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Original Paper

Adhesion Molecules in High-grade Soft Tissue Sarcomas: Correlation to Clinical Outcome

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The extracellular matrix (ECM) forms a framework for cell adhesion, but it also regulates growth and differentiation. Normal and malignant cells interact with the ECM via specific receptors, the integrins. To explore the mechanisms of growth and spread in soft tissue sarcomas the expression of the major ECM molecules and their corresponding integrin receptors were studied by immunohistochemistry in high-grade soft tissue sarcomas: malignant fibrous histiocytoma (20 cases), malignant peripheral nerve sheath tumour (17 cases) and synovial sarcoma (21 cases). The expression pattern was compared with cell proliferation and clinical outcome. Integrins were found to be expressed according to histological pattern. In synovial sarcomas, the epithelial component showed a high α_2 but negative or minimal detection of α_5 expression, while a weak α_2 expression and a moderate α_5 expression were found in the spindle cell component. No α_2 expression was detected in malignant fibrous histiocytoma, and minimal α_5 expression was detected in malignant schwannoma. The α_6 expression levels were positively correlated with the occurrence of metastases in all types of sarcomas studied. The expression of ECM molecules was downregulated and irregular in most tumours. In conclusion, the divergent integrin expression pattern could be useful in the diagnosis and classification of soft tissue sarcomas. Furthermore, since high laminin receptor expression correlates with occurrence of metastases, it could become a useful prognostic marker.

Key words: integrins, extracellular matrix, sarcomas, prognosis
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INTRODUCTION

An important determinant of cell function is the ability of cells to interact with the extracellular matrix (ECM), including the basement membrane (BM) [1]. This interaction is mediated by a family of adhesion molecules called integrins, which play an important role in cell growth, differentiation and migration [2, 3]. The integrins are membrane bound molecules, heterodimers composed of an α- and a β-chain. The β_1-chain can associate with several different α-chains, and depending on the α-chain, the heterodimers with β_1 form the major receptors for several ECM components [4]. Thus, the $\alpha_2\beta_1$ integrin is the major receptor for type IV collagen, the $\alpha_6\beta_1$ integrin is the major receptor for laminin and the $\alpha_5\beta_1$ integrin is the major receptor for fibronectin. The integrins containing a β_3-chain bind to vascular components [5], such as thrombospondin and vitronectin, $\alpha_v\beta_3$ being the major vitronectin receptor.

Integrin expression is important in normal cell functions. In tumours, their expression pattern is of importance for tumoral behaviour. A disorganisation of BM components, or changes in integrin expression, seem to be associated with a malignant phenotype and a more aggressive tumour behaviour [6, 7]. In carcinomas, the deregulation of $\alpha_2\beta_1$ is frequently seen [8] and in several tumours, the expression of $\alpha_6\beta_1$ is associated with tumour invasion and metastases [9]. In non-neoplastic tissues, the expression level of the fibronectin receptor $\alpha_5\beta_1$ is proportional to the amount of fibronectin matrix deposited around the cells [10]. In malignant transformation, $\alpha_5\beta_1$ expression is often reduced, with loss of

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the fibronectin matrix, which dramatically increases the ability of malignant cells to invade the surrounding tissues [11].

Previous studies have emphasised the fact that integrin expression is related to cell type [12]. Based on this finding, it has been shown that integrin expression can be used as a cell lineage marker which can be useful in tumour diagnosis and classification. The integrin expression pattern has also been reported to be of use as a prognostic marker in epithelial tissues and tumours [13]. The aim of this study was to evaluate the expression level and pattern of ECM components: laminin, type IV collagen, fibronectin and vitronectin and their corresponding integrin receptors in high-grade malignant musculoskeletal tumours, known to produce ECM. The possible use of integrin expression in the histopathological diagnosis of sarcomas was also studied. Furthermore, the expression pattern of adhesion molecules was compared with the proliferation index, the occurrence of metastases, and clinical outcome, to determine whether adhesion molecule expression could be of use as a prognostic marker.

