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Altered expression of urokinase-type plasminogen activator and plasminogen activator inhibitor in high-risk soft tissue sarcomas

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Summary. In recent years, classification of soft-tissue sarcomas (STS) has improved with cytogenetic analyses, but their clinical behavior is still not easily predictable.

The aim of this study was to detect alterations in the urokinase-type plasminogen system, involved in tumor growth and invasion, by comparing mRNA levels of its components with those of paired normal tissues, and relating them with patient clinical course. Real-time PCR was performed on human STS cell lines and tissues from highly malignant STS, including leiomyosarcomas and malignant fibrous histiocytomas, to evaluate the expression of urokinase-type plasminogen activator (uPA), uPA receptor (uPAR) and plasminogen activator inhibitor-1 (PAI-1). Immunohistochemistry of gene products was also performed.

Median mRNA values of all genes studied were higher in tumors than in paired normal tissues. In agreement with data on STS cell lines, significant up-regulation for uPA and PAI-1 genes compared to reference values was seen. Moreover, different levels of expression were related to histotype and metastatic phenotype. There was accordance between uPA mRNA and protein expression, while immunodetection of PAI-1 product was weak and scattered.

Clearly, the controversial role of PAI-1 protein requires further biological analyses, but evident involvement of uPA/PAI-1 gene overexpression in STS malignancy may highlight a molecular defect useful in discriminating STS high-risk patients.

Key words: Soft-tissue sarcomas, mRNA expression, uPA system, Immunohistochemistry

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Introduction

Soft tissue sarcomas (STS) are a heterogeneous group of tumors with features so similar that differential diagnosis is often uncertain. Recently, cytogenetic analyses have improved their classification, but histotype-specific behavior is not yet predictable (Dirix and van Oosterom, 2002). STS represent <1% of cancers in adults and 15% to 20% are poorly differentiated with aggressive and metastatic clinical course. Malignant fibrous histiocytoma (MFH) has an uncertain origin and is highly aggressive with a metastatic incidence ranging from 30% al 50% depending on histological subtype (Campanacci, 1999). Nowadays, the generally accepted view is that MFH is a diagnosis of exclusion for undifferentiated pleomorphic sarcomas. Leiomyosarcoma (LMS) has the phenotypic features of smooth-muscle differentiation and originates, in most cases, from vessel walls. Histologically, it is characterized by a proliferation of spindle cells, with elongated nuclei and eosinophilic cytoplasm. Pleomorphic appearance with highly anaplastic round cells is common; survival is approximately 30% (Campanacci, 1999). Genetically, they belong to the complex karyotype tumors with genomic instability, lack of specific alterations and high frequency of mutations in genes involved in p53 and RB pathways (Helman and Meltzer, 2003). New cytogenetic and molecular genetic techniques have led to a biological characterization of STS subtypes useful for diagnosis and prognosis, limiting the use of the term "MFH" to undifferentiated pleomorphic sarcoma (Fletcher et al., 2002).

Surgery is usually the treatment of choice for patients with high-grade STS, but less than 50% survive five-years. An Italian randomized trial revealed a positive impact of intensified adjuvant chemotherapy on disease-free survival (DFS) and overall survival (OS) of patients with high-risk extremity STS (Frustaci et al.,

2001). The investigation of biological factors that influence growth and spread of tumor cells might help in the selection of high-risk patients to whom adequate therapies should be administered. Tumor growth and metastasis involve molecular interactions between tumor cells and surrounding normal tissue. Several steps are involved in these processes, but degradation of extracellular matrix is an essential pre-requisite for growth of primary tumors, metastatic spread and neoangiogenesis (Mignatti and Rifkin, 1993). In particular, among the proteases involved, the urokinase-type plasminogen activation system has been described as frequently implicated in the process of degradation of extracellular matrix during tumor cell proliferation and metastasis (Stetler-Stevenson et al, 1996; Duffy, 2004).

This system includes the urokinase-type plasminogen activator (uPA), its receptor (uPAR) and its inhibitor, plasminogen activator inhibitor type 1 (PAI-1). Binding of uPA to uPAR activates protease and catalyzes conversion of plasminogen to plasmin, which subsequently activates type IV collagenase, or directly degrades extracellular matrix proteins such as fibrin, laminins and proteoglycans). Proteolytic activity of both uPA and uPAR is regulated by PAI-1 that is able to react with uPAR-bound uPA (Andreasen et al., 2000; Ploug, 2003; Gils and Declerck, 2004).

Deregulation of uPA system enzymatic activity has been associated with poor prognosis in many human tumors (Memarzadeh et al., 2002; Seetoo et al., 2003; Gamberi et al., 2004). In order to associate specific molecular defects with histological and clinical aspects, we analyzed uPA proteolytic system in STS cell lines and in a selected series of tissues from patients with high-grade MFH and LMS. Positive involvement of uPA system in poorly differentiated STS determines a strong biological connection between two histologically different tumors, leading to important clinical implications.

