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**Histology and Histopathology**

*From Cell Biology to Tissue Engineering*

# Prognostic role of *XTP1/DEPDC1B* and *SDP35/DEPDC1A* in high grade soft-tissue sarcomas

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**Summary.** Background. The outcome of patients with metastatic soft tissue sarcoma (STS) remains unfavourable and new therapeutic strategies are needed. The aim of this study was to determine the role of RhoGAP, *XTP1/DEPDC1B* and *SDP35/DEPDC1A,* as possible prognostic markers, to be used to identify candidate patients for more effective and personalized therapies.

Materials-Methods. *SDP35/DEPDC1A* and *XTP1/DEPDC1B* transcriptional levels were evaluated by Real-Time PCR in 86 primary STS and 22 paired lung metastasis. 17 normal tissues were used as control. Protein expression was evaluated by tissue microarray, including 152 paraffin-embedded STS samples and by western blot in 22 lung metastases and paired primary STS.

Non-parametric and parametric analysis were used to establish the differences in gene and protein expression and prognostic factors were tested with Kaplan Meier and Cox's regression analyses.

Results. *SDP35/DEPDC1A* and *XTP1/DEPDC1B* gene were down-regulated in adjacent normal tissues while sarcoma specimens presented high mRNA levels, significantly related to metastasis-free survival. Gene expression further increased in paired metastatic lesions. Immunohistochemical staining showed a variable expression in intensity and distribution, with a significantly higher probability of metastatic disease in patients up-regulating *SDP35/DEPDC1A*. Western blotting assessed high levels of proteins in STS specimens and indicated a stronger expression of SDP35/DEPDC1A in metastases when compared to primary tumours. Multivariate analyses highlighted that *SDP35/DEPDC1A* abundance, grade III and no history of radiation therapy were significant independent risk factors.

Conclusions. Our results demonstrated that increased expression of *SDP35/DEPDC1A* and *XPT1/DEPDC1B* correlates with metastatic progression and identified *SDP35/DEPDC1A* as an independent marker for prediction of poor prognosis.

**Key words:** Sarcoma, Metastasis, Gene and protein expression, Prognosis, Biomarkers

## Introduction

Soft tissue sarcomas (STS) are a heterogeneous group of mesenchymal tumours. Surgery combined with radiation and chemotherapy has increased the 5-year disease-free and overall survival in localized STS, while clinical outcome of patients with advanced disease remains strongly unfavourable. Thus, identification of novel biomarkers useful in recognizing more aggressive phenotypes and in devising new therapeutic strategies are necessary. Previous studies based upon differential gene profiling (Kanehira et al., 2007; Kretschmer et al., 2011; Obara et al., 2012) identified up-regulation of *XTP1/DEPDC1B*, belonging to Ras-homology (Rho) GTPases family (Peck et al., 2002; Moon and Zheng,

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2003) and DEP domain-containing proteins (also called *DEPDC1B* or *BRCC3*), in STS cell lines, when screened against their normal counterpart healthy cells (Gazziola et al., 2003). Other investigations identified the closest homologue *SDP35/DEPDC1A* (also called *DEPDC1*) to be up-regulated in certain carcinomas (Kanehira et al., 2007). This suggests that these unique RhoGAPs may influence tumour progression, participating in the regulation of diverse cellular phenomena, such as actin microfilament dynamics, maintenance of cell shape and polarity, cell migration, intracellular membrane trafficking, gene transcription, cell-cycle progression and apoptosis (Moon and Zhang, 2003; Kassambara et al., 2013). *SDP35/DEPDC1A* and *XTP1/DEPDC1B* were found weakly or negatively expressed in a set of normal human tissues (Kanehira et al., 2007) and their de novo expression or up-regulation may represent potential biomarkers in various cancers (Okayama et al., 2012; Kassambara et al., 2013; Yuan et al., 2014). In bladder cancer cells, down-regulation of the two *SDP35/DEPDC1A* transcriptional variants by RNAi suppresses cell growth, implicating its control in cell division (Kanehira et al., 2007). Recent data suggest that *SDP35/DEPDC1A* and *XTP/DEPDC1B* may control cell-cycle progression through modulation of cell-cycle genes and/or coordination of de-adhesion events necessary for cell division (Marchesi et al., 2014; Mi et al., 2015). Lack of both genes promotes a delay in transition to mitotic progression with accumulation of cells at G2 phase. In addition, *SDP35/DEPDC1A* interacts with *ZNF224*, a zinc finger transcriptional repressor involved in DNA epigenetic modification and represses the negative regulator of NF-kB signalling pathway leading to suppression of apoptosis (Harada et al., 2010). The role of *XTP1/DEPDC1B* in tumour progression appears associated with the interplay between de-adhesion events and cell-cycle progression that promotes mitosis with a behaviour similar to that of CDK1/cyclin B. In turn, the lack of *XTP1/DEPDC1B* restores adhesion-dependent mechanisms delaying progression into M phase (Matthews et al., 2012). In this study we aimed at investigating if altered expression of *SDP35/DEPDC1A* and *XTP1/DEPDC1B* correlates with metastatic progression of malignant STS, such as to assert the relevance of the molecules as novel prognostic biomarkers.