MATERIALS AND METHODS

Materials

This study was carried out on tissue samples from surgically removed primary high-grade soft tissue sarcomas. The study included 20 storiform-pleomorphic malignant fibrous histiocytomas (MFH), 17 malignant peripheral nerve sheath tumours (MPNST) and 21 synovial sarcomas (SS), of which 7 were spindle cell monophasic and 14 biphasic. From each tumour a representative sample was fixed in 2% paraformaldehyde in phosphate buffered saline (PBS) and embedded in glycolmethacrylate for the immunohistochemical studies. Normal adjacent tissue was used as a control.

All remaining tumour tissue was fixed in formalin and embedded in paraffin. All tumours were diagnosed at the Rizzoli Orthopaedic Institute by a team of pathologists particularly expert in the diagnosis of sarcomas. Appropriate immunohistochemistry with a panel of antibodies, such as S-100, desmin, smooth muscle actin, vimentin and cytokeratin, was always performed for differential diagnosis. Broders' criteria for grading was used [14] and only grade 3 and 4 tumours were included in this study.

Antibodies

The antibodies used to study the expression of integrin chains and ECM molecules are listed in Table 1. For the determination of the amount of proliferating cells the Ki67 antibody (Dako A/S, Copenhagen, Denmark) was used at a 1:25 dilution.

Immunohistochemistry

The resin blocks were stored at −20°C. Sections (2 μm) were mounted on poly-L-lysine pretreated glass slides. The slides were dried at 37°C for 2 h and stored at 4°C. The immunohistochemical study was performed using the streptoavidin–biotin peroxidase system (sABC), without removing the resin. The sections were rinsed three times with PBS, pretreated with trypsin for 10 min at 40°C and incubated with the blocking serum for 20 min at room temperature. The primary antibodies were then added and incubated for 2 h at 37°C. After washing the sample twice in PBS, they were exposed to the conjugate secondary antibody and the streptoavidin–biotin complex (Autoprobe III, Biomeda) for 30 min at room temperature. The reaction was detected by 3-amino-9-ethylcarbazole (Sigma No. A-5754; Sigma Chemical Co., St. Louis, Missouri, U.S.A.) solution (0.2 mg/ml in 0.05 M acetate buffer containing 0.03% H_2O_2) for 30 min at room temperature. The sections were again rinsed twice with PBS and counterstained with Mayer's haematoxylin or methyl green. The intensity of the immunoreaction was scored as: − (negative), + (weak), ++ (moderate), +++ (strong). Negative controls were performed for each staining series.

Clinical follow-up and statistics

The follow-up, ranging from 3 to 132 months, was dated from the histological diagnosis. Disease-free patients had a minimum follow-up of 3 years. Data concerning survival and the occurrence of metastases were recorded, as well as the time interval between diagnosis and the detection of metastases. The relationship between integrin subunit expression and the percentage of Ki67-positive cells was assessed by linear regression analysis.

The correlation between the number of tumour cells expressing the laminin receptor (LR), and the occurrence of metastases was assessed by the chi-square test.

The patients were divided using a cut-off for LR expression of 35% corresponding to the median value of α_6-positive cell percentage of the population. Further cut-offs of 20% and 50% of α_6-positive cells were used. The difference between the disease-free survival curves was assessed by log-rank test.

RESULTS

Malignant fibrous histiocytoma (MFH)

In MFH, moderate to strong β_3 integrin expression was observed, whereas surrounding non-neoplastic tissues were completely negative. In several of the MFHs, the β_3 subunit

Table 1. *Antibodies used in this study*

Target structure	Clone	Dilution	Source
Integrins			
α_2	P1E6	1:50	Oncogene Science, Uniondale, New York, U.S.A.
α_5	P1D6	1:50	Oncogene Science, Uniondale, New York, U.S.A.
α_6	G0H3	1:80	Immunotech, Marseille, France
α_v	AMF6	1:50	Immunotech, Marseille, France
β_3	SZ21	1:80	Immunotech, Marseille, France
Extracellular matrix molecules			
Laminin	LAM-89	1:100	Sigma, St Louis, Missouri, U.S.A.
Type IV collagen	COL-94	1:200	Sigma, St Louis, Missouri, U.S.A.
Vitronectin	VNS-3	1:50	Takara, Otsu, Japan
Fibronectin	IST-4	1:250	Sigma, St Louis, Missouri, U.S.A.

