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Expression profiles in malignant fibrous histiocytomas: Clues for differentiating 'spindle cell' and 'pleomorphic' subtypes

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ABSTRACT

We analysed 21 samples of malignant fibrous histiocytoma (MFH) distinguished into the two principal morphological categories ('spindle cell' and the 'pleomorphic' subtypes). The aim of our study was to verify if a distinction between the two subclasses of MFH in terms of expression/activation of protein profiles could support and extend the morphological criteria. For this purpose, we carried out an immunohistochemical and immunoblotting analysis of proteins that could be relevant in sarcoma biology and potential diagnostic and therapeutical targets such as matrix metalloproteinases (MMPs) and molecules related to adhesive and proliferative properties. Our analysis revealed that MMP-1, MMP-9 expression and p27(kip1) cytoplasmic localisation can be considered valid parameters in the classification and potential explanation of the aggressive behaviour of this non-homogeneous group of MFH.

1. Introduction

Soft tissue sarcomas (STS) comprise a heterogeneous group of mesenchymal tumours. Malignant fibrous histiocytoma (MFH) has been regarded as the most common STS in adult life but it has been plagued by controversy in terms of both histogenesis and validity as a clinicopathological entity.[1,2] The trend for some pathologists to diagnose MFH less frequently than other subtypes may result from different diagnostic criteria reflecting the concept of MFH as a common morphological manifestation of a variety of poorly differentiated STS, resulting in the diagnosis of MFH after a process of exclusion. A schematic morphological approach to STS considers a final distinction into three categories on the basis of cell shape: round cell morphology, spindle cells admixed with other mesenchymal elements (pleomorphic) and predominantly spindle cell morphology.[3] MFHs can be found within the 'spindle cell' and the 'pleomorphic' subtypes.[3] These two principal morphological categories result useful as they indicate different clinical outcomes, being the pleomorphic type the more aggressive form of the tumour. MFHs are tumours consisting of an admixture of fibroblastic, histiocytic and undifferentiated cells arranged in a storiform growth pattern. The undifferentiated cell may represent a progenitor with capacity to differentiate into histiocytic and fibroblastic cells but the relationship between histiocytic-like cells and true macrophages/histiocytes remains debatable. In fact, some authors consider histiocytic-like cells present in MFH as normal infiltrating macrophages induced by chemoattractants.[4] By sharpening the distinction between sarcoma

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Table 1 – Patient clinical features

Case number	F/M	Age	Extent of disease	Site of primary
'Pleomorphic' MFH				
17	M	71	Local recurrence	Extremity
40	M	85	Local recurrence	Extremity
117	F	67	Local recurrence	Extremity
127	F	63	Metastases (sub cutis)	Extremity
129	M	69	Primary disease	Extremity
139	F	33	Metastases (lung)	Extremity
147	M	30	Local recurrence	Retroperitoneal
148	M	50	Metastases (lung)	Extremity
158	M	70	Metastases (lung)	Extremity
175	F	64	Local recurrence	Extremity
209	F	34	Metastases (back)	Extremity
212	F	34	Metastases (lung)	Extremity
228	M	71	Metastases (lung)	Retroperitoneal
251	M	58	Primary disease	Extremity
'Spindle' MFH				
31	F	86	Local recurrence	Breast
83	F	87	Local recurrence	Breast
169	M	79	Primary disease	Extremity
182	F	60	Local recurrence	Extremity
183	M	57	Primary disease	Gastric
187	M	80	Local recurrence	Extremity
264	F	33	Local recurrence	Extremity

types and including functional criteria in the classification, it is likely that genes that define biologically specific features can provide a better characterisation of distinct groups. The products of these genes might include diagnostic markers related to tumour histogenesis as well as targets for new therapies.

While certain tumours exhibit fairly consistent and predictable histiotype-specific behaviour, other lesions, in particular MFH, present with a broad range of clinical behaviour not immediately predictable from histological typing alone. New expression profiles of these poorly differentiated adult STS could be very useful to improve our understanding of MFHs and their biology and origin. Recent reports on gene expression profiles of STS using cDNA microarray technologies provided new insights into MFH characterisation[5–7] and suggested MFHs as a pleomorphic subtypes amongst STS.

Tumour growth and metastasis involve molecular interactions between tumour cells and the surrounding normal tissues. Several steps are involved in this process, but degradation of the extracellular matrix (ECM) is an essential prerequisite for expansive growth of primary tumours, metastatic spread and neoangiogenesis. In particular, amongst the proteases involved, the MMP family is frequently implicated in the process of ECM degradation.[8,9] The proper management of the ECM represents a specialised function of mesenchymal cells and includes cell proliferation and migration required for ECM restoration in physio- and pathological situations. While MMP activity is very important in many malignancies such as carcinomas,[10] little is known on the role of MMPs in STS and in particular in MFHs.

The aim of our study was to verify if a distinction between the two major subclasses of MFH ('spindle cells' and 'pleomorphic') in terms of expression/activation of MMPs

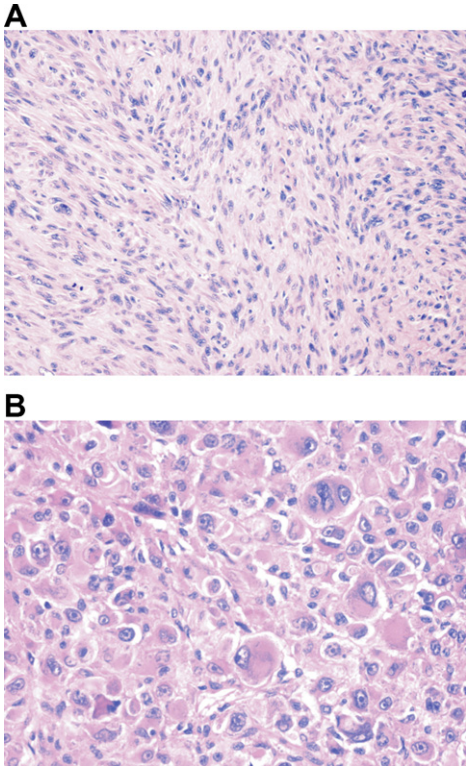

Fig. 1 – MFHs display marked pleomorphism, often with bizarre giant tumor cells, admixed with spindle and rounded histiocyte-like cells, sometimes with foamy cytoplasm. The spindle cells are prominent in (A) whereas the pleomorphic cells prevail in (B). Haematoxylin–eosin staining. Original magnification 100× (A), 200× (B).

and proteins related to adhesive and proliferative properties could support morphological criteria and provide insight to explain the more aggressive behaviour of the pleomorphic form.