## Materials and methods

### *Tumour specimens*

152 patients diagnosed at the Rizzoli Orthopaedic Institute from 1985 to 2011 with grade II and III STS according to the Fédération Nationale des Centres de Lutte Contre le Cancer grading system were included in this study (Table 1). Selection criteria included deep localisation of the primary tumour mass in the extremities or trunk, a diameter >5 cm, no local relapses at diagnosis, no previous radiation or chemotherapy. Diagnosis based on histological, cytogenetic and immunohistochemical criteria (Fletcher et al., 2013) was confirmed by independent pathologists. Clinical follow-up was calculated from the date of the first diagnosis to the clinical event, or to the last day of follow-up. Minimum follow-up for metastasis-free survival was 5 years. Frozen samples of 86 of the 152 primary STS (Table 1) and 22 paired metastatic lesions were available for Real Time PCR analysis. 17 adjacent normal samples were used as control.

*SDP35/DEPDC1A* and *XTP/DEPDC1B* protein levels were determined by western blot in 22 lung metastases and paired primary STS, while their immunoreactivity was evaluated in paraffin embedded specimens of 131/152 primary STS (Table 2). 21 archival samples were excluded because the results were not evaluable for sample damage during the procedure.

For all tumour specimens the percentage of neoplastic cells in reference histological sections was ≥90% with no detectable signs of substantial intralesional lymphocyte infiltrations.

All samples were managed in accordance with the authorisations issued by the Rizzoli Orthopaedic Institute's Ethical Committee and following informed patient consent.

### *RNA isolation and Real-Time PCR*

Total RNA was extracted from frozen specimens using the TRIzol Reagent followed by DNase digestion (Life Technologies, Foster City, CA, USA) according to the manufacturer's protocol. Reverse transcription of mRNA was performed from 0.5 µg of total RNA using High Capacity cDNA Archive kit (Life Technologies) according to the manufacturer's instructions. cDNA from human samples was analyzed by RT-PCR performed in triplicate using the ABI PRISM 7900 Sequence Detector (Life Technologies). PCR mixtures contained: 1.25 µl of target or endogenous Reference Assay Mix 20X, 22.5 ng DNA diluted in 11.25 µl $H_2O$, 12.5 µl TaqMan Universal Master Mix 2X (Life Technology) to yield a 25 µl 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. Expression levels of *XTP1/DEPDC1B* and *SDP35/DEPDC1A* were quantified using the $2^{-\Delta\Delta CT}$ comparative method. The amount of target gene was normalized to an endogenous reference (GAPDH) and relative to a calibrator (cDNA from healthy lymphocytes) using TaqMan Gene Expression Assays (ID: Hs00293551m1, ID: Hs00854841g1 and ID: Hs99999905 m1, respectively; Applied Biosystems Inc.). Genes were considered up-regulated when values were >1±SD and under-expressed when <1±SD. Standard deviation of each $2^{-\Delta\Delta ct}$ value was less than 0.2, in accordance with the consensus guidelines for RT-