was strongly and uniformly expressed on giant cells, while the expression was moderate to strong in mononuclear cells (Figure 1). In contrast, α_v showed a weaker and more focal distribution within the tumours, and no α_v expression could be seen on the giant cells. Most MFHs showed a weak to moderate staining for α_6 and α_5, whereas α_2 subunit expression was not detected. Of the ECM proteins, laminin and fibronectin were irregularly and focally expressed, no type IV collagen expression was seen. Vitronectin expression was moderate and uniform (Table 2). No statistically significant correlation was found between the integrin expression and number of Ki67-positive cells.

Malignant peripheral nerve sheath tumour (MPNST)

Compared with non-neoplastic nerve tissue, the cells of MPNST showed a strong β_3 expression. α_v, its counterpart in the vitronectin receptor, was also expressed by tumour cells. In contrast, the α_2 integrin expression was low in tumour cells (Figure 2), much lower than in the corresponding normal tissues. Minimal or no α_5 expression was detected in MPNST. Of the ECM proteins, both laminin and type IV collagen were expressed around the neoplastic cells, but, the expression was weak and irregular compared with non-neoplastic nerve tissue or blood vessels in which the expression of both these molecules is uniform. Both fibronectin and vitronectin were only focally and weakly expressed (Table 2).

When comparing cell proliferation with integrin expression, a negative, statistically significant correlation was found between α_2 expression and Ki67-positive cells ($r = 0.89$, $P < 0.001$). In contrast, although the distribution was irregular, the expression of α_6 appeared to be higher in tumours with > 25% Ki67-positive cells (Figure 3).

Table 2. Expression of integrin subunits and extracellular matrix components in soft tissue sarcomas

	MFH	MPNST	SS EP	SS SP
α_2	–	+	++/+++	+/–
α_6	+/++	++	++	+
α_v	+	+	++	+
β_3	++/+++	+++	+	+
α_5	+/++	+/–	–	++
LM	+	++	++	+
IV COLL	–	+	+	–
FN	+	+	+	++
VN	++	+	+	+

MFH, malignant fibrous histiocytoma; MPNST, malignant peripheral nerve sheath tumour; SS, synovial sarcoma; EP, epithelial cells; SP, spindle cells; LM, laminin; IV COLL, type IV collagen; FN, fibronectin; VN, vitronectin. –, no expression; +/–, minimal detection; +, weak; ++, moderate; +++, strong.

Synovial sarcoma (SS)

The integrin chains were distributed according to the different histological patterns of SS. In biphasic tumours, the laminin and type IV collagen receptors were strongly and uniformly expressed in the epithelial component. α_2 was moderately to strongly expressed in the epithelial areas, whereas almost no α_2 expression was seen in the spindle cell component (Figure 4), and α_5 was only expressed in the spindle cell component. The ECM proteins also showed a similar differentiation-dependent distribution. In epithelial areas, a low expression of both laminin and type IV collagen was seen in contrast to the spindle cell component, in which a moderate expression of fibronectin was observed.

