
CMA	HHF35	1/50	Dako	−
MyoD1	5.8A	1/200	Dako	+
SM22α	Polyclonal (rabbit)	1/500	H Yamamura et al[16]	+

CMA, common muscle actin; SMA, smooth muscle actin.

MM). Fourteen cases were classified as predominantly fibroblastic/fibrohistiocytic (storiform-pleomorphic) type, four as predominantly histiocytic or xanthomatous type, and one as giant cell type. Two cases categorised as the first two types each showed pronounced inflammatory cell infiltrations. No myxoid variants were seen in the present series. All 19 cases were histologically high grade. We also examined 11 cases of conventional osteosarcoma and 11 cases of MFH of soft tissue treated over the same period, as control groups. The location of the primary tumours in osteosarcoma cases included femur (seven cases; distal, six; proximal, one), proximal tibia (two cases), humerus (one case), and ilium (one case). All cases were high grade and the histological subtype was osteoblastic in nine cases, chondroblastic in one case, and telangiectatic in one case. With regard to the cases of soft tissue MFH, in two cases each the primary location was the thigh, leg, shoulder, and buttock, and in one case each it was the back, groin, and retroperitoneum. There were five cases of grade 2 and six of grade 3 and the histological subtype was storiform-pleomorphic variant in eight cases and myxoid variant in three.

Immunohistochemistry

Routinely processed, formalin fixed, paraffin wax embedded tissue specimens were obtained from biopsies and/or surgical resections of the primary tumours in all 19 cases. Based on the examination of haematoxylin and eosin stained slides, one representative paraffin wax block sample from each case was chosen for immunohistochemical staining using the avidin–biotin complex (ABC) method. Table 1 lists the antibodies for muscle markers used in our study. Monoclonal antibodies against calponin (clone hCP) and α-SMA (clone 1A4) were purchased from Sigma (St Louis, Missouri, USA). The specificity of the clone hCP monoclonal antibody to the basic (h1) calponin isoform has been verified by immunoblot analysis with alkaline phosphatase staining (Bio-Rad, Hercules, California, USA), as described previously.[15] Monoclonal antibodies against desmin (clone D33), common muscle actin (clone HHF35), and MyoD1 (clone 5.8A) were purchased from Dako (Carpinteria, California, USA). A polyclonal antibody

specific for the SM22α protein was generated in rabbits, as described previously.[16] The 4 µm thick sections mounted on poly-L-lysine coated microslides were dewaxed in xylene, dehydrated through graded alcohol, and immersed in 70% methanol with H_2O_2 to inhibit any endogenous peroxidase activity. Then, if necessary, antigen retrieval was performed for several antibodies (those directed against calponin, desmin, MyoD1, SM22α) using a commercial 400 W microwave oven (Toshiba ERT 330; Tokyo, Japan) four times for five minutes each in a 10mM citrate buffer (pH 7.0).[17] The sections were reacted with the primary antibody overnight at 4°C. They were then incubated with biotinylated antimouse or antirabbit immunoglobulin for 30 minutes, and subsequently stained using the ABC method. Diaminobenzidine was used as a chromogen, and the slides were counterstained with haematoxylin. Appropriate positive and negative controls were included in each staining procedure.

Tumour cells were considered positive if definite cytoplasmic staining distant from the edge of the section or areas of necrosis was observed. The extent of immunostaining in each specimen was scored by two independent observers (TU and MM) according to the number of stained tumour cells, as follows: ++, > 50% positive; +, 10–50% positive; −, < 10% positive or no staining.