**Table 1.** Detailed patient characteristics.

| patients* | gender | age | histotype | grade | metastasis site | outcome | XTP1 IHC grading | SDP35 IHC grading |
|---|---|---|---|---|---|---|---|---|
| 1 | M | 64 | UPS | II | multiple locations | DOD | 4 | 4 |
| 2 | M | 54 | UPS | III | NM | ALIVE | 0 | 1 |
| 3 | M | 63 | UPS | III | NM | ALIVE | 1 | 1 |
| 4 | M | 61 | UPS | II | lung | DOD | 1 | 1 |
| 5 | F | 73 | UPS | III | lung | ALIVE | 1 | 1 |
| 6 | F | 54 | UPS | III | lung | DOD | 0 | 0 |
| 7 | M | 64 | UPS | III | lung | DOD | 1 | 4 |
| 8 | M | 62 | UPS | III | NM | ALIVE | 0 | 0 |
| 9 | M | 38 | UPS | III | NM | ALIVE | 0 | 0 |
| 10** | F | 43 | UPS | III | NM | ALIVE | nv | nv |
| 11 | F | 67 | UPS | III | NM | DEAD | 1 | 4 |
| 12 | F | 72 | UPS | III | lung | DOD | 1 | 4 |
| 13 | F | 76 | UPS | III | lung | DOD | 1 | 5 |
| 14 | M | 38 | UPS | III | lung | DOD | 1 | 4 |
| 15 | M | 62 | UPS | III | lung | ALIVE | 1 | 1 |
| 16 | F | 66 | FS | III | lung | ALIVE | 4 | 5 |
| 17 | M | 71 | LMS | III | lung | DOD | 1 | 4 |
| 18 | M | 57 | LMS | III | lung | ALIVE | 0 | 1 |
| 19 | M | 63 | LMS | III | lung | DOD | 4 | 1 |
| 20** | F | 76 | LMS | III | multiple locations | DOD | nv | nv |
| 21 | M | 34 | LMS | III | lymph nodes | DOD | 1 | 5 |
| 22 | M | 62 | LMS | III | lung | DOD | 4 | 1 |
| 23 | M | 66 | LMS | III | lung | DOD | 0 | 1 |
| 24 | M | 35 | LMS | III | lung | DOD | 0 | 1 |
| 25 | M | 55 | LMS | III | lung | DOD | 4 | 5 |
| 26** | M | 78 | LMS | II | NM | ALIVE | nv | nv |
| 27 | M | 38 | LMS | II | NM | ALIVE | 0 | 0 |
| 28 | F | 62 | UPS | II | lymph nodes | DOD | 0 | 0 |
| 29 | M | 43 | LMS | III | NM | ALIVE | 0 | 4 |
| 30 | M | 52 | LMS | III | lung | DOD | 1 | 5 |
| 31 | F | 79 | LMS | III | NM | DEAD | 0 | 0 |
| 32 | F | 57 | LMS | III | lung | DOD | 1 | 1 |
| 33** | M | 76 | LMS | III | lymph nodes | DOD | nv | nv |
| 34** | F | 66 | UPS | III | lung | DOD | nv | nv |
| 35 | F | 68 | LMS | III | lung | ALIVE | 4 | 5 |
| 36** | F | 74 | LMS | III | liver | DOD | nv | nv |
| 37 | F | 42 | LMS | III | lung | DOD | 1 | 4 |
| 38 | F | 71 | LMS | III | lung | DOD | 0 | 4 |
| 39** | M | 49 | LS | II | lymph nodes | DOD | nv | nv |
| 40** | M | 42 | LS | II | NM | ALIVE | nv | nv |
| 41 | M | 56 | LS | III | NM | ALIVE | 1 | 1 |
| 42 | M | 69 | LS | II | lung | DOD | 4 | 1 |
| 43** | M | 47 | LS | II | NM | ALIVE | nv | nv |
| 44 | F | 73 | LS | II | NM | DEAD | 1 | 1 |
| 45 | F | 49 | LS | II | NM | ALIVE | 4 | 4 |
| 46 | F | 40 | LS | II | NM | ALIVE | 0 | 0 |
| 47 | M | 42 | LS | III | NM | ALIVE | 0 | 2 |
| 48** | F | 85 | LS | II | NM | DEAD | nv | nv |
| 49 | M | 62 | LS | III | lung | DOD | 4 | 5 |
| 50** | F | 75 | FS | III | multiple locations | DOD | nv | nv |
| 51** | M | 54 | FS | III | NM | ALIVE | nv | nv |
| 52** | F | 29 | FS | III | vertebrae | DOD | nv | nv |
| 53** | M | 18 | UPS | III | vertebrae | DOD | nv | nv |
| 54** | M | 76 | FS | II | NM | DEAD | nv | nv |
| 55** | F | 82 | FS | III | lung | ALIVE | nv | nv |
| 56 | M | 45 | LS | III | femur | DOD | 4 | 5 |
| 57 | M | 46 | LS | III | lung | DOD | 2 | 4 |
| 58** | F | 58 | LS | II | lung | DOD | nv | nv |
| 59** | M | 53 | LS | III | lung | DOD | nv | nv |
| 60 | M | 44 | LS | III | lung | DOD | 1 | 4 |
| 61 | M | 43 | LS | III | lung | ALIVE | 4 | 4 |
| 62 | M | 44 | LS | III | liver | DOD | 2 | 4 |
| 63 | M | 42 | LS | III | lung | ALIVE | 3 | 5 |
| 64 | M | 53 | LS | III | osseous | DOD | 1 | 4 |
| 65 | M | 59 | LS | III | NM | ALIVE | 4 | 4 |
| 66 | M | 31 | LS | III | lung | DOD | 1 | 1 |
| 67** | M | 63 | LS | II | NM | ALIVE | nv | nv |
| 68 | M | 69 | LS | III | lymph nodes | ALIVE | 1 | 1 |
| 69 | F | 32 | LS | III | NM | ALIVE | 1 | 4 |
| 70 | M | 41 | LS | II | NM | ALIVE | 0 | 4 |
| 71 | M | 42 | LS | II | NM | ALIVE | 3 | 1 |
| 72 | F | 66 | LS | II | NM | DEAD | 2 | 0 |
| 73** | M | 44 | LS | III | lymph nodes | ALIVE | nv | nv |
| 74 | M | 78 | LS | II | osseous | DOD | 1 | 0 |
| 75 | M | 55 | LS | II | NM | ALIVE | 1 | 1 |
| 76 | M | 59 | LS | II | NM | ALIVE | 0 | 0 |