Materials and methods

Cell lines

MFH cells lines, NTI-MFH-4 from primary tumor and NTI-MFH-2 from lung metastasis as well as LMS cell lines, NTI-LMS-8 from primary tumor and NTI-LMS-12 from lung metastasis were previously established and well characterized at National Cancer Institute, CRO-IRCCS, Aviano, Italy.

Cells were routinely cultured in Iscove's modified Dulbecco's medium, supplemented with 100U/ml penicillin, 100μg/ml stretomycin (Life Technology, Paisley, Scotland, UK) and 10% inactivated fetal calf serum (FCS) (Sigma-Aldrich, St. Louis, Missouri, USA).

Tumor specimens

Primary tumor samples from 38 high-grade STS, including 16 MFH and 22 LMS (Table 1) referred at the Rizzoli Orthopaedic Institute were considered. For each sample both frozen, paraffin-embedded material and paired normal tissue were available. In all specimens the percentage of tumor cells estimated after hematoxylin-eosin staining of tissue sections adjacent to those used for the study was equal or more than 90%, with a high tumor/stroma cell ratio. All tumors were diagnosed at the Rizzoli Institute by a group of expert pathologists. Diagnoses were based on hematoxylin-eosin stained samples according to WHO classification. MFH is a diagnosis of exclusion, thus this subgroup included poorly differentiated pleomorphic sarcomas. For differential diagnosis of LMS, immunohistochemical analysis with desmin, smooth muscle actin, muscle specific actin was performed. Selection criteria were primary tumors deeply localized with the major diameter greater than 5 cm, high-grade according to the Fédération Nationale des Centres de Lutte contre le Cancer (FNCLCC), spindle cell or pleomorphic sarcomas, no local relapse and no chemotherapy. Only 7/38 patients were treated with neoadjuvant radiotherapy and the majority of resections presented adequate margins (radical/wide).

Patients

Selected patients (22 males and 16 females) with adequate histological material and complete clinical data were included. Age ranged from 34 to 79 years with a median of 60 years. Clinical follow-up was evaluated considering time to metastasis, metasisis-free survival (MFS) and OS. MFS and OS were calculated from the date of diagnosis to the metastatic event or the last day of follow-up. 10 patients were metastasis-free and 11 out of 28 presented metastases at diagnosis (time to metastasis designed as 0). Follow-up ranged from 0 to 102 months, with a median of 9.5 months. Minimal follow-up for metastasis-free patients was 3 years (Table 1).

RNA extraction and reverse transcription

Total RNA was extracted from cells (≈ 3.000.000), normal and tumor frozen tissue (≈ 150mg) and from peripheral blood lymphocytes using TRIzol Reagent (Invitrogen, Carlsbad CA) and stored at -80°C in RNA secure reagent (Ambion, Inc, Austin TX). Concentration of total RNA was measured with spectrophotometer and the A260/A280 ratio of RNA was ≈ 1.6. Purity and quality were identified by gel electrophoresis.

Reverse transcription of mRNA was carried out in 100μl final volume from 400ng total RNA using High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA) according to manufacturer's instructions.

Gene expression analysis by real-time and evaluation of PCR results

Quantitative PCR reaction was performed on cDNA, by ABI PRISM 7900 Sequence Detector (PE Applied

Biosystems, Foster City CA, USA), with TaqMan technology (Orlando et al.,1998; Lehmann et al., 2000). Expression of target genes, uPA, uPAR and PAI-1 was quantified using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) according to manufacturer's protocol (Table 2a).

PCR mixture contained 1.25 ml Target or Endogenous Reference Assay Mix 20X, 22.5 ng DNA diluted in 11.25 ml of distillate water, 12.5 ml TaqMan Universal Master Mix 2X (Applied Biosystems, Foster City, CA) in a 25 ml final reaction volume. Following activation of UNG (Uracil-N-Glycosylase) for 2 min at 50°C and of AmpliTaq Gold DNA polymerase for 10 min at 95°C all genes were amplified by 45 cycles for 15 seconds at 95°C and for 1 min at 60°C.

For each sample, cDNA was analyzed in duplicate

A negative control for each gene expression assay was included in every run in order to exclude false-positive results.

For calculation of gene expression we used $2^{-\Delta\Delta CT}$ comparative method (Winer et al., 1999). The amount of target was normalized to an endogenous reference (GAPDH) and relative to a calibrator (cDNA from lymphocytes).

Each gene was considered up-regulated when the value was $> 1 \pm$ SD and under-expressed when the value was $< 1 \pm$ SD. Value 1 corresponds to fluorescence emission in each amplification reaction for target and reference in pool of lymphocytes.

Standard deviation of each $2^{-\Delta\Delta ct}$ value are less than 0.2, according to protocol required for data reliability.

Immunohistochemistry (IHC)

IHC was chosen to test uPA, uPAR and PAI-1 protein expression distribution to verify the percentage of positive tumor cells. 5 micron sections from formalin-fixed, paraffin-embedded material were deparaffinized,

Table 1. Clinical table.