2. Materials and methods

2.1. Antibodies

The following primary antibodies were utilised in this study: mouse monoclonal and rabbit polyclonal antibodies anti MMP-9 were provided by Chemicon International (Temecula, CA, USA); mouse monoclonal anti-MMP-2, anti-MMP-1 and anti-TIMP-1 antibodies were from Oncogene Research Products (San Diego, CA, USA); rabbit polyclonal anti-FAK, anti-p27(kip1) and anti-c-Src, goat polyclonal anti-CDK4 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); rabbit polyclonal anti-Akt, anti-phospho-Akt (ser473), anti-phospho-Src (Tyr527), anti-Stat-3 and anti-IGF-I Receptor β were provided by Cell Signalling Technology Inc. (Beverly, MA, USA); mouse monoclonal anti phosho-Stat-3 (Tyr705) was from Upstate (Upstate, Millipore, Milan, Italy); mouse monoclonal anti-CDK2 was provided by BD Transduction Laboratories (Becton Dickinson Italia S.p.A, Milan, Italy).

2.2. Tissue samples and histological classification

For this study, 21 cases of archival tissue samples embedded in paraffin and fresh frozen tissues of surgically resected specimens from patients with MFH were used. The histological diagnosis was based not only on histological features on haematoxylin–eosin stain, but also on immunohistochemical analysis according to the description in the WHO classification.[11] Amongst these 21 MFH cases, on the basis of predominant cell shape 14 were considered as 'pleomorphic' (pMFH) and 7 as 'spindle' (sMFH) having round cell morphology and predominantly spindle cell morphology, respectively. For 11 pMFH and 6 sMFH cases paired adjacent non-neoplastic tissue were obtained. Table 1 shows the clinical pathological features of the patients.

2.3. Immunohistochemical technique

Immunohistochemistry was performed with the avidin–biotin–peroxidase complex (ABC-px) as previously described.[12] For all samples, negative controls included omission of primary antibody.

2.4. Analysis of immunohistochemical staining

The interpretation of immunohistochemical staining was expressed as follows: – negative (less than 10% of the cells stained), + weak positive (11–50% of the cells stained), ++ moderate positive (51–80% of the cells stained) and +++ strong positive (more than 80% of the cells stained).

2.5. Western blotting

Lysates obtained from tissue samples were loaded onto a SDS–polyacrylamide gel (4–15% polyacrylamide gradient) (20 μg/lane) subjected to electrophoresis under reducing conditions and transferred to a nitrocellulose membrane. The membranes were saturated with TBS buffer (20 mM Tris

Table 2 – Frequency and staining intensity of MMP-1, MMP-2 and MMP-9 in malignant fibrous histiocytomas

	pMFHs	%	sMFHs	%
MMP-1				
Positive	9/9	100	2/4	50
Negative	0/9	0	2/4	50
Staining intensity				
+	2/9	22	2/2	100
++	3/9	33	0/2	0
+++	4/9	45	0/2	0
MMP-2				
Positive	5/9	55	2/4	50
Negative	4/9	45	2/4	50
Staining intensity				
+	3/5	60	2/2	100
++	2/5	40	0/2	0
+++	0/5	0	0/2	0
MMP-9				
Positive	11/11	100	2/4	50
Negative	0/11	0	2/4	50
Staining intensity				
+	0/11	0	2/2	100
++	6/11	54	0/2	0
+++	5/11	46	0/2	0