**Table 1.** Detailed patient characteristics (Continuation).

| patients* | gender | age | histotype | grade | metastasis site | outcome | XTP1 IHC grading | SDP35 IHC grading |
|---|---|---|---|---|---|---|---|---|
| 77 | M | 43 | LS | II | lung | ALIVE | 0 | 2 |
| 78 | F | 67 | LS | III | lung | DOD | 1 | 2 |
| 79 | M | 35 | LS | II | NM | ALIVE | 0 | 1 |
| 80 | M | 29 | LS | II | NM | ALIVE | 1 | 1 |
| 81 | F | 36 | LS | II | NM | ALIVE | 2 | 0 |
| 82 | M | 39 | LS | II | NM | ALIVE | 3 | 4 |
| 83 | M | 31 | LS | II | lymph nodes | DOD | 1 | 1 |
| 84** | M | 28 | LS | II | NM | ALIVE | nv | nv |
| 85 | M | 45 | LS | II | osseous | DOD | 0 | 0 |
| 86 | F | 58 | LS | II | NM | ALIVE | 0 | 1 |
| 87 | M | 50 | FS | III | NM | ALIVE | 4 | 0 |
| 88 | M | 33 | UPS | III | NM | ALIVE | 4 | 3 |
| 89 | M | 56 | LMS | III | NM | DEAD | 5 | 2 |
| 90 | M | 38 | FS | III | lung | ALIVE | 4 | 3 |
| 91 | M | 53 | LMS | III | vertebrae | ALIVE | 4 | 4 |
| 92 | M | 54 | FS | III | NM | ALIVE | 5 | 5 |
| 93 | M | 50 | FS | III | NM | ALIVE | 5 | 4 |
| 94 | M | 15 | LMS | III | lung | ALIVE | 4 | 4 |
| 95 | M | 15 | FS | II | NM | ALIVE | 0 | 0 |
| 96 | M | 31 | LMS | III | NA | ALIVE | 5 | 4 |
| 97 | M | 65 | UPS | III | osseous | DOD | 0 | 0 |
| 98 | M | 48 | FS | III | NM | ALIVE | 4 | 3 |
| 99 | M | 22 | LMS | III | NM | ALIVE | 4 | 3 |
| 100 | M | 15 | FS | III | NM | ALIVE | 3 | 0 |
| 101 | M | 35 | FS | III | NM | ALIVE | 5 | 5 |
| 102 | M | 21 | UPS | III | NM | DOD | 4 | 3 |
| 103 | M | 32 | FS | III | NM | ALIVE | 5 | 5 |
| 104 | M | 72 | FS | III | NM | DEAD | 4 | 4 |
| 105 | M | 50 | FS | III | lung | ALIVE | 1 | 3 |
| 106 | M | 79 | LMS | III | osseous | DOD | 4 | 5 |
| 107 | M | 58 | FS | III | osseous | DOD | 4 | 3 |
| 108 | M | 63 | UPS | III | NM | ALIVE | 5 | 5 |
| 109 | M | 56 | FS | III | NM | ALIVE | 5 | 5 |
| 110 | M | 71 | LMS | III | NM | ALIVE | 4 | 5 |
| 111 | M | 44 | FS | III | NM | ALIVE | 5 | 5 |
| 112 | M | 17 | UPS | III | multiple locations | DOD | 0 | 2 |
| 113 | M | 55 | LMS | III | multiple locations | ALIVE | 4 | 4 |
| 114 | M | 62 | LMS | III | osseous | ALIVE | 4 | 4 |
| 115 | M | 68 | FS | III | NM | ALIVE | 4 | 3 |
| 116 | M | 28 | FS | III | NM | ALIVE | 3 | 5 |
| 117 | M | 48 | FS | III | multiple locations | ALIVE | 5 | 5 |
| 118 | M | 45 | FS | III | lung | ALIVE | 4 | 4 |