	Sex	Age	Tumor	Site	M/NM	Follow-up months	Outcome	Neoadjuvant terapy	Size (cm)	Resection margins
1	M	72	MFH storiform pleomorphic	thigh	M	98	DOD		9.0	Wide
2	M	54	MFH storiform pleomorphic	leg	NM	77	NED		5.5	Wide
3	M	63	MFH storiform pleomorphic	thigh	NM	95	NED		6.0	Wide
4	M	61	MFH storiform pleomorphic	shoulder blade	M	37	AWD		14.0	Wide
5	F	73	MFH storiform pleomorphic	thigh	M	5	AWD		5.5	Wide
6	F	54	MFH storiform pleomorphic	thigh	M	84	DOD		6.5	wide
7	M	64	MFH storiform pleomorphic	thigh	M	9	DOD		20.0	Marginal
8	M	62	MFH storiform pleomorphic	arm	NM	55	NED		14.5	Wide
9	M	38	MFH storiform pleomorphic	thigh	NM	47	NED		5.5	Wide
10	F	43	MFH storiform pleomorphic	thigh	NM	48	NED		20.0	Wide
11	F	67	MFH storiform pleomorphic	pelvic girdle	NM	7	DOD		18.0	Radical
12	F	72	MFH storiform pleomorphic	thigh	M	26	DOD		6.0	Radical
13	F	76	MFH storiform pleomorphic	thigh	M	0	DOD		18.0	Wide
14	M	38	MFH storiform pleomorphic	thigh	M	0	DOD		36.0	Marginal
15	M	62	MFH storiform pleomorphic	pelvic girdle	M	10	NED		24.0	Radical
16	F	66	MFH storiform pleomorphic	thigh	M	25	DOD		20.0	Radical
17	M	71	LMS conventional	thigh	M	0	DOD	radioterapy	15.0	Wide
18	M	57	LMS pleomorphic	forearm	M	0	AWD		6.0	Wide
19	M	63	LMS pleomorphic	arm	M	5	DOD		11.0	Wide
20	F	76	LMS pleomorphic	thigh	M	0	DOD		16.0	Marginal
21	M	34	LMS pleomorphic	thigh	M	0	DOD	radioterapy	10.0	Wide
22	M	62	LMS conventional	thigh	M	0	DOD		10.0	Wide
23	M	66	LMS conventional	forearm	M	0	AWD	radioterapy	10.0	Wide
24	M	34	LMS pleomorphic	spine	M	0	DOD	radioterapy	14.0	Wide
25	M	55	LMS conventional	thigh	M	2	DOD	radioterapy	22.0	Wide
26	M	78	LMS conventional	leg	NM	67	NED		18.0	Wide
27	M	38	LMS pleomorphic	leg	NM	102	NED	radioterapy	66.0	radical
28	F	61	LMS pleomorphic	thigh	M	17	AWD		8.0	Wide
29	M	43	LMS pleomorphic	thigh	M	63	AWD		19.0	Wide
30	M	52	LMS pleomorphic	thigh	M	5	DOD		6.5	Wide
31	F	79	LMS conventional	thigh	NM	80	DOD		16.0	Wide
32	F	57	LMS conventional	thigh	M	0	DOD		5.5	Wide
33	M	76	LMS pleomorphic	thigh	M	12	DOD		5.5	Wide
34	F	66	LMS pleomorphic	leg	NM	15	DOD		16.0	Wide
35	F	68	LMS pleomorphic	thigh	M	24	DOD		9.0	Wide
36	F	74	LMS conventional	thigh	M	0	DOD		20.0	Wide
37	F	42	LMS conventional	thigh	M	5	DOD	radioterapy	23.0	Wide
38	F	71	LMS pleomorphic	leg	M	7	AWD		10.5	Wide

M: metastatic tumors; NM: non-metastatic tumors; DOD: died of disease; AWD: alive with disease; NED: no evidence of disease.

rehydratated and immunostained with monoclonal antibodies (Table 2b). Expression level of all studied proteins was assessed with avidin-biotin-peroxidase complex method (BIOMEDA, Foster City CA). Negative controls were performed by omitting the primary antibody. Human breast carcinoma was used as positive control. Positivity of IHC reaction was scored negative or minimal (less than 10% of positive cells), weak (10%-25%), moderate (26%-50%), strong (>51%).

Statistical analysis

All biological data were shown as median (m) and 25^{th}-75^{th} percentile for their strong non-Gaussian distribution. Non parametric Mann Whitney U-test and Wilcoxon analysis were performed to compare gene expression in unpaired and paired samples respectively; p values < 0.05 were considered to be statistically significant. MFS and OS were calculated by Kaplan-Meier analysis and comparison of curves was performed by Breslow's test.

Results

Gene expression in STS cell lines

uPA and PAI-1 genes were up-regulated with regard to the calibrator, defined as 1, in all cell lines. However, their behavior showed an opposite trend in relation to cell phenotype. uPA $2^{-\Delta\Delta ct}$ values were higher in cell lines from metastases than in primary tumors (Table 3). Increased uPA expression in metastasis was 2.3-fold for LMS cells and 1.6-fold for MFH. On the contrary, PAI-1 mRNA levels were lower in metastatic cells of 31% and 6.6% respectively compared to the primary tumor cells. Conversely, uPAR gene was under-expressed in all cell lines, with slightly increased mRNA levels only in LMS cells from lung metastasis.