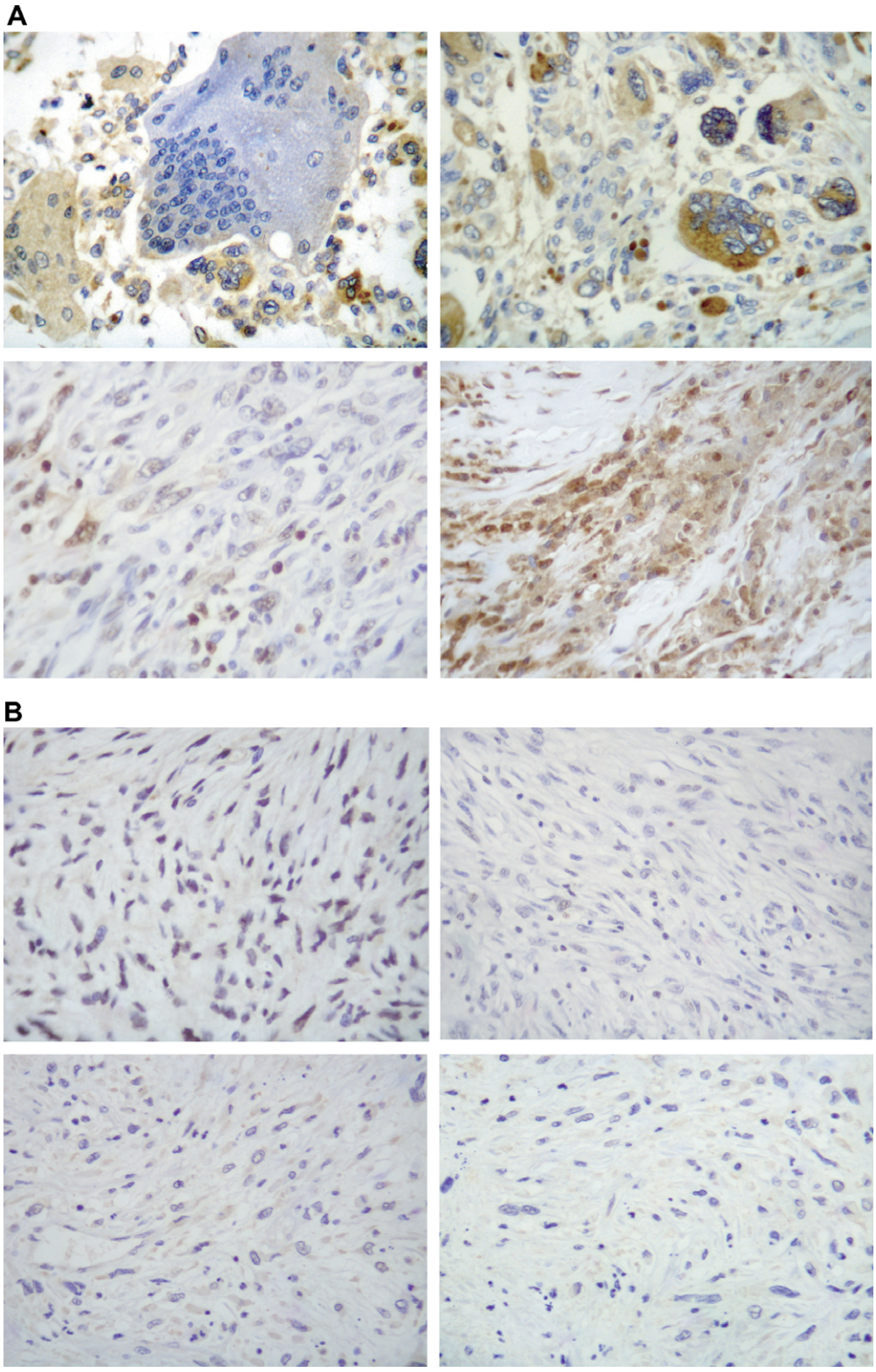

Fig. 2 – Immunohistochemical analysis of frozen representative sections of pMFHs (A) and sMFHs (B) stained for MMP-1. The expression of MMP-1 is strong in multinucleated cells in pMFHs and prevalently associated to inflammatory cells in sMFHs. Original magnification 200×.

and 0.15 M NaCl) containing 0.1% Tween-20 (TBST) and 5% non-fat dry milk for 2 h at room temperature and then incubated with primary antibodies at 4 °C overnight. After extensive washing in TBST, the membranes were incubated with HRP-conjugated appropriate secondary antibodies (Amersham, GE Healthcare, Milan, Italy) and then revealed with the ECL Plus chemiluminescence kit (Amersham, GE Healthcare).

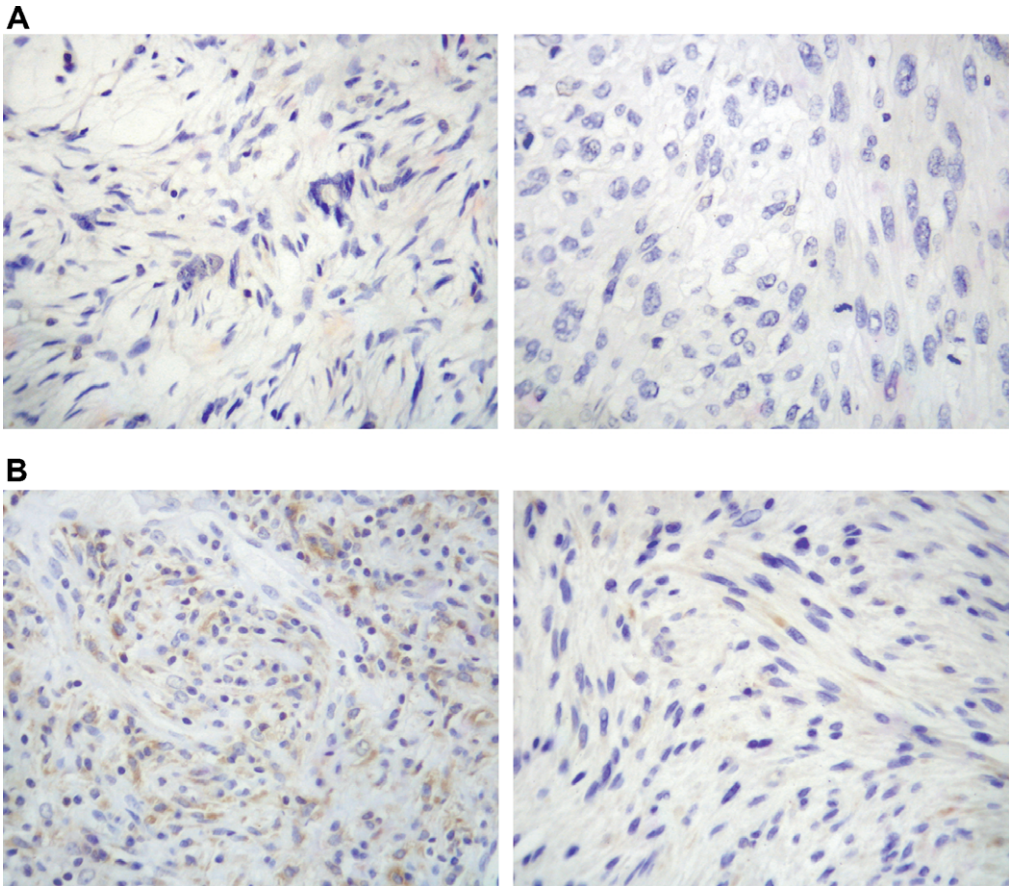

Fig. 3 – Immunohistochemical analysis of frozen representative sections of pMFHs (A) and sMFHs (B) stained for MMP-2. Original magnification 200×.

2.6. *Statistical analyses*

Statistical significance was evaluated by the Mann–Whitney's *U* non-parametric test with significance taken as $p < 0.05$.

3. Results

3.1. *Pathological findings*

The cases included in this study represent examples of undifferentiated high grade sarcomas, alternatively termed pleomorphic malignant fibrous histiocytomas. According to the WHO classification, they do not show a definable line of sarcomatous differentiation by using current technology. These tumours have marked cytological and nuclear pleomorphism, often with bizarre giant tumour cells, admixed with spindle cells and rounded histiocytic-like cells, sometimes with foamy cytoplasm.

From files of our sarcoma database, we have isolated two groups of the so-called PMFHs with clearly distinct morphologies and similar immunohistochemical profiles. In one group, the spindle cells were prominent (Fig. 1A), whereas, in the other group, the pleomorphic cells prevailed (Fig. 1B). In the present study, the 'spindle cell' (sMFH) and the 'pleomorphic' (pMFH) tumours were comparatively analysed for differential expression of biological determinants that could potentially distinguish the two groups. Twelve standard markers were used altogether including antigens of specific lineage (keratins, vimentin, desmin, smooth muscle actin, CD57, CD68/KP-1 and S100). In selected cases HMB45 (reactive in malignant melanoma), CD34, CD31, CD99 and CD117 were investigated. From this analysis all samples resulted positive for vimentin and negative for the other markers with few exceptions: very weak positivity was detected for EMA antigen (1 sample), CD68 (9 samples), smooth muscle actin (HHF35, 2 samples; 1A4, 3 samples), CD34 (1 sample), CD99 (1 sample) and CD117 (1 sample). Since no clear distinction could be detected between sMFH and pMFH in terms of differential positivity for the various markers pMFHs and sMFHs were still conventionally distinguished on the basis of morphological features as reported in Fig. 1.