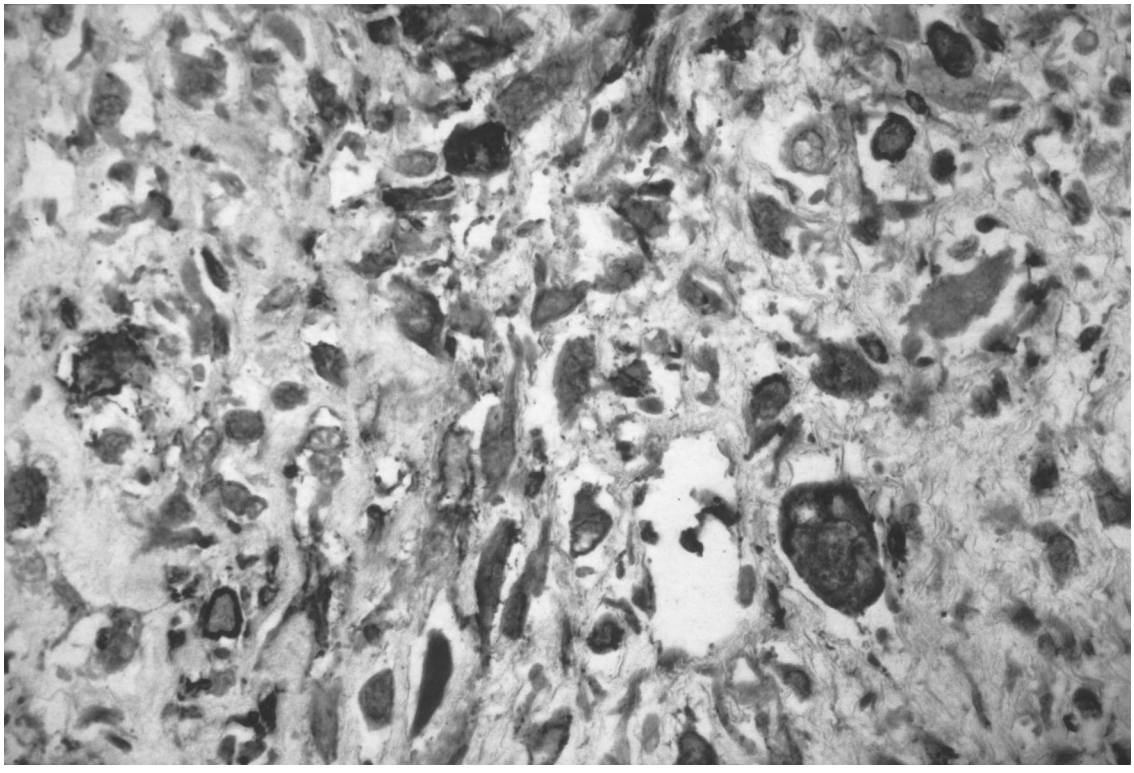

Figure 1. In malignant fibrous histiocytoma the β_3 integrin subunit is expressed by mononuclear and giant cells (×400).