Patient follow-up

Survival curves of 38 patients showed 26.3% MFS rate and 39.4.% OS rate

A significant difference was seen between the two histological subtypes, MFH and LMS, in terms of MFS (37.5% and 18.1% respectively, p=0.01), while Breslow's test was not significant for OS (50% and 32% respectively, p=0.06) (Fig. 1a,b). Nine out of 11 cases with metastases at diagnosis were LMS and in both subtypes the majority of adverse events occurred within the third year.

Gene expression in STS tissues

uPA and uPAR gene expression

All tumors showed high expression of uPA gene compared to calibrator, with $2^{-\Delta\Delta ct}$ values ranging from 8.85 to 7595.1, while uPAR mRNA was more than the

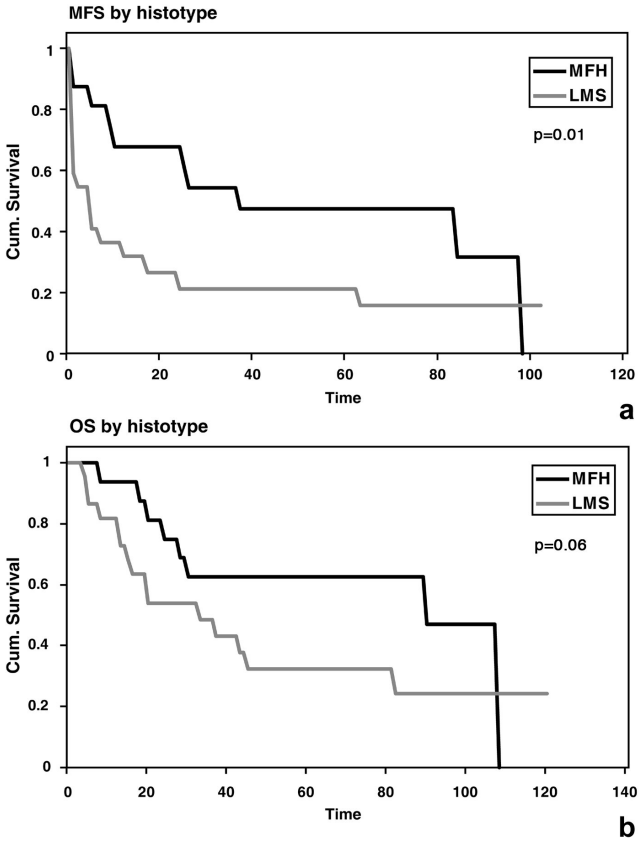

Fig. 1. MFS **(a)** and OS **(b)** curves according to histotype. Comparison of survival curves was performed by Breslow's test.

Table 2.a. TaqMan Gene Expression Assays®.

GAPDH	Assay ID: Hs99999905_m1
uPA	Assay ID: Hs00170182_m1
uPAR	Assay ID: Hs00182181_m1
PAI-1	Assay ID: Hs00167155_m1

Table 2.b. Antibodies used in the study.

uPA	Anti-uPA,(C-20): sc-6830, Santa Cruz Biotechnology: dil 1:40
uPAR	Anti-uPAR,(CD 87): 3936, D.B.A. dil 1:10
PAI-1	Anti-PAI-1,(M-20): sc-6644, Santa Cruz Biotechnology: dil 1:50

calibrator only in 16/38 tumor samples (from 0.01 to 26.0). Moreover, mRNA median values of both uPA and uPAR were significantly higher in tumors than in normal tissue (m=331, 25^{th}-75^{th}=108-958 vs m=23, 25^{th} - 75^{th}=4-62; p=0.0005 for uPA and m=0.9, 25^{th}-75^{th}=0.4-2.0 vs m=0.02, 25^{th}-75^{th}=0.01-0.10; p=0.0005 for uPAR) (Fig. 2a,b), with a 13- and 16-fold median increase respectively (Table 4). In addition, subdividing the 38 STS into histotypes, LMS showed a stronger expression than MFH for both genes (p=0.003) (Table 5).

Although there was no association between mRNA levels and time to metastases, uPA expression was slightly higher in metastatic than in non-metastatic tumors (1010±310 vs 801±511) (Fig. 3), while no differences were seen for uPAR gene expression (3.9±1.2 vs 3.1±2.5 respectively). In accordance with mRNA data, a moderate to strong immunoreactivity for uPA protein (Fig. 4) was seen in the most of the tumor samples consistent with high mRNA expression of the correspondent gene. Otherwise, only 7 STS moderately immunoreacted to uPAR protein in less than 50% of tumor cells (Table 6). The scarce stromal cells present in the samples had a minimal positivity for both proteins.

PAI-1 gene expression

Concerning PAI-1, tumor $2^{-\Delta\Delta ct}$ values ranged from 4.4 to 4240.4, with a significant up-regulation compared to calibrator. Moreover, the marked difference in gene expression between tumor and normal tissue (respectively m=109, 25^{th}-75^{th}=25-243 and m=1.3, 25^{th}-75^{th}=0.2-4.5, p= 0.0005) (Fig 2c) was consistent with the median increase of 58-fold in tumor (Table 4). According to histotypes and clinical follow-up, LMS had

Table 3. $2^{-\Delta\Delta CT}$ values in STS cell lines.