| 119 | M | 42 | UPS | III | NM | ALIVE | 0 | 0 |
| 120 | M | 43 | UPS | III | lung | DOD | 3 | 5 |
| 121 | M | 82 | UPS | III | multiple locations | DOD | 3 | 4 |
| 122 | M | 64 | UPS | III | lung | DOD | 4 | 5 |
| 123 | F | 34 | UPS | III | lung | DOD | 3 | 5 |
| 124 | M | 54 | UPS | III | NM | DEAD | 4 | 5 |
| 125 | M | 46 | UPS | III | NM | ALIVE | 4 | 5 |
| 126 | F | 62 | UPS | III | lung | DOD | 0 | 4 |
| 127 | F | 88 | UPS | III | lung | ALIVE | 2 | 4 |
| 128 | M | 65 | UPS | III | NM | ALIVE | 3 | 5 |
| 129 | M | 71 | UPS | III | lung | DOD | 5 | 4 |
| 130 | M | 52 | UPS | III | lung | DOD | 4 | 4 |
| 131 | M | 64 | UPS | III | lung | ALIVE | 5 | 4 |
| 132 | M | 56 | UPS | III | NM | ALIVE | 5 | 5 |
| 133 | F | 79 | UPS | III | NM | DEAD | 3 | 4 |
| 134 | M | 55 | UPS | III | NM | ALIVE | 2 | 5 |
| 135 | M | 61 | UPS | III | lung | DOD | 2 | 4 |
| 136 | M | 38 | UPS | III | lung | DOD | 0 | 4 |
| 137 | M | 69 | UPS | III | NM | DEAD | 3 | 4 |
| 138 | F | 57 | UPS | III | NM | ALIVE | 5 | 4 |
| 139 | M | 70 | UPS | III | NM | DEAD | 0 | 5 |
| 140 | M | 71 | UPS | III | multiple locations | DOD | 4 | 5 |
| 141 | M | 76 | UPS | III | NM | ALIVE | 3 | 3 |
| 142 | F | 63 | FS | III | multiple locations | ALIVE | 4 | 4 |
| 143 | M | 50 | UPS | III | NM | ALIVE | 3 | 4 |
| 144 | F | 54 | UPS | III | multiple locations | DOD | 5 | 5 |
| 145 | F | 70 | UPS | III | multiple locations | DOD | 5 | 5 |
| 146 | M | 58 | UPS | III | NM | ALIVE | 3 | 4 |
| 147 | M | 70 | UPS | III | lung | DOD | 3 | 4 |
| 148 | F | 72 | UPS | III | NM | DEAD | 2 | 5 |
| 149 | F | 76 | UPS | III | multiple locations | DOD | 4 | 4 |
| 150 | M | 62 | UPS | III | NM | ALIVE | 2 | 4 |
| 151 | F | 43 | UPS | III | NM | ALIVE | 1 | 5 |
| 152 | F | 67 | UPS | III | NM | DEAD | 3 | 5 |

*The first 86 patients had RT-PCR analysis. **Patients without IHC expression. UPS, undifferentiated pleomorphic sarcoma; LMS, Leiomyosarcoma; LS, Liposarcoma; FS, Fibrosarcoma; NM, no metastasis; DOD, Dead of disease; DEAD, Dead of other causes; nv, not available.

PCR data reliability.

### *Tissue microarray (TMA) construction and immunohistochemistry (IHC)*

Protein expression was evaluated by IHC on TMA (3D Histech Ltd, Budapest, Hungary) performed in 152 paraffin-embedded STS samples and in normal tissues by spotting duplicated samples for each patient.