RT-PCR analysis

We also performed RT-PCR analysis for the expression of the basic (h1) and neutral (h2) calponin, α-SMA, and SM22α genes on frozen tumour samples that were available from four cases of MFH of bone. Tumour tissues were frozen immediately after surgical removal and stored at −80°C until extraction of RNA. Total RNA was extracted from the tumour tissues using the Isogen RNA extraction kit (Nippon Gene, Toyama, Japan). RT of 2 µg of total RNA was carried out using the reaction mixture "ready to go you prime first strand beads" (Pharmacia, Uppsala, Sweden) in the presence of 0.2 µg of random hexamer primer. After 60 minutes of incubation at 37°C, 0.5 µM of each of the forward and reverse primers, 200 µM of each dNTP mixture, and 2.5 U of Taq DNA polymerase (Pharmacia) were added to 8 µl of the first strand

Table 2 Immunoreactivity for muscle markers in MFH of bone, in comparison with those in osteosarcoma and MFH of soft tissue

	MFH of bone (n=19)			Osteosarcoma (n=11)			MFH of soft tissue (n=11)		
	++	+	−	++	+	−	++	+	−
α-SMA	2	7	10 (47%)	0	5	6 (45%)	2	1	8 (27%)
Calponin	1	8	10 (47%)	1	3	7 (36%)	0	1	10 (9%)
SM22α	7	11	1 (95%)	1	6	4 (64%)	1	1	9 (18%)
Desmin	0	1	18 (5%)	0	1	10 (9%)	0	2	9 (18%)
MyoD1	0	2	17 (11%)	0	0	11 (0%)	0	0	11 (0%)
HHF35	0	3	16 (16%)	0	0	11 (0%)	0	3	8 (27%)

++, >50% positive; +, 10–50% positive (including focal/scattered +); −, <10% positive or no staining. The figures in parenthesis are the percentages of positive cases.
HHF35, antibody to common muscle actin; MFH, malignant fibrous histiocytoma; SMA, smooth muscle actin
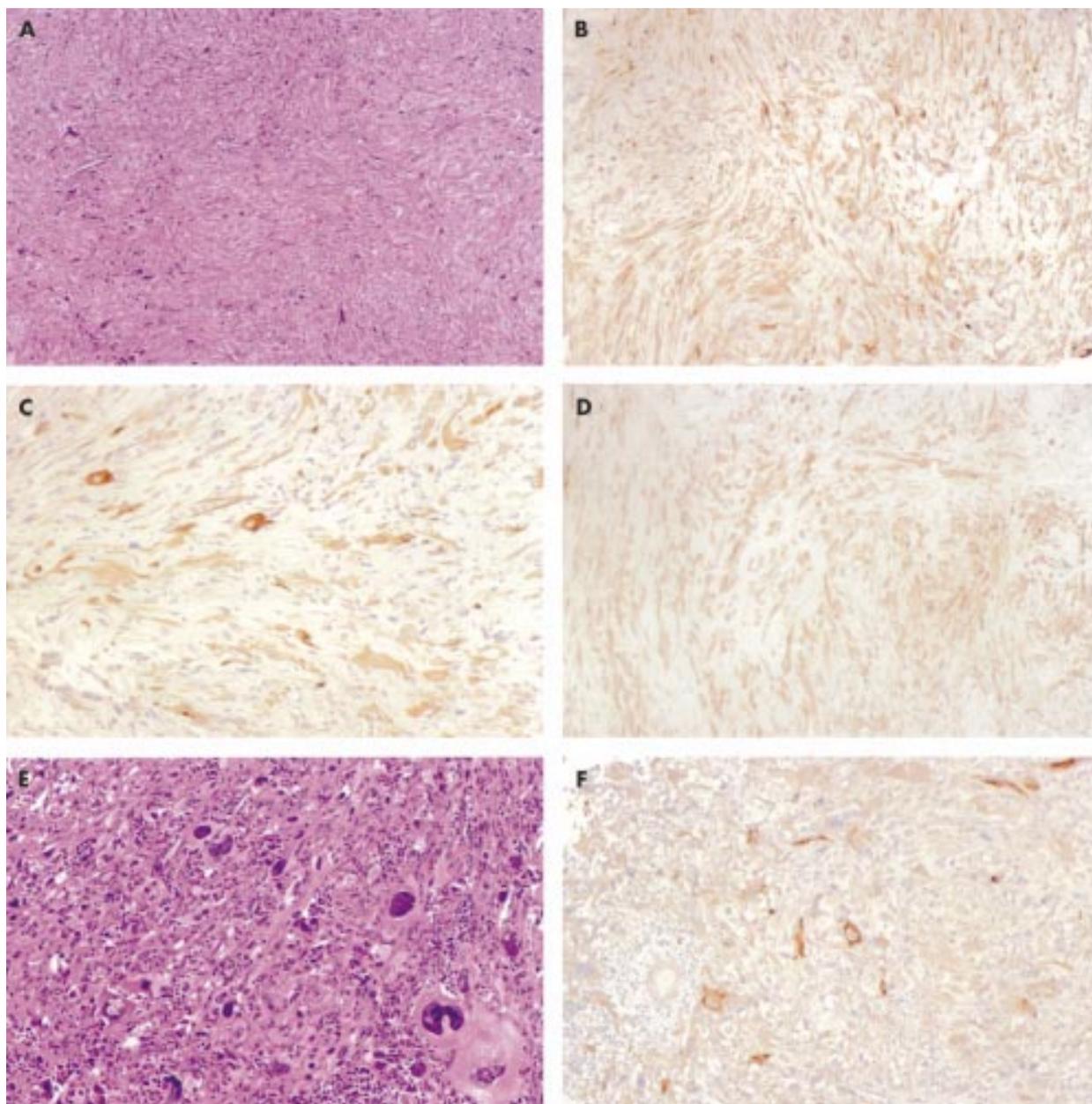
Figure 1 (A) Storiform-pleomorphic microscopic appearance in malignant fibrous histiocytoma (MFH) of bone arising in the right distal femur of a 55 year old man (haematoxylin and eosin stain). Diffuse immunohistochemical positive reactivity with (B) calponin, (C) α-SMA, and (D) SM22α in the same patient as (A). (E) Pleomorphic pattern with pronounced inflammatory cell infiltration in MFH of bone arising in the left distal femur of a 15 year old boy (haematoxylin and eosin stain). (F) Scattered tumour cells show positive cytoplasmic reactivity with calponin in the same patient as (E).