CELL LINES	uPA	uPAR	PAI-1
Primary NTI-MFH- 4	25.10±0.01	0.18±0.04	15128.7±0.32
Lung meta NTI-MFH -2	66.72±0.15	0.1±0.02	1013.41±0.05
Primary NTI-LMS-12	133.89±0.18	0.27±0.00	8051.26±0.20
Lung meta NTI-LMS-8	442.64±0.03	1.11±0.08	2521.38±0.17

Endogenous reference gene: GAPDH; Calibrator: healthy lymphocyte pool ($2^{-\Delta\Delta CT}$ =1).

Table 4. Distribution of increased gene expression in STS samples.

	Median	25^{th}	75^{th}
uPA	13.5 - fold	2.9	38.6
uPAR	16.4 - fold	5.8	45.4
PAI-1	58.7 - fold	19.1	302

Data are expressed as n-fold difference relative to normal tissue.

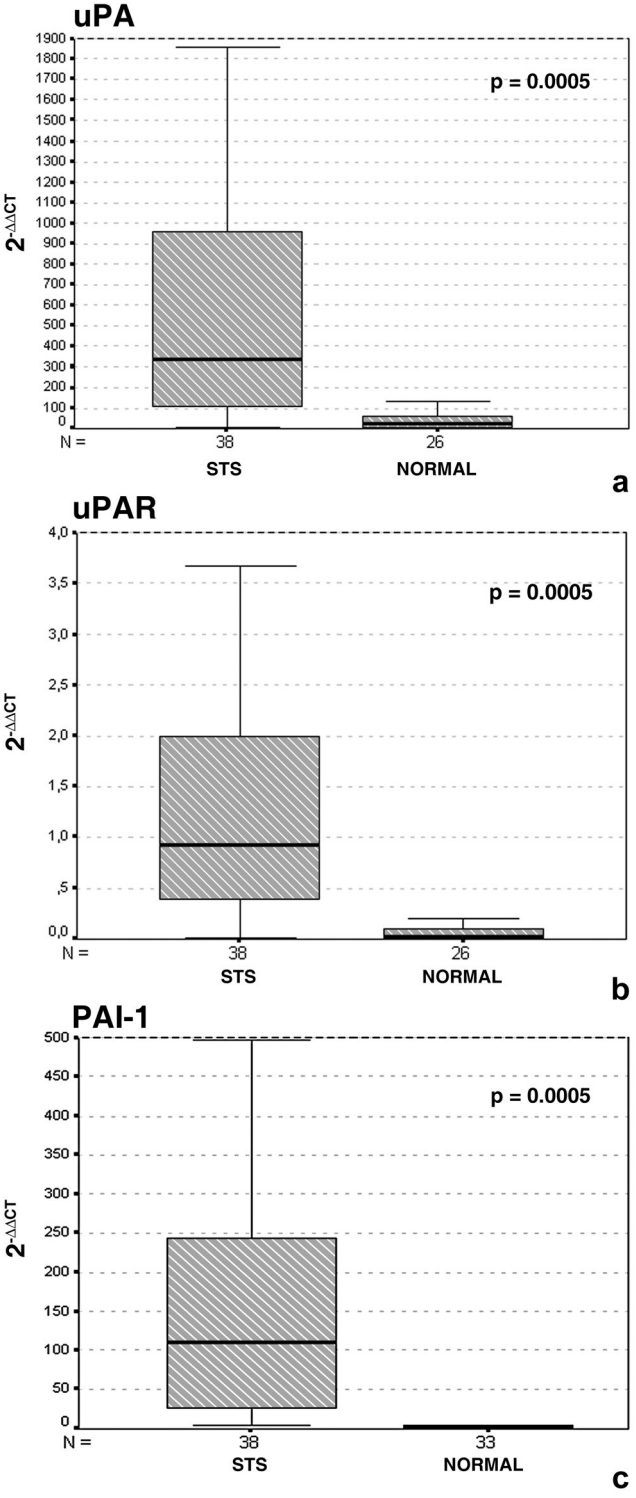

Fig. 2. Non-Gaussian distribution of gene expression **(a, b, c)** in tumor tissue and paired normal tissue. In STS mRNA values are distributed in a larger range and towards significantly higher levels than in normal tissue.

a slightly higher dispersion of mRNA values compared to MFH (Table 5), and PAI-1 mRNA expression was 6-fold higher in metastatic compared to non-metastatic tumors (669.3 ± 231vs 93.5±32.9) (Fig. 3). At the protein level, weak and scattered PAI-1 immunoreactivity was seen in all samples both in tumor and stromal cells (Table 6).