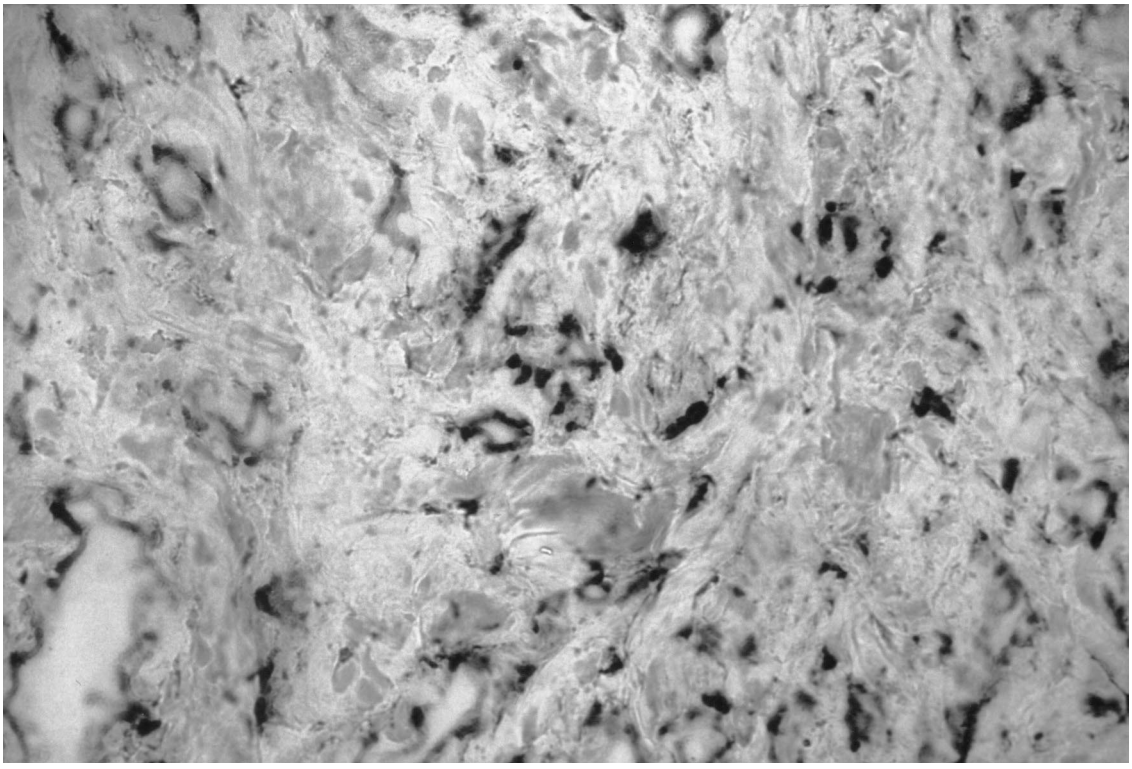

Figure 2. In malignant peripheral nerve sheath tumour (grade IV), α_2 is expressed only by scattered tumour cells (×400).

When comparing the integrin expression and proliferation index, a negative and statistically significant correlation was found between α_5 integrin expression and Ki67-positive cells ($r = -0.88$, $P < 0.001$) (Figure 5).

Integrin expression and clinical outcome

In order to define the relationship between $\alpha_6\beta_1$ integrin/LR expression and incidence of metastasis, the 58 patients were divided into two groups using a cut-off of 35% of α_6-positive cells, which corresponded to the median value of all samples examined, irrespective of tumour type. The first group included 27 cases (7 MFH, 9 SS and 11 MPNST) with less than 35% α_6-positive cells, the second group included 31 cases (13 MFH, 12 SS and 6 MPNST) with 35% α_6-positive cells or more. 33 of the 58 patients developed metastases, while the other 25 were disease-free. 27 (82%) of the 33 patients who developed metastases had $\geq 35\%$ α_6-positive cells. Only 4 (16%) of the 25 non-metastatic patients had more than 35% α_6-positive cells. A significantly different disease-free survival (DFS) estimate was observed between the two groups, as DFS was 77% for the patients with $< 35\%$ α_6-positive cells and 13% for the patients with $\geq 35\%$ α_6-positive cells (chi-square = 22.2; $P < 0.001$). In most cases, metastases occurred within the first 30 months from the histological diagnosis. A significant decrease in time from diagnosis to metastasis ($P < 0.001$) was found in the group with a percentage of α_6-positive cells $\geq 35\%$ (Figure 6). Within each type of tumour considered, a significantly higher DFS was found in the patients with less than 35% α_6-positive cells compared with those with 35% or more (Table 3). Also, when random cut-off points were used, the trend in DFS was significantly different (Table 4).

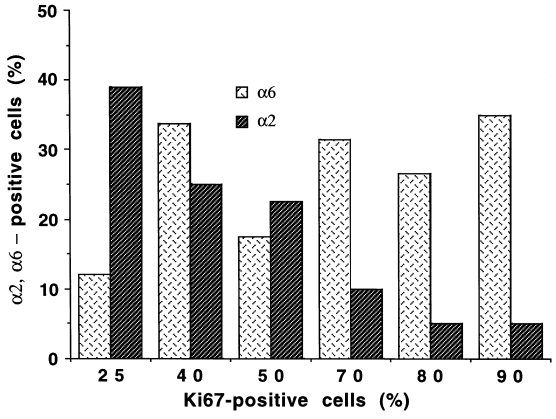

Figure 3. The correlation between the number of tumour cells expressing α_2 or α_6 and the proliferation rate measured by Ki67 expression, in malignant schwannoma.