reaction mixture, and then the total volume was adjusted to 50 µl with water. The parameters used for the amplification were 30 cycles of denaturation (94°C, 40 seconds), annealing (60°C, 30 seconds), and polymerisation (72°C, 90 seconds). Sequences of the selected forward and reverse 5' to 3' primers used, and predicted products sizes were as follows: basic (h1) calponin, GAGTGTGCAGACGGAACTTCAGCC (forward), GTCTGTGCCCAACTTGGGGTC (reverse), 671 bp; neutral (h2) calponin, CTGCAGAGCGGGGTGGACATTGGC (forward), GCCGGCCTCCTCCTGGTAGTAAGG (reverse), 519 bp; α-SMA, CCAGCTATGTGAAGAAGAAGAGG (forward), GTGATCTCCT-TCTGCATTCGGT (reverse), 965 bp; SM22α, CGCGAAGT-GCAGTCCAAAATCG (forward), GGGCTGGTTCTTCTTCAAT-GGGG (reverse), 928 bp; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), CCCATCACCATCTTCCAGGA (forward), TTGTCATACCAGGAAATGAGC (reverse), 731 bp. The

correctness of these primers has been confirmed previously.[18] Linearity of the PCR products for calponins and SM22α was obtained between 25 and 35 cycles, and for α-SMA and GAPDH between 20 and 30 cycles. As a negative control, PCR reactions were conducted with each set of primers, but RNA was omitted in the RT reactions. After 1% agarose gel electrophoresis in the presence of 0.5 µg/ml of ethidium bromide, the PCR products were revealed by ultraviolet irradiation and the image captured and measured by Eagle Eye II still video system (Stratagene, La Jolla, California, USA). Variations in signal intensities between different agarose gels were corrected by using those of the molecular weight markers in each gel analysed. To assess the relative levels of expression, the signal intensity was subjectively graded by two independent observers (HY, KT) in a blind manner, from (−), (+), (++), to (+++), indicating negative expression and low, intermediate,
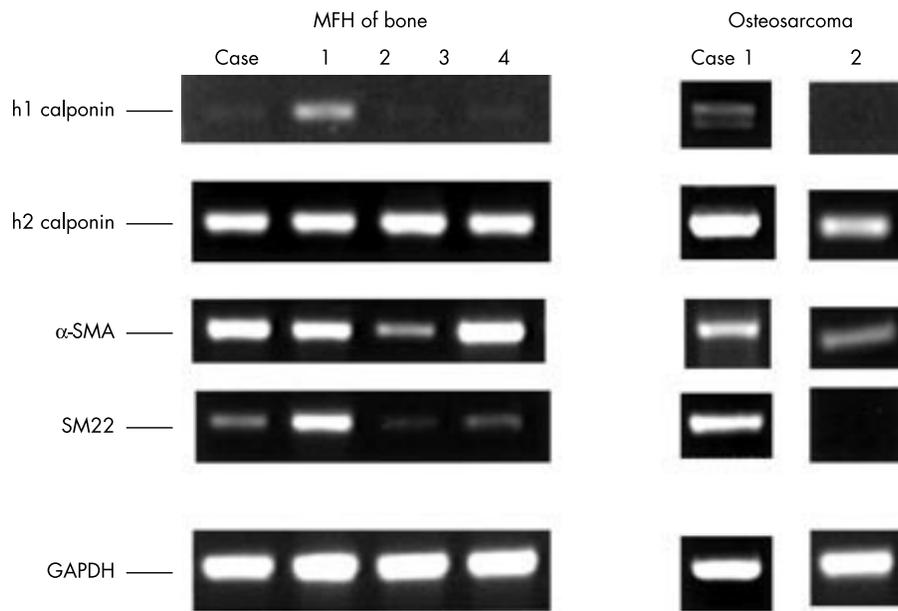
Figure 2 Reverse transcription polymerase chain reaction analysis for the expression of basic (h1) and neutral (h2) calponin isoforms, α smooth muscle actin (α-SMA), and SM22α genes in four cases of malignant fibrous histiocytoma (MFH) of bone and two representative cases of osteosarcoma. GAPDH; glyceraldehyde-3-phosphate dehydrogenase.

and high levels of expression, respectively. Negative results were confirmed at least twice.

RESULTS
Immunohistochemical analysis

Table 2 summarises the results of the immunohistochemical staining. Positive reactivity for α-SMA, calponin, and SM22α was found in nine, nine, and 18 of 19 cases of MFH of bone, respectively, although only one, two, and three cases of MFH of bone showed positive staining for desmin, MyoD1, and HHF35 (common muscle actin), respectively. Similarly, the 11 osteosarcoma cases were relatively frequently positive for α-SMA (five cases), calponin (four cases), and SM22α (seven cases), but showed less frequent positive staining for desmin (one case), MyoD1 (none), and HHF35 (none). In contrast, the 11 cases of MFH of soft tissue cases were rarely positive for all of these muscle markers. Figure 1 shows the histological findings of two representative cases of MFH of bone with their immunohistochemical staining for calponin, α-SMA, and SM22α. There was no correlation between the expression pattern of these muscle markers and the histological subtype in MFH of bone.

Expression of mRNA for muscle markers in MFH of bone

Figure 2 shows the results of the RT-PCR analysis for the expression of the basic (h1) and neutral (h2) calponin isoforms, α-SMA, and SM22α genes in four cases of MFH of bone and two representative cases of osteosarcoma. An mRNA transcript of the basic (h1) calponin gene was expressed in all of the four cases of MFH of bone at various levels, namely: (++) in one case and (+) in three cases. The smooth muscle differentiation specific genes, including α-SMA and SM22α, were also expressed in all these cases to various degrees. In contrast, a case of osteoblastic osteosarcoma (case 2 in fig 2) showed complete lack of expression of basic (h1) calponin and SM22α mRNA transcripts, with only an intermediate level of expression of α-SMA mRNA. In contrast, the mRNA transcript for the neutral (h2) calponin, which is known to be expressed in both smooth muscle and non-smooth muscle tissues,[15][19] was uniformly expressed in all tumour samples examined.