3.2. *MMP expression – immunohistochemical analysis*

The frequency and staining intensity of MMPs in our cases of MFH are given in Table 2. MMP-1 was positive in all pMFH and in 50% sMFH samples examined. The staining intensity for MMP-1 ranged from moderate to strong in pMFHs and weak or moderate in sMFHs. Representative examples of MMP-1 staining in pMFHs (Fig. 2A) and sMFHs (Fig. 2B) are depicted.

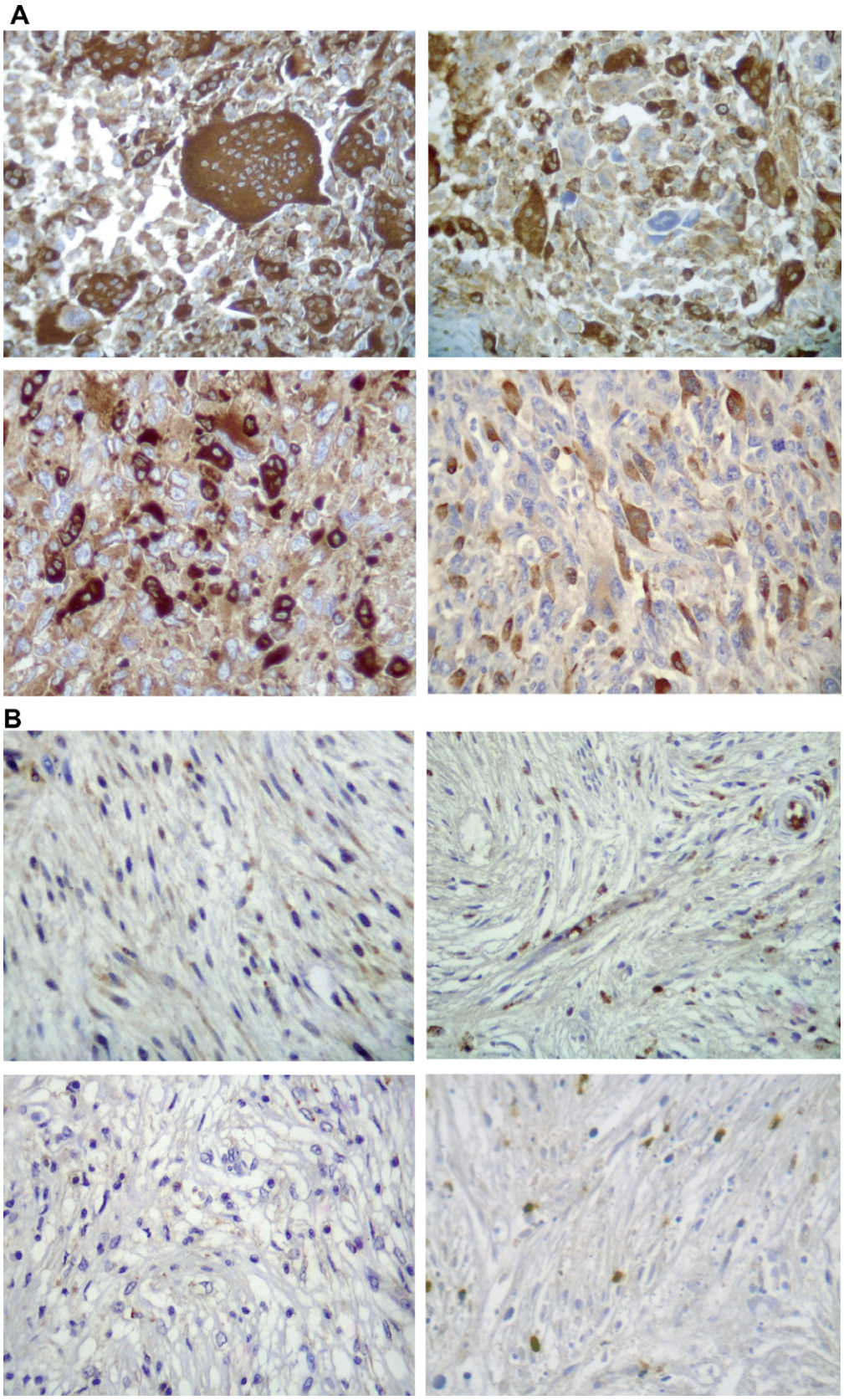

Fig. 4 – Immunohistochemical analysis of frozen representative sections of pMFHs (A) and sMFHs (B) stained for MMP-9. MMP-9 is expressed at very high levels in multinucleated giant cells only in pleomorphic MFHs. Original magnification 200×.

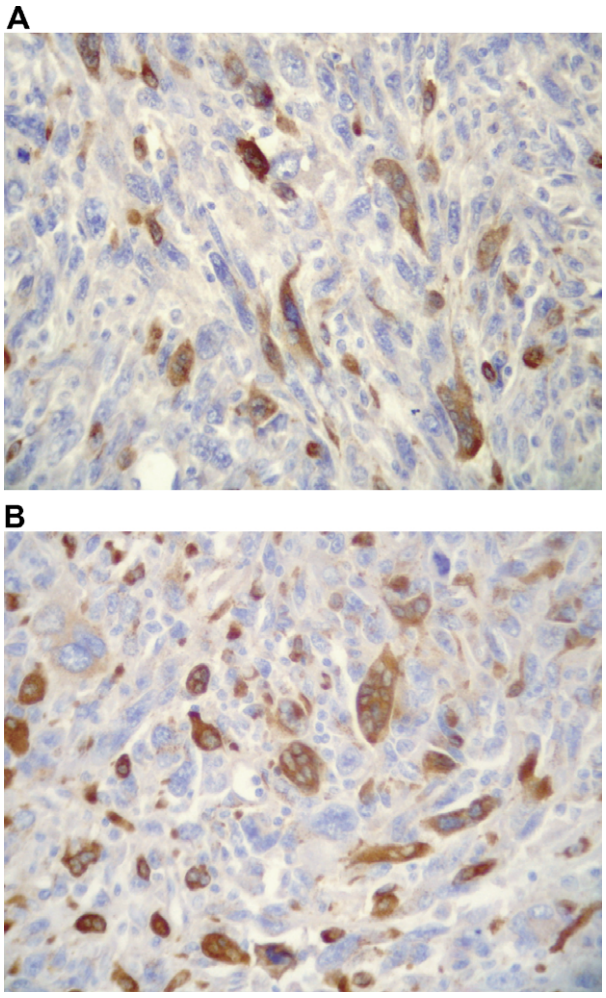

Fig. 5 – MMP-9 expression in a representative pMFH sample. A monoclonal (A) and a polyclonal antibody (B) against MMP-9 were used. In both conditions MMP-9 is localised above all in multinucleated cells and the degree of positivity was high. Original magnification 200×.