Conclusions

STS consist of numerous histotypes with few biological predictive parameters, and prognosis is determined by pathological and clinical aspects such as size, location, grading, necrosis, and surgical margins. Thus, detection of molecular markers is necessary to understand biological aggressiveness and associates molecular defects to specific histological subtypes, useful in defining target therapies (Borden et al., 2003). Some authors suggested a new method for STS classification based on transcriptional profile and indicated that a large number of genes may contribute to differentiate these tumors (Nielsen et al., 2002). However, the same study underlined absence of a clear distinction between MFH and a peculiar group of LMS not expressing genes involved in muscle structure and function.

Table 5. $2^{-\Delta\Delta ct}$ median values in STS histotypes.

	MFH			LMS			p*
	Median	25th	75th	Median	25th	75th	
uPA	210	39.6	367	601	246	1978	0.003
uPAR	0.71	0.1	0.9	1.7	0.6	12.7	0.003
PAI-1	109	21.9	139	109	25	1227	ns

* Mann Whitney test. Endogenous reference gene: GAPDH; Calibrator: healthy lymphocyte pool ($2^{-\Delta\Delta ct}$ =1).

Table 6. Immunohistochemistry distribution.

	Tumor	M/NM	uPA	uPAR	PAI-1
1	MFH storiform pleomorphic	M	+	-	+
2	MFH storiform pleomorphic	NM	++	-	+/-
3	MFH storiform pleomorphic	NM	++	-	+/-
4	MFH storiform pleomorphic	M	+++	++	+/-
5	MFH storiform pleomorphic	M	+++	-	+/-
6	MFH storiform pleomorphic	M	+	-	-
7	MFH storiform pleomorphic	M	++	-	+
8	MFH storiform pleomorphic	NM	++	-	+
9	MFH storiform pleomorphic	NM	++	+	+/-
10	MFH storiform pleomorphic	NM	+++	++	+/-
11	MFH storiform pleomorphic	NM	+++	++	-
12	MFH storiform pleomorphic	M	++	-	+/-
13	MFH storiform pleomorphic	M	++	++	-
14	MFH storiform pleomorphic	M	++	-	-
15	MFH storiform pleomorphic	M	++	-	-
16	MFH storiform pleomorphic	M	++	++	+/-
17	LMS conventional	M	++	++	+
18	LMS pleomorphic	M	++	-	-
19	LMS pleomorphic	M	+	+	+
20	LMS pleomorphic	M	++	-	+/-
21	LMS pleomorphic	M	+/-	-	+/-
22	LMS conventional	M	++	-	+/-
23	LMS conventional	M	+	-	+/-
24	LMS pleomorphic	M	++	-	+/-
25	LMS conventional	M	+	-	+
26	LMS conventional	NM	++	-	-
27	LMS pleomorphic	NM	+/-	-	-
28	LMS pleomorphic	M	++	-	+
29	LMS pleomorphic	M	++	-	+/-
30	LMS pleomorphic	M	+	-	+/-
31	LMS conventional	NM	++	-	+/-
32	LMS conventional	M	++	-	+/-
33	LMS pleomorphic	M	++	-	+/-
34	LMS pleomorphic	NM	+++	++	+
35	LMS pleomorphic	M	+++	+	+/-
36	LMS conventional	M	+++		-
37	LMS pleomorphic	M	++		+
38	LMS conventional	M	++		+/-

M: metastatic tumors; NM: non-metastatic tumors; (+/-) scattered; (+): weak; (++): moderate; (+++): strong

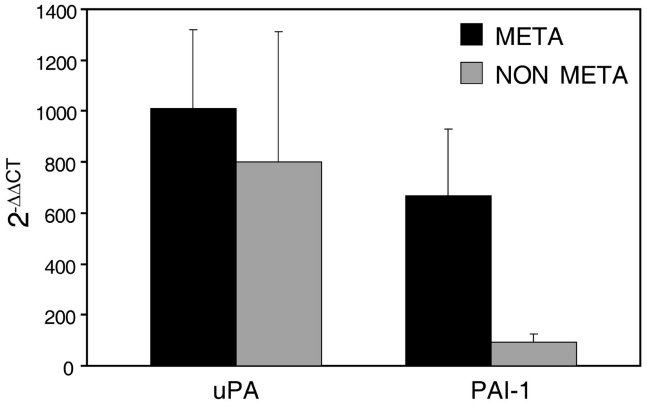

Fig. 3. uPA and PAI-1 mRNA mean values in metastatic and non-metastatic groups.

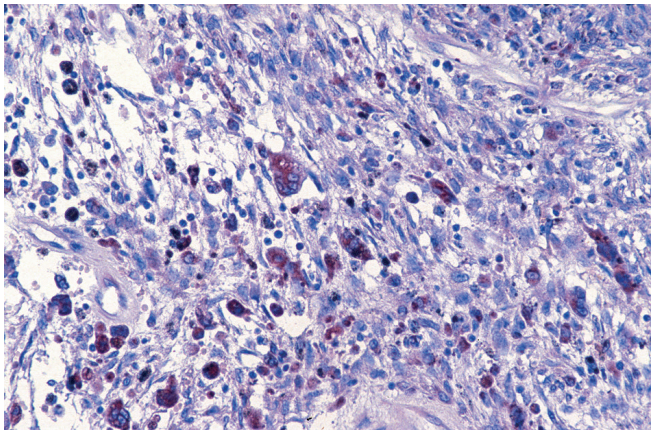

Fig. 4. Immunoreactivity of uPA protein in MFH (IHC, x 40).