DISCUSSION

We demonstrated, using both immunohistochemistry and RT-PCR analysis, that MFH of bone shows relatively frequent expression of smooth muscle markers, including α-SMA, calponin, and SM22α. Of these markers, both calponin, specifically as basic (h1) calponin, and SM22α are newly recognised, and are the earliest specific markers of differentiated smooth muscle cells.[20–22] We have previously reported the relatively frequent expression of basic (h1) calponin (10 of 17 cases), SM22α (14 of 17 cases), and α-SMA (13 of 17 cases) mRNA transcripts in human osteosarcoma by RT-PCR analysis, and the expression of the basic (h1) calponin gene was significantly correlated with a favourable prognosis in this disease.[18] We have also analysed the expression of basic (h1) calponin by immunohistochemical means in a series of soft tissue sarcomas, and only one of 11 cases of MFH showed positive reactivity for basic (h1) calponin.[23] These findings show that MFH of bone has a similar pattern of expression of smooth muscle differentiation specific markers to that seen in osteosarcoma, rather than to that seen in soft tissue MFH. In fact, we treat patients with MFH of bone in a similar manner to those with primary osteosarcoma. The comparable immunohistochemical findings presented in our study support this interpretation. With regard to the role of the antitumour activity of basic (h1) calponin, Horiuchi et al recently reported its possible role as a tumour suppressor in experimental human uterine leiomyosarcoma.[24] However, in our present series no significant correlation between the expression of basic (h1) calponin and the prognosis of MFH of bone could be found (data not shown).

> "Malignant fibrous histiocytoma of bone has a similar pattern of expression of smooth muscle differentiation specific markers to that seen in osteosarcoma"

Although MFH of bone is a distinct clinicopathological entity, its true histogenesis is still debatable. Galmiche et al recently reported that human bone marrow stromal cells express various kinds of smooth muscle specific markers, including α-SMA and calponin, suggesting that they originate from mesenchymal cells that differentiate along a vascular smooth muscle differentiation pathway.[25] Interestingly, these "smooth muscle specific" markers are broadly expressed in a variety of tissues, such as normal and malignant breast tissue,[26] salivary gland pleomorphic adenoma,[27] and myofibroblastic sarcoma,[28] and are postulated as novel myofibroblastic/myoepithelial differentiation molecular markers. From these observations, it is strongly suggested that
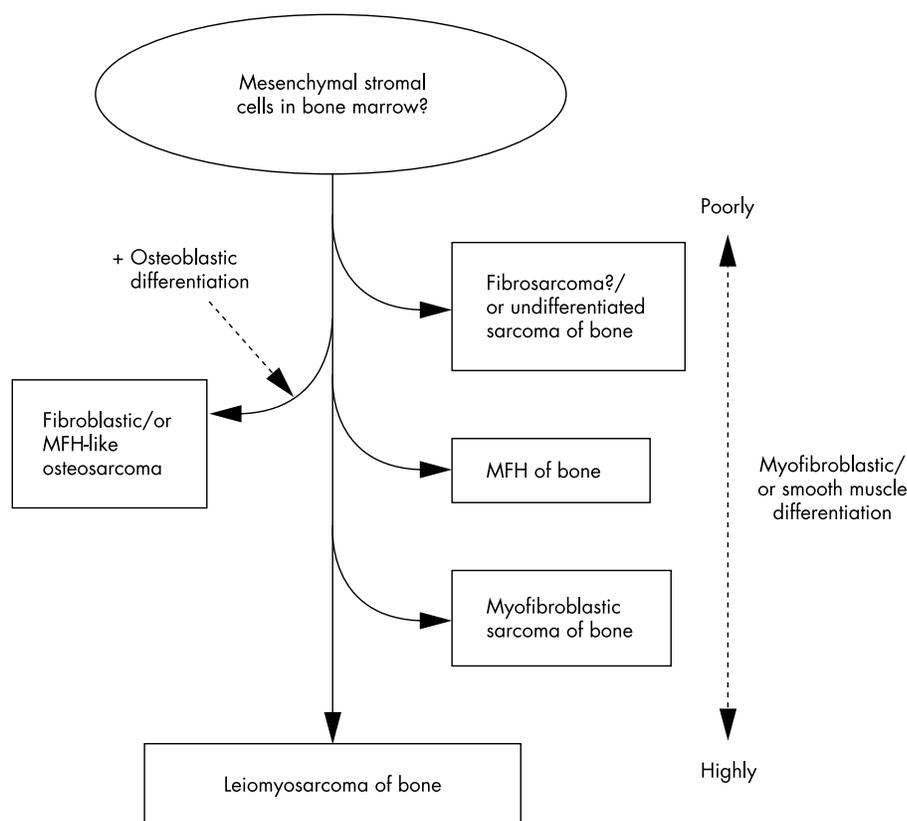
Figure 3 Hypothetical model of classification for spindle cell/or pleomorphic sarcomas arising in bone according to a spectrum of different degrees of myofibroblastic or smooth muscle cell differentiation. MFH, malignant fibrous histiocytoma.