Notably, MMP-1 was expressed very strongly in multinucleated cells and less intensely in other neoplastic or stromal cells (Fig. 2A). Amongst multinucleated cells we could recognise different levels of expression for MMP-1 or non-homogenuos stained areas within a single multinucleated cell (Fig. 2A). On the contrary, the positive pattern in sMFHs was prevalently associated to infiltrated inflammatory cells well distinguishable based on their morphology (Fig. 2B).

The expression of MMP-2 was detected in about 50% samples irrespective of the morphological phenotype. The intensity of the immunostaining for MMP-2 ranged from weak to moderate (Fig. 3 and Table 2).

As reported for MMP-1 expression, also MMP-9 was positive in all pMFH and in only 50% sMFH samples examined. Representative examples of MMP-9 expression and localisation in pMFHs (Fig. 4A) and sMFHs (Fig. 4B) are shown. The immunostaining pattern for MMP-9 indicated a very strong positive signal for this protease only in pMFHs (Fig. 4A and Table 2). In particular in 5 out of 11 pMFHs the MMP-9 expression was very high whereas in 6 samples the intensity was moderate. In sMFHs, the MMP-9 expression was weak and prevalently associated to inflammatory infiltrated cells (Fig. 4B). It was also evident that in pMFH all multinucleated cells were highly reactive for MMP-9. However, true histiocytic or tumour cells were not distinguishable. In some cases to confirm the very strong MMP-9 reactivity of this type of cells, we utilised different antibodies including a monoclonal. An example of highly positive multinucleated cells in pMFHs is shown (Fig. 5).

3.3. *MMP expression – immunoblotting analysis*

Quantitative MMP expression profiles were evaluated by immunoblotting in tissue extracts derived from surgical explants. Fourteen pMFH and 7 sMFH specimens displayed a strong signal for the presence of MMP-1, which was highly expressed above all in pMFHs (Fig. 6A, upper). On the contrary, MMP-2 was present only in some samples and as a faint band (Fig. 6A, upper). In 3 pMFH samples (#127, #148 and #175) the presence of the activated form of MMP-2 was detected (Fig. 6A, upper). MMP-9 expression was detected with a strong positive signal in all pMFH samples whereas 2 out of 7 samples of sMFHs were completely negative (Fig. 6A, upper). Furthermore, the activated form of the enzyme was detectable only in pMFHs (9 out 14 samples). Interestingly, the MMP-9 activated form was present in 5 out of 7 samples all of metastatic patients (Table 2). In samples #127, #148 and #175, the activated forms of both MMP-2 and MMP-9 were present and MMP-1 was well expressed. Densitometric analyses normalised for β-actin were carried out to quantify expression of MMPs. Significative differences were revealed between the two subtypes of MFHs for the expression of MMP-9 and MMP-1 that were both produced at higher levels in pMFHs (Fig. 6A, lower). Furthermore, MMP-9 and MMP-1 expression was analysed by immunoblotting in neoplastic and, whenever possible, normal tissues (Fig. 6B). In 8 out of 11 pMFH samples, MMP-9 was produced at higher levels in tumour than in the normal counterpart. Moreover, it is of note that the activated form of the enzyme was present only in the tumour specimens, but for sample #175 in which both forms were present even if at a higher level in tumour tissue. Three out of the 6 sMFH samples expressed a higher quantity of MMP-9 in tumour than in normal tissues (Fig. 6B), although the activated form was not detectable. On the other hand, the levels of MMP-1 were quite similar in normal and tumour samples for both MFH groups (Fig. 6A). These findings suggested that amongst the MMPs examined, MMP-9 could have a specific role in the biology and tumour progression of pMFHs but not in sMFHs. TIMP-1, a natural inhibitor of MMPs, was not detected in our samples (data not shown).

3.4. *Protein expression in MFH specimens*

To contribute to the understanding of the molecular pathology of these MFH subtypes the expression and activation of proteins related to adhesive and proliferative properties were investigated.

FAK, whose overexpression and/or activation correlate with tumour development and progression in many types of cancer,[13] was expressed in all samples except in two pMFH