The overall survival estimate was 89% for the group of patients with $< 35\%$ α_6-positive cells and 74% for those with $\geq 35\%$, but the difference was not statistically significant. No significant correlations were found between the other integrin receptors and the occurrence of metastasis.

DISCUSSION

Although high-grade soft tissue sarcomas are a heterogeneous group of tumours, they share several common features. Most tumours form ECM and are composed of spindle cells. Many of these high-grade tumours can have very similar morphology and, therefore, the differential diagnosis can be uncertain. Surgery is usually the treatment of choice, but less than 50% of patients with high-grade sarcomas have a 5-year survival. Adjuvant therapies, such as pre- and postoperative chemotherapy, have dramatically improved prognosis in osteosarcoma and Ewing's sarcoma, but conflicting results

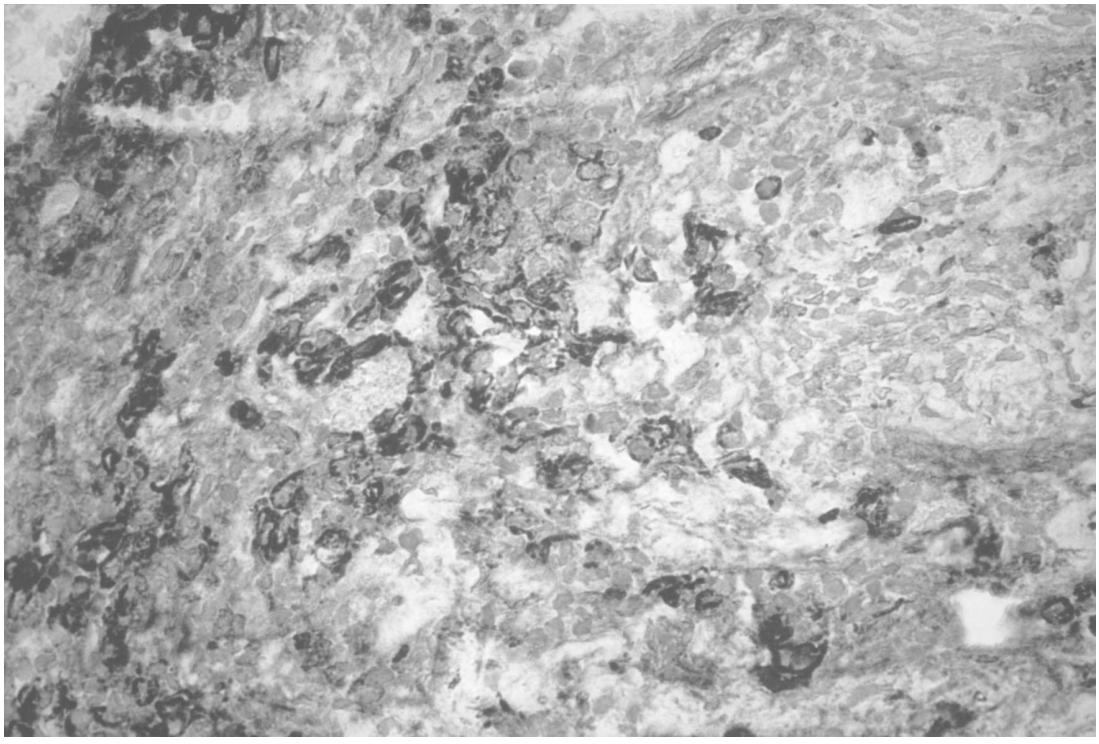

Figure 4. In synovial sarcoma, a moderate to strong α_2 expression is seen in the epithelial component, whereas only a focal and weak expression is seen in the spindle cell component (×400).

have been reported for other sarcoma types. To develop new treatment strategies, it is of utmost importance to understand tumour biology, and in particular those factors influencing growth and spread, such as the ability of tumour cells to interact with ECM.