Sections were incubated with rabbit polyclonal anti-DEPDC1B (anti-XTP1) and anti-DEPDC1A (anti-SDP35) antisera (Novus Biologicals, Littleton CO, USA), diluted 1:500 and 1:1000 respectively. Sections were then washed and incubated with the streptavidin-biotin peroxidase DAB detection systems (Dako, Glostrup, Denmark) according to the manufacturer's protocol. Staining was arbitrarily scored for intensity (i.e. 0=no expression; 1=weak expression; 2=moderate expression; 3=strong expression) and for the percentage of positive cells (negative; 1=10-49%; 2≥50%) seen within the lesion. Cut-off levels of the sum of scores were applied as 0 for negative, 1-3 for weakly positive, and 4-5 for moderate to strong positivity in more than 50% of tumour cells which is considered significant protein up-regulation. Immunostaining of 21 tumour samples was not evaluable.

### *Western Blotting (WB)*

Cell lysates from frozen tissue were resolved by SDS-PAGE on 6% gels, transferred onto Immobilon membranes (GE Healthcare, Little Chalfont, UK ), which were blocked with a 5% milk solution and incubated with either anti-SDP35/DEPDC1A (Novus Biologicals 1:5,000 dilution) or anti-XTP1/DEPDC1B (Biorbyt, Cambridge, UK; 1:250 dilution). The signal was visualized by using the Immobilon Western Chemiluminiscent HRP substrate (Millipore, Billerica, MA, USA) and quantified by densitometric analysis (GS-800 imaging densitometer and Quantity One software; Bio-Rad, Hercules, CA, USA). STS cell lines SW872, SK-LMS-1 and HT1080 which express constitutively *XTP1/DEPDC1B* and *SDP35/DEPDC1A* were used as positive control. A rabbit anti-actin polyclonal antiserum (Sigma Chemical Co., St. Louis, MO, USA) was used as a loading control.

**Table 2.** Clinical characteristics of STS patients with IHC and RT-PCR data available.

| | IHC (131) | RT-PCR (86) |
|---|---|---|
| Gender | No. | No. |
| Male | 100 | 57 |
| Female | 31 | 29 |
| Median age (yr - range) | 55 | 55.5 |
| Site No. | No. | |
| Upper Limbs | 11 | 5 |
| Lower Limbs | 107 | 77 |
| Axial Skeleton | 13 | 4 |
| Total median follow up (months) | 43 | 47 |
| Metastasis median time (months) | 24 | 18 |
| Histology | No. | No. |
| UPS | 54 | 18 |
| LMS | 25 | 20 |
| LS | 33 | 42 |
| FS | 19 | 6 |
| Outcome | No. | No. |
| Alive | 59 | 36 |
| Dead of Disease | 72 | 50 |
| Metastasis | 69 | 52 |
| Local relapses | 22 | 16 |
| Adjuvant Treatments | No. | No. |
| Chemotherapy | 21 | 17 |
| Radiotherapy | 68 | 54 |

### *Statistical analysis*

mRNA expression levels were reported as median within the 25-75$^{th}$ percentile for their strong non-Gaussian distribution. Non-parametric Wilcoxon's, Kruskal-Wallis, and Mann-Whitney U tests were used to establish the differences in gene expression levels in paired and unpaired surgical specimens respectively. Chi-square ($\chi^2$) test with Fisher's exact p value was used to correlate protein expression with clinical parameters. Probability of metastasis-free survival (MFS) was performed according to Kaplan-Meier method and statistical difference between survival curves was sought using Log-Rank test. The prognostic factors were tested with Cox's regression analyses (Hazard ratios and 95% CI). All statistical analyses were performed using the SPSS software (SPSS Ic., Chicago, IL), and p values ≤0.05 were considered significant.

## Results

### *Clinical characteristics of STS patients*

152 patients with complete clinical follow-up and all relevant histopathological information were admitted to the study (Tables 1, 2).

81 patients developed metastases during follow-up and 75 died of disease. Average period of overall survival (OS) and metastasis-free survival (MFS) was respectively 170 (95% CI=140-200) and 142.5 months (95% CI=110.79-174.39) with a median of 95 (95% CI=20.8-169) and 38 months (95% CI=0.00-94.83). Patients with grade II STS, patients under 60 years of age and patients who had undergone radiation had a significantly more favourable 5-year OS and MFS than the others (Table 3).