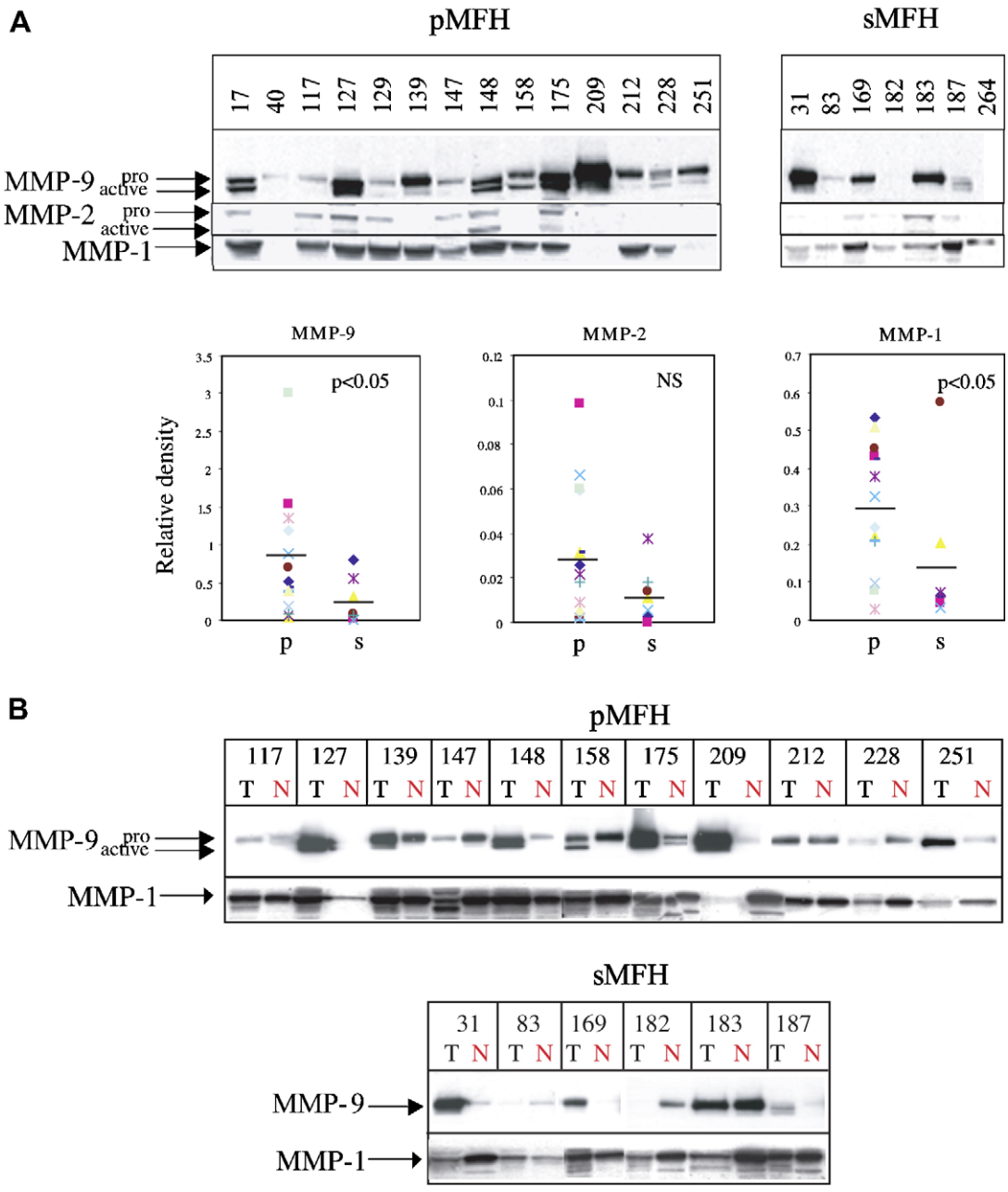

Fig. 6 – MMP-9 expression in tissue extracts. (A) Immunoblotting of pMFH (left) and sMFH (right) samples for the presence of MMP-9, MMP-2 and MMP-1. Densitometric analyses were standardised for β-actin content. (B) MMP-9 and MMP-1 expression in pMFH and sMFH samples with paired normal tissues.

and one sMFH samples. FAK autophosphorylation (Y397) was a rare event and did not represent a clue for discriminating MFH subtypes (Fig. 7A and B).

Akt appeared strongly activated (Fig. 7A), since the signal for the phosphorylated protein was very intense in all specimens. Densitometric analysis demonstrated that the ratio between pAkt and Akt was generally higher in pMFH than in sMFH subgroup but the difference was not significant (Fig. 7B). Src was highly phosphorylated in all samples and the ratio between p-Src and Src was not different in the two MFH populations (Figs. 7A and B). Also expression of Stat-3 or IGF-1Rβ was not useful to distinguish pMFH and sMFH specimens (Figs. 7A and B).

Next, we examined the expression levels of proteins involved in cell cycle such as CDK2, p21 and p27. The CDK2 levels were not significantly different in the two groups being low or moderate in all specimens (Figs. 7A and B).

p21 and p27(kip1) were both expressed in our samples and significant differences in expression levels were noticed only for p21, which was more strongly expressed in sMFH samples (Figs. 7A and B).

Since cytoplasmic expression of p27(kip1) affects microtubule (MT) stability following cell adhesion on ECM constituents[14] and low cytoplasmic p27(kip1) expression correlates with the metastatic phenotype of human sarcomas *in vivo*,[14] we next investigated subcellular localisation of p27(kip1) in immunohistochemistry. We examined nine samples of pMFHs and they all displayed reactivity for the protein except one patient. The intensity was variable but the localisation of the protein resulted either cytoplasmic or nuclear in all samples (Fig. 8A). On the contrary, when present, p27(kip1) was only nuclear in sMFHs (Fig. 8B).

4. Discussion

In this study, we suggest that MMP-1 and MMP-9 expression levels and the presence of the active MMP-9 form as well as p27 localisation could provide important clues to distinguish sMFH from pMFH amongst the non-homogenous group of MFHs.

Different components of the main signalling pathways known to be involved in the invasive and metastatic processes of STS were not useful for discriminating MFHs: the expression levels of IGF-1Rβ, FAK, Stat-3, Akt, Src and CDK2 and their activation status were not significantly different. While Stat-3 and Akt were reported to be frequently activated in bone/soft tissue tumours[15] and elevated levels of FAK were suggested to play a role in tumour development,[16,17] in the present series of MFHs only the expression level of p21 was significantly higher in sMFHs than in pMFHs, indicating a potential stronger cell cycle inhibition in the less aggressive MFHs.

pMFHs expressed MMP-9 and MMP-1 at elevated levels as revealed by immunostaining and Western blotting analyses, whereas sMFHs were weakly positive for both MMPs. Recent evidences demonstrated that STS (MFH, leiomyosarcomas, synovial sarcomas and fibrosarcomas) express high levels of MMP-9.[18] Since MMP-9 expression was correlated with progression and metastatic processes, it was suggested that MMP-9 targeting could represent an alternative therapeutic intervention. Benassi et al. examined the expression of MMP-2, MMP-9 and TIMP-2 in synovial sarcomas, liposarcomas and malignant peripheral nerve sheath tumours (MPNST)[19] and in a further study found that MMP-2 expression or reduced TIMP-2 expression was associated to a less favourable histological grade and poor prognosis.[20] Roebuck et al.[21] suggested that the aggressive and invasive characteristics of STS are not due to significant changes in the production of individual MMPs but are more likely associated to multiple modifications of MMP production and activation. In this latter study, MMP-9 expression and activation were ascribed to the inflammatory cells, including macrophages, neutrophils and osteoclasts that are present in tissue adjacent areas of tumour necrosis.[21]

Our data adds more information about MMP-9 expression and its production by MFHs: the level of positivity for this enzyme clearly differentiated sMFHs from pMFHs. High positivity for MMP-9 was associated to a pleomorphic appearance, whereas low or even absent expression was associated to