The present study showed that in high-grade soft tissue sarcomas, ECM components are irregularly expressed and distributed, confirming disorganisation of extracellular matrix reported in other malignant tumours [15]. A certain tumour-specific expression pattern was noted, as type IV collagen was only expressed by MPNST and the epithelial component of SS. However, in MPNST a clear downregulation of type IV collagen and laminin expression was also seen in comparison with the non-neoplastic nerve tissue. Fibronectin expression was weak and focal in both MPNST and MFH, whereas in SS the spindle cell component showed a moderate, but irregular expression. It has been previously reported that down-regulation of fibronectin is associated with an increased aggressiveness of tumour cells [16], and the finding that in sarcomas fibronectin expression was decreased is in accordance with this.

Alterations in integrin expression and regulation cause loss of adhesion mechanisms and of signal transduction from cells to ECM and vice versa [17]. Integrin distribution has been studied in several tumour types. In renal cell carcinomas, the expression pattern is dependent on the histological grade [18] and in neoplastic breast lesions, α_v and α_6 integrin subunit expression is associated with the more malignant histological types [19]. The present study shows that sarcomas have a certain tumour type-specific and differentiation-specific expression pattern. In MFH and in the spindle cells of SS, a moderate α_5 expression was seen, whereas in MPNST, almost no expression was detected. In contrast, α_2 was

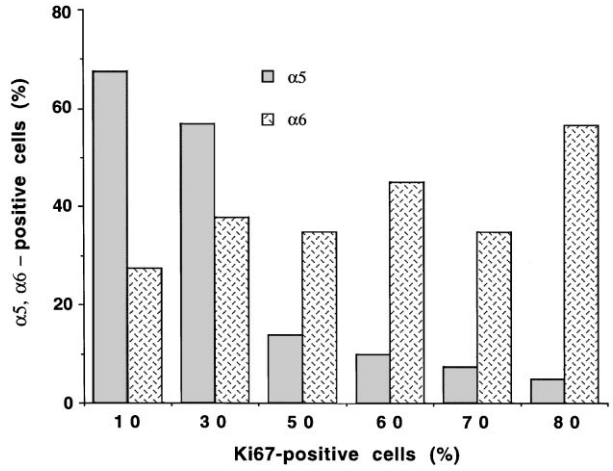

Figure 5. The percentage of tumour cells expressing α_6 or α_5 compared with the Ki67 values in synovial sarcoma.

Table 3. *Relationship between laminin receptor (LR) cell positivity and clinical behaviour in different tumour types*

	LR cell positivity < 35%		LR cell positivity > 35%		
	Relapsed *n* (%)	Disease-free *n* (%)	Relapsed *n* (%)	Disease-free *n* (%)	*P*⋆
MPNST	3/9 (33)	8/8 (100)	6/9 (67)	/	0.009
MFH	2/13 (15)	5/7 (71)	11/13 (85)	2/7 (29)	0.022
SS	1/11 (9)	8/10 (80)	10/11 (91)	2/10 (20)	0.002

⋆Fisher's Exact test. LR, laminin receptor; MPNST, malignant peripheral nerve sheath tumour; MFH, malignant fibrous histiocytoma; SS, synovial sarcoma.

Table 4. Disease-free survival in relation to laminin receptor (LR) cell positivity

	LR cell positivity < 50% *n* (%)	LR cell positivity > 50% *n* (%)	LR cell positivity < 20% *n* (%)	LR cell positivity > 20% *n* (%)
Relapsed	17 (41)	16 (94)	4 (25)	29 (69)
Disease-free	24 (59)	1 (6)	12 (75)	13 (31)
Total	41	17	16	42
	11.52		7.46	
*P**	< 0.001		< 0.01	

*Fisher's Exact test. LR, laminin receptor.

expressed in MPNST and in the epithelial areas of SS, but was hardly seen in the spindle cell component of SS and not detected in MFH. α_5 and α_2 could thus be useful in the differential diagnosis of sarcomas, α_2 expression ruling out MFH and α_5 expression ruling out MPNST. In SS, a different pattern of integrins was seen according to the different histological components. This is in accordance with previous studies that have shown that the integrin pattern, being a cell lineage marker rather than a marker differentiating benign and malignant cells, may be useful in recognising heterogeneous cell subpopulations in tumours [12].