Accordingly, Kaplan Meier analysis based on grade, age and radiation therapy showed that the difference between OS (respectively log-rank=9.7, p=0.002, log-rank=8.8, p=0.001, log-rank=12, p=0.002) and MFS curves (log-rank=11.43; p=0.001, log-rank=9.30, p=0.002; log-rank=19.21, p=0.0005, respectively) was

statistically significant (Fig. 1a-c).

Gender, size and site of primary lesions and chemotherapeutic treatment did not correlate with metastatic progression.

### *XTP1/DEPDC1B and SDP35/DEPDC1A mRNA expression*

mRNAs expression levels ($2^{-\Delta\Delta ct}$) of *XTP1/DEPDC1B* and *SDP35/DEPDC1A* were evaluated in 86 out of the 152 primary STS specimens (Table 1) and in 22 paired metastases. 17 normal tissues adjacent to primary tumours used as control showed gene underexpression ($2^{-\Delta\Delta ct}$ median levels respectively of 0.20 and 0.38) .

In contrast, primary STS presented gene up-regulation (173.5 for *XTP1/DEPDC1B* and 195.5 for *SDP35/DEPDC1A*) with slightly higher mRNA median values in grade III (214.50; $25^{th}$-$75^{th}$=75.75-3081.50 for *SDP35/DEPDC1A* and 195.0; $25^{th}$-$75^{th}$=29.75-3202.25 for *XTP1/DEPDC1B*) compared to grade II (148.5; $25^{th}$-$75^{th}$=18.75-8174.0 and 94.5; $25^{th}$-$75^{th}$=14.25-901.50 respectively).

Concerning the histotype, LMS and FS primary lesions had higher expression of SDP35/ *DEPDC1A* (843.0 and 232.5 respectively), than LS and UPS (164.5 and 130 respectively), while FS had higher $2^{-\Delta\Delta CT}$ levels of *XTP1/DEPDC1B* (1121.0) than LMS (215.5), LS (205.0) and UPS (51.5). No statistical difference was seen by Kruskal-Wallis test in the distribution across histotype (p=0.20).

Paired Wilcoxon's analysis showed that the metastatic lesions had significantly higher mRNA expression levels than the corresponding paired primary tumours (respectively 3595 and 299, p=0.02 for *XTP1/DEPDC1B*; 1354 and 366, p=0.05 for *SDP35/DEPDC1A*) (Fig. 2a,b).

Interestingly, the median levels of both RhoGAPs were significantly higher in primary tumours of the 52 metastatic patients when compared to the non-metastatic (respectively, 258.0 and 66.0 for *XTP1/DEPDC1B*, Mann Whitney p=0.03; 621.5 and 59.5 for *SDP35/DEPDC1A*, Mann Whitney p=0.01) (Fig. 2c,d). By setting the *XTP1/DEPDC1B* and *SDP35/DEPDC1A* expression cut-off at 173 and 195, corresponding to respective median values, we found that patients with mRNA levels above cut-off, had a worse prognosis (Table 3), with a significantly higher probability of metastatic event than patients with lower values (log-rank=6.45; p=0.01 for *XTP1/DEPDC1B*; log-rank=16.48 p<0.0005 for *SDP35/DEPDC1A*; Fig. 3a,b). Gene expression had no effect on OS.

### *XTP1/DEPDC1B and SDP35/DEPDC1A protein expression*

Immunohistochemistry was available in 131/152 primary STS specimens (Table 2).