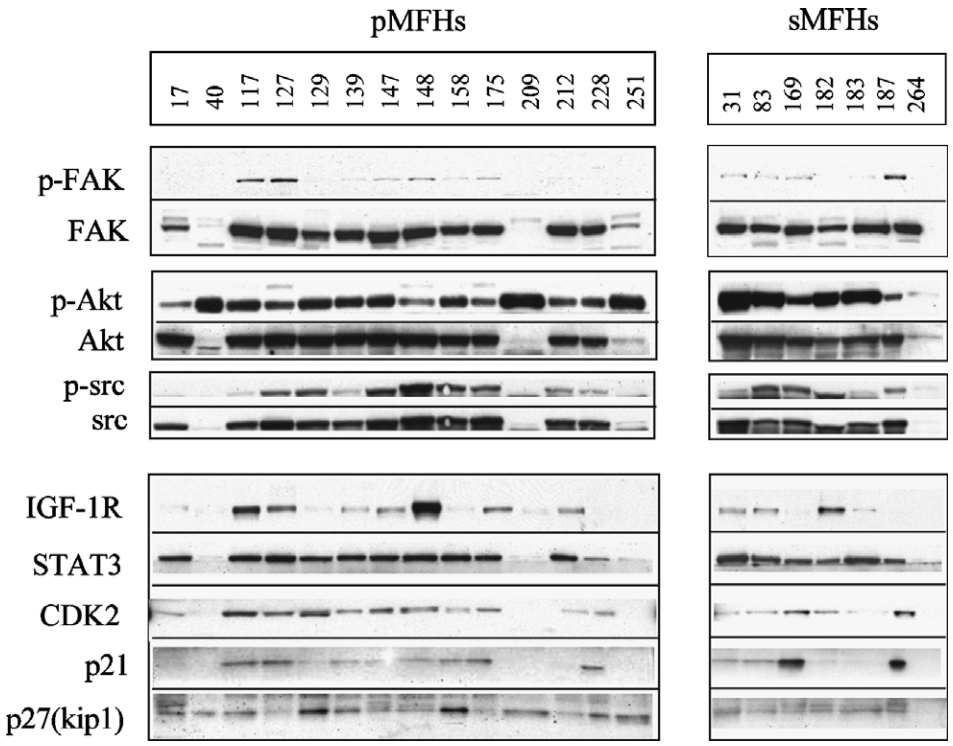

Fig. 7 – (A) Protein expression levels evaluated by immunoblotting of pMFHs (left) and sMFHs (right). (B) Densitometric analyses for protein expression were standardised for β-actin content. For activated status, the level of phosphorylated protein was standardised with the level of unphosphorylated protein.

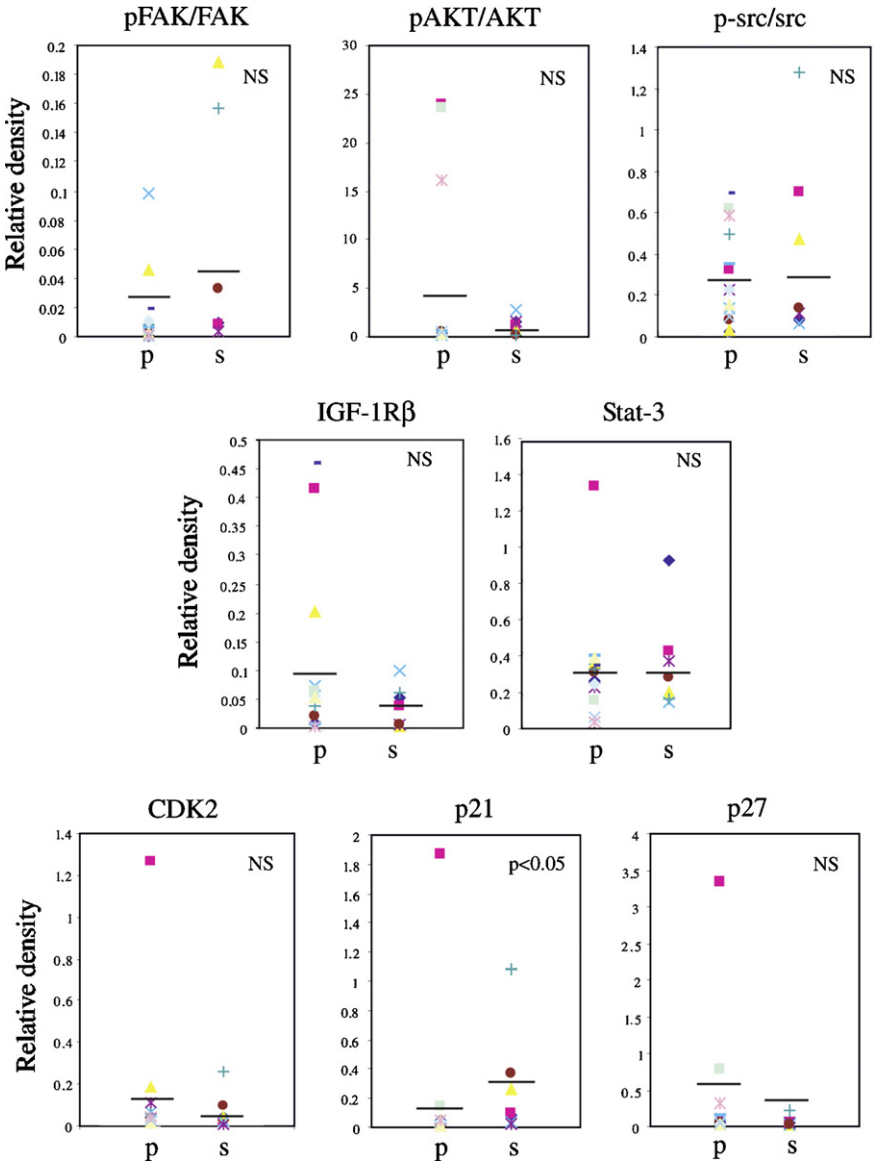

Fig. 7 *(continued)*

'spindle' cell enriched samples. In pMFH specimens, MMP-9 expression was detected in stromal as well as in tumour cells with a very high intensity associated prevalently to multinucleated cells. On the contrary, MMP-9 reactivity was associated to inflammatory cells in sMFH where very rarely neoplastic and multinucleated cells expressed MMP-9. Western blotting analyses confirmed the immunostaining results for MMPs and the demonstration of the active form of MMP-9 only in pMFH extracts indicated that the two groups of MFHs could be diversified based on the functional status of this enzyme. The real lineage of this type of cells has been controversial: are the histiocytic-like cells and multinucleated giant cells of neoplastic or reactive origin? Many authors suggest that histiocytic-like cells in MFH should be considered as a reactive monocyte/macrophage cell rather than as a proper neoplastic element. Here, multinucleated giant cells were highly positive also for MMP-1. MMP-1 and MMP-9 are enzymes produced by both macrophages and osteoclasts but also by a variety of tumour cells. However, while the reactivity for MMPs, especially for MMP-1, has different intensity in multinucleated giant cells in sMFHs versus pMFHs, it is not appropriate to ascribe to a particular lineage this type of cells on the basis only of different grade of reactivity for MMPs.