The β_3 and α_v integrins form the vitronectin receptor and overexpression of this receptor seems to be associated with a more invasive behaviour of tumours [20]. The β_3 integrin, considered a marker of macrophages [21], showed a strong expression in most of the giant cells of MFH which lack α_v expression. In contrast, the mononuclear cells of MFH expressed both vitronectin receptor chains. This might indicate diverging differentiation of giant cells and mononuclear cells in MFH. Those giant cells of MFH that show strong expression of β_3 were probably only reactive, non-neoplastic cells or osteoclast-like giant cells.

The fraction of proliferating cells in a tumour, evaluated by the immunohistochemical detection of Ki67 antigen, is often associated with histological grade and prognosis [22]. The Ki67 index is usually low in well-differentiated low-grade tumours and high in poorly-differentiated high-grade tumours [23, 24]. In MPNST, when comparing the Ki67 index with integrin expression, an inverse correlation between α_2 expression and the percentage of proliferating cells was seen, suggesting that a downregulation of type IV collagen receptor is related to cell transformation and proliferation. In SS, the decrease of α_5 expression was inversely proportional to the increase of the Ki67 index in the spindle cell component. The α_5 chain forms a complex with the β_1 subunit which recognises the arginine, glycine, and aspartic acid sequence in fibronectin [25] and participates in matrix formation [26]. It has been shown that malignant transformation reduces $\alpha_5\beta_1$ receptors and the consequent matrix loss contributes to the migratory and invasive properties of the cells [27]. This study suggests that this mechanism is also of importance in soft tissue sarcomas.

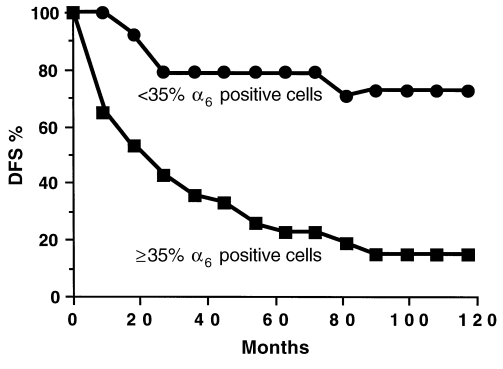

Figure 6. The probability of disease-free survival at different time intervals in the two groups, those with < 35% and those with ≥ 35% laminin receptor expressing tumour cells.

The possible prognostic role of LR expression in relation to the disease-free interval and overall survival is debated. Although some authors claim that cells with high metastatic capacity bind less to laminin [28] and patients with tumours expressing the LR have a lower risk of metastasis [29], other studies have demonstrated that LR expression is associated with a high metastatic potential [30, 31]. Our study strongly supports the latter view, as it is shown that, at least in soft tissue sarcomas, LR expression is an important predictor for the risk of metastasis. Patients with a lower LR expression had a lower incidence of metastasis compared with those with a high expression. The difference of the DFS estimate was also statistically significant within each tumour type. Moreover, the detection of a small number of LR-positive cells within the tumour was associated with a longer interval to metastasis and a decreased rate of metastasis. Although high LR expression was a predictor of metastases, the LR expression level did not predict overall survival. This may be explained by the fact that all metastases were located in the lung, and patients underwent metastectomy. Therefore, the occurrence of metastases did not influence survival during follow-up. To detect differences in overall survival, a longer follow-up is needed.

In conclusion, the tumour-specific distribution pattern of ECM components and integrins seen in sarcomas may be useful in the diagnosis of these tumours. The irregular distribution of ECM molecules and the altered regulation of integrin subunits are related to cell proliferation and aggressive behaviour of the sarcomas studied. In particular the α_6 chain of the LR was positively correlated with metastasis and therefore can be a useful marker for tumour aggressiveness.

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