To define multiple molecular aspects associated with an increased risk of metastases, we studied the expression of uPA proteolytic system in a selected series of highly malignant MFH and LMS. Our previous findings associated biological aggressiveness of human high-risk STS to deregulation in MMP proteolytic pathway (Benassi et al., 2003). Moreover, the occurrence of lung metastases in usually benign giant cell tumor (GCT) is associated with alterations of the uPA proteolityc system, thus defining biological markers in discriminating a more aggressive phenotype (Gamberi et al., 2003).

In the present study, uPAR was minimally expressed in STS cell lines and in the majority of tumor specimens, although median mRNA levels were higher than in paired normal tissues. These results lead to many biological considerations, but uPAR under-expression limits its clinical significance.

On the contrary, strong biological and clinical aggressiveness in our series of STS from patients with high-risk of metastases, appeared to be associated with increased expression of uPA and PAI-1 mRNA compared to both calibrator and normal paired tissue. Our studies on STS cell lines confirmed up-regulation of both genes, with increased expression levels depending on histotype and metastatic phenotype (Table 3). In agreement with these data, in tumor tissues uPA expression was significantly higher in LMS with a metastasis rate of 81.8% than in MFH with a metastasis rate of 56%. In addition, subdividing the 38 STS according to follow-up, 25% increased mRNA values were found in metastatic rather than non-metastatic tumors, associated with high protein expression. A previous study supported the role of uPA as a prognostic indicator in a wide series of STS, relating protein levels to tumor characteristics and behavior (Choong et al., 1996). While uPA is a well-known prognostic marker in many tumors (Duffy et al., 1999), the role of PAI-1 in tumor growth and invasion is still under investigation. In the present study, the overexpression of PAI-1 gene in both STS cell lines and tumor tissues supports the evidence of neoplastic biological activity, depending on cell type and molecular interactions (Stefansson et al., 2003; Durand et al., 2004; Romer et al., 2005). In accordance with data that correlate PAI-1 levels with poor prognosis (Schmitt et al., 1997; Duffy, 2002), we found higher mRNA levels in primary tumors from patients who developed metastases during follow-up compared to tumors from metastasis-free patients, suggesting a relevant role of PAI-1 in the early phase of the metastatic process. However, the weak and scattered distribution of PAI-1 protein in tumor cells, emphasizes the importance of further integrated analyses to understand the biological effects on clinical outcome.

In conclusion, this study indicates that in highly malignant STS, MFH and LMS, overexpression of uPA and PAI-1 genes at mRNA level may have a potential biological value in discriminating high-risk patients, with important clinical implications for cancer progression and therapy.

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References

Andreasen P.A., Egelund R. and Petersen H.H. (2000). The plasminogen activation system in tumor growth, invasion and metastasis. Cell. Mol. Life Sci. 57, 25-40.

Benassi M.S, Magagnoli G., Ponticelli F., Pazzaglia L., Zanella L., Gamberi G., Ragazzini P., Ferrari C., Mercuri M. and Picci P. (2003). Tissue and serum loss of metalloproteinase inhibitors in high grade soft tissue sarcoma. Histol. Histopathol. 18, 1035-1040.

Borden E.C., Baker L.H., Bell R.S., Bramwell V., Demetri G.D., Eisenberg B.L., Fletcher C.D., Fletcher J.A., Ladany M., Meltzer P., O'Sullivan B., Parkinson D.R., Pisters P.W., Saxman S. (2003). Soft tissue sarcomas of adults: state of translational science. Clin. Cancer Res. 9, 1941-1956.

Campanacci M. (1999). Soft tissue tumors. In: Bone and soft tissue tumors. 2nd edn. Springer-Verlag. Wien, New York. pp 909-1199.

Choong P.F., Ferno M., Akerman M., Willen H., Langstrom E., Gustafson P., Alvegard T. and Rydholm A. (1996). Urokinase-plasminogen-activator levels and prognosis in 69 soft-tissue sarcomas. Int. J. Cancer 69, 268-272.

Dirix L.Y. and van Oosterom A.T. (2002). Gene-expression profiling to classify soft tissue sarcomas. Lancet 359, 1263-1264.

Duffy M.J. (2002). Urokinase plasminogen activator and its imhibitor PAI-I, as prognostic markers in breast cancer: from pilot to level I evidence studies. Clin. Chem. 48, 1194-1197.

Duffy M.J. (2004). The urokinase plasminogen activator system: role in malignancy. Curr. Pharm. Des. 10, 39-49.

Duffy M.J., Maguire T.M., McDermott E.W. and O'Higgins N.(1999). Urokinase plasminogene activator: a prognostic marker in multiple types of cancer. J. Surg. Oncol. 71, 130-135.