The localisation of p27(kip1) represented another useful marker: in pMFH specimens p27(kip1) was present both in the nucleus and in the cytoplasm, in sMFHs the protein was expressed only into the nucleus. The role of p27(kip1) expression as a diagnostic or prognostic marker for a wide variety of neoplasms is under intense investigation. An inverse correlation between p27(kip1) and the degree of malignancy has been observed, and low p27(kip1) expression has been shown to correlate with aggressive clinical behaviour and decreased survival in a variety of human malignancies,[22] including synovial sarcomas,[23,24] malignant peripheral nerve sheath tumours,[25] liposarcomas,[26] and Kaposi sarcomas.[27] In a study

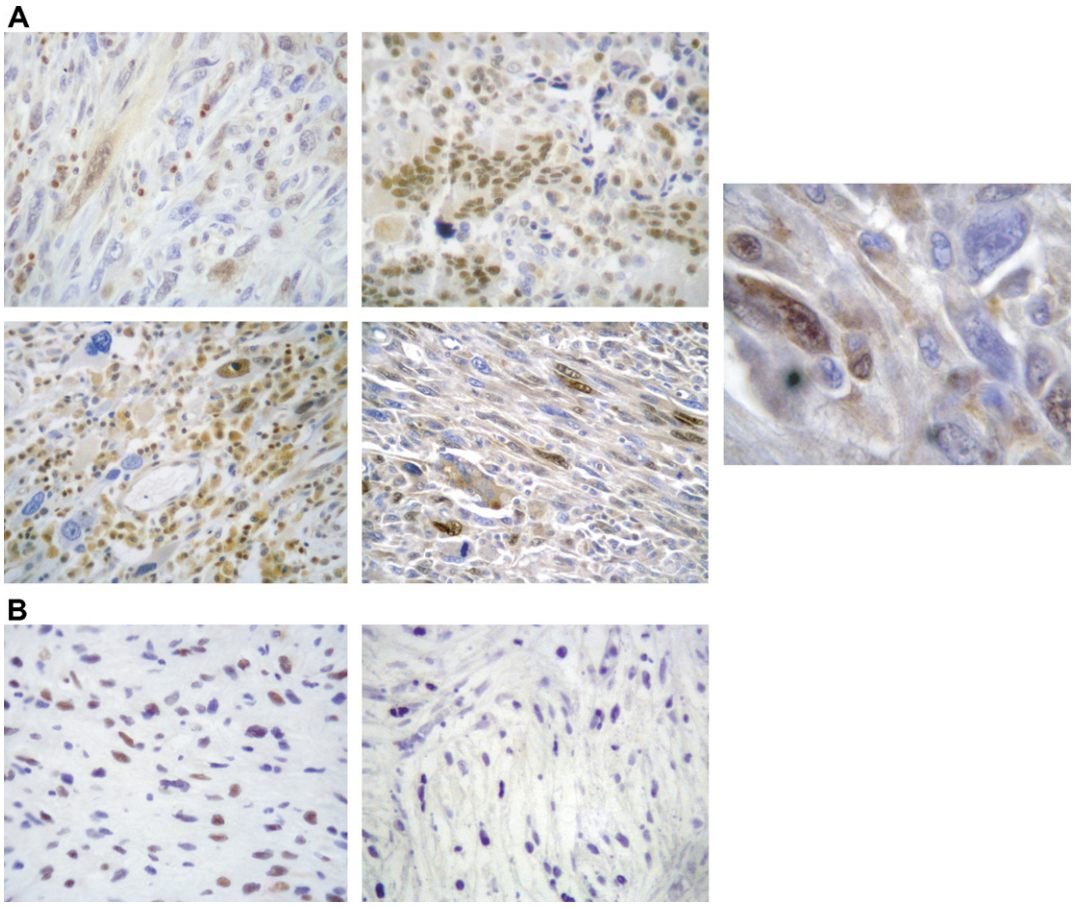

Fig. 8 – p27(kip1) staining of pMFHs (A) and sMFHs (B). p27(kip1) was present both in the nucleus and in the cytoplasm of pMFH cells (on the right a typical field at higher magnification). p27(kip1) localised only in the nucleus in sMFH cells. Original magnification 200×.

describing the altered expression of the Rb pathway proteins in STS, amongst 23 MFH analysed out of a large sample of STS only one resulted positive for p27(kip1) in immunohistochemistry .[28] We found reactivity for p27(kip1) in a high percentage of MFH samples both by immunohistochemistry and Western blotting analyses. The discrepancy between the present study and that of Sabah et al.[28] is striking, as the cut-off to define a positive phenotype was 10% immunoreactive tumour cells in both studies. One explanation could be in the modality of tissue sections: standard histological sections versus tissue microarray; alternatively, Sabah et al. could have overlooked the cytoplasmic expression; finally, the quality of the antibodies used could have prevented the detection of low or moderately positive cells. Since the cytoplasmic expression of p27(kip1) affects microtubule stability, there is a functional link between proliferation and invasion of tumour cells based on the distinct activities played by p27(kip1) in the different subcellular compartments.[14] Taking into account all these findings both expression and localisation of p27(kip1) have to be considered as additional parameters of aggressiveness in MFH.

In conclusion, this study has revealed that MMP-1, MMP-9 and p27(kip1) could be considered valid parameters in the classification of the non-homogeneous group of MFHs. Further studies including larger number of samples are necessary to establish these proteins as target for preventing tumour progression.

Conflict of interest statement

None declared.

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