MFH of bone derives from mesenchymal stromal cells in bone marrow and has a more myofibroblastic differentiation than soft tissue MFH.

Primary leiomyosarcoma of bone is an extremely rare malignant bone tumour that was first described by Evans and Sanerkin in 1965,[29] and recently reviewed by Antonescu et al.[30] It is defined as a malignant mesenchymal tumour primarily arising in bone, which is composed predominantly of spindle cells that definitely exhibit smooth muscle differentiation. Although its existence is still controversial,[1 11] it is gradually being accepted as a true primary bone tumour as a result of refinements in immunohistochemical techniques and advances in the more precise interpretation of electron microscopic examinations. Immunohistochemically, the tumour cells generally show strong and diffuse positive reactivity for α-SMA (100%) and common muscle actin (HHF35) (85%), and less frequently positive reactivity for desmin (50%) (data on the positivity for calponin and SM22α are not available).[30] However, immunohistochemical staining for these smooth muscle markers can be confusing because considerable numbers of cases of MFH of bone also show positive reactivity for these markers, as shown in our present study. Moreover, in a high grade, poorly differentiated leiomyosarcoma arising in bone, the differential diagnosis from MFH, fibrosarcoma, and myofibroblastic sarcoma of bone may be quite difficult or impossible even by electron microscopic examination. Thus, it might be more practical to hypothesise that these primary malignant tumours arising in bone are a consecutive entity in a spectrum of different degrees of smooth muscle or myofibroblastic differentiation, rather than to prescribe them as independent clinicopathological entities (fig 3), although there is no convincing evidence that the expression pattern of smooth muscle markers is highly conserved during the pathogenesis of these sarcomas. Antonescu et al have also suggested that vascular smooth muscle cells or multipotential mesenchymal stem cells (that is, bone marrow stromal cells) capable of smooth muscle dif-

Take home messages

- Smooth muscle/myofibroblastic markers, including α smooth muscle actin, calponin, and SM22α, are expressed relatively frequently in malignant fibrous histiocytoma (MFH) of bone compared with soft tissue MFH
- This pattern of expression is similar to that seen in osteosarcoma
- MFH of bone may have a bone marrow stromal cell origin, with a common histogenetic pathway to that of primary leiomyosarcoma of bone

ferentiation could be possible origins for intraosseous leiomyosarcoma,[30] supporting our hypothesis. Moreover, a recent cytogenetic study using a comparative genomic hybridisation technique in leiomyosarcomas at various sites including bone, compared with MFH, demonstrated that both types of tumours had a similar pattern of recurrent genomic imbalances, suggesting that MFH is a morphological modulation in the tumour progression of other sarcomas, particularly leiomyosarcoma.[31]

In conclusion, we have demonstrated the relatively frequent expression of smooth muscle/myofibroblastic markers, including α-SMA, calponin, and SM22α, in MFH of bone, which is similar to the pattern seen in osteosarcoma rather than that seen in soft tissue MFH. Our present study also suggests a possible bone marrow stromal cell origin for MFH of bone, with a common histogenetic pathway to that of primary leiomyosarcoma of bone.

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