Durand M.K., Bodker J.S., Christensen A., Dupont D.M., Hansen M., Jensen J.K., Kjelgaard S., Mathiasen L., Pedersen K.E., Skeldal S., Wind T. and Andreasen P.A. (2004). Plasminogen activator inhibitor-I and tumour growth, invasion, and metastasis. Thromb. Haemost. 91, 438-449.

Fletcher C.D.M., Unni K.K. and Mertens F. (2002). So-called fibrohistocytic tumors and Smooth muscle tumors. In: WHO classification of Tumors: pathology and genetics of tumors of soft tissue and bone. Fletcher C.D.M., Unni K.K. and Mertens F. (eds). IARC Press. Lyon. pp 109-131.

Frustaci S., Gherlinzoni F., De Paoli A., Bonetti M., Azzarelli A., Comandone A., Olmi P., Buonadonna A., Pignatti G., Apice G., Zmerly A., Serraino D. and Picci P. (2001). Adjuvant chemotherapy for adult soft tissue sarcomas of the extremities and girdles: results of the Italian randomized cooperative trial. J. Clin. Oncol. 19, 1238-1247.

Gamberi G., Serra M., Ragazzini P., Magagnoli G., Pazzaglia L., Ponticelli F., Ferrari C., Zanasi M., Bertoni F., Picci P. and Benassi

M.S. (2003). Identification of markers of possible prognostic value in 57 giant cell tumors of bone. Oncol. Rep 10, 351-356.

Gamberi G., Benassi M.S., Ragazzini P., Pazzaglia L., Ponticelli F., Ferrari C., Balladelli A., Mercuri M., Gigli M., Bertoni F. and Picci P. (2004). Proteases and interleukin-6 gene analysis in 92 giant cell tumors of bone. Ann. Oncol. 15, 498-503.

Gils A. and Declerck P.J. (2004). Structure, function and pathophysiological relevance of plasminogen activator inhibitor-1. Thromb. Haemost. 91, 425-437.

Helman L.J. and Meltzer P. (2003). Mechanisms of sarcoma development. Nature Rev. 3,685-694.

Lehmann U., Glockner S., Kleeberger W., Feist H., von Wasielewski R. and Kreipe H. (2000). Detection of gene amplification in archivial breast cancer specimens by laser-assisted microdissection and quantitative Real-Time polymerase chain reaction. Am. J. Pathol. 156, 1855-1864.

Memarzadeh S., Kozak K.R., Chang L., Natarajan S., Shintaku P., Reddy S.T. and Farias-Eisner R. (2002). Urokinase plasminogen activator receptor: prognostic biomarker for endometrial cancer. Proc. Natl. Acad. Sci. USA 99, 10647-1052.

Mignatti P. and Rifkin D.B. (1993). Biology and biochemistry of proteinases in tumor invasion. Physiol. Rev. 73, 161-195.

Nielsen T.O., West R.B., Linn S.C., Alter O., Knowling M.A., O'connell J.X., Zhu S., Fero M., Sherlock G., Pollack J.R., Brown P.O., Botstein D. and van de Rijn M. (2002). Molecular characterisation of soft tissue tumours: a gene expression study. Lancet 359, 1301-1307.

Orlando C., Pinzani P. and Pazzagli M. (1998). Developments in quantitative PCR. Clin. Chem. Lab. Med. 36, 255-269.

Ploug M. (2003). Structure-function relationship in the interaction between the urokinase-type plasminogen activator and its receptor. Curr. Pharm. Des. 9, 1499-1528.

Romer M.U., Kirkebjerg Due A., Knud Larsen J., Hofland K.F., Christensen I.J., Buhl-Jensen P., Almholt K., Lerberg Nielsen O., Brunner N. and Lademann U. (2005). Indication of a role of plasminogen activator inhibitor type I in protecting murine fibrosarcoma cells against apoptosis. Thromb. Haemost. 94, 859-866.

Schmitt M., Harbeck N., Thomssen C., Wilhelm O., Magdolen V., Reuning U., Ulm K., Hofler H., Janicke F. and Graeff H. (1997). Clinical impact of the plasminogen activator system in tumor invasion and metastasis: prognostic relevance and target for therapy. Thromb. Haemost. 78, 285-296.

Seetoo D.Q., Crowe P.J., Russell P.J. and Yang J.L. (2003). Quantitative expression of protein markers of plasminogen activation system in prognosis of colorectal cancer. J. Surg. Oncol. 82,184-193.

Stefansson S., McMahon G.A., Petitclerc E. and Lawrence D.A. (2003). Plasminogen activator inhibitor-1 in tumor growth, angiogenesis and vascular remodeling. Curr. Pharm. Des. 9, 1545-1564.

Stetler-Stevenson W.G., Hewitt R. and Corcoram M. (1996), Matrix Metalloproteinases and tumor invasion: from correlation and casuality to the clinic. Sem. Cancer. Biol. 7, 147-154.

Winer J., Jung C.K., Shackel I. and Williams P.M. (1999). Development and validation of Real-Time quantitative reverse trascriptase-polymerase chain reactionfor monitoring gene expression in cardiac myocytes in vitro. Anal. Biochem. 270, 41-49.

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