Up-regulation (moderate to strong staining intensity

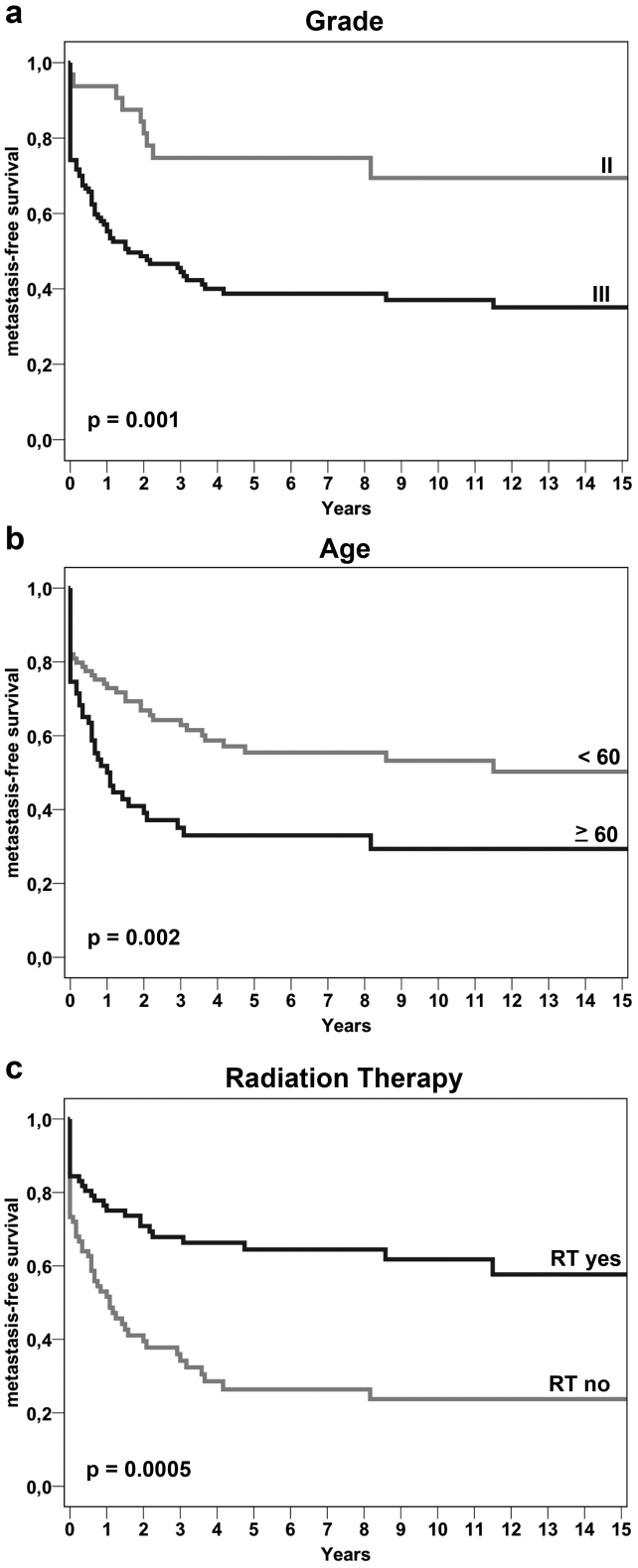


**Fig. 1.** 5-year MFS probability curves (Kaplan-Meier analysis) for 152 STS patients stratified according to grade **(a)**, age **(b)** and previous radiation therapy **(c)**. MFS was found to be significantly higher in patients with grade II, patients under 60 years of age and in patients who had received previous radiation treatment.

**Table 3.** Univariate analysis for 5-year OS and MFS.

| Variable | | Patient N. 152 | 5-year OS% | p | 5-year MFS% | p |
|---|---|---|---|---|---|---|
| Age | <60 | 89 | 64 | 0.003 | 55 | 0.002 |
| | >60 | 63 | 43 | | 33 | |
| Site | Extremity | 138 | 58 | 0.09 | 47 | 0.89 |
| | Axial | 14 | 30 | | 38 | |
| Histology | LMS | 29 | 50 | 0.07 | 32 | 0.09 |
| | UPS | 57 | 48 | | 43 | |
| | FS | 24 | 45 | | 40 | |
| | LS | 42 | 77 | | 64 | |
| Chemotherapy * | No | 125 | 53 | 0.1 | 38 | 0.5 |
| | Yes | 26 | 66 | | 49 | |
| Radiation therapy | No | 75 | 38 | 0.001 | 26 | 0.0005 |
| | Yes | 77 | 69 | | 64 | |
| Grade | II | 32 | 86 | 0.002 | 74 | 0.01 |
| | III | 120 | 47 | | 39 | |
| XTP Protein ** | Negative | 80 | 62 | 0.3 | 48 | 0.8 |
| | Positive | 51 | 49 | | 44 | |
| SDP Protein ** | Negative | 53 | 71 | 0.07 | 58 | 0.05 |
| | Positive | 78 | 50 | | 39 | |
| XTP mRNA*** | Up to | 43 | 66 | 0.2 | 54 | 0.01 |
| | Over | 43 | 50 | | 29 | |
| SDP mRNA *** | Up to | 43 | 54 | 0.3 | 62 | 0.0001 |
| | Over | 43 | 68 | | 21 | |

* unknown in 1 patient. ** 131 patients. *** 86 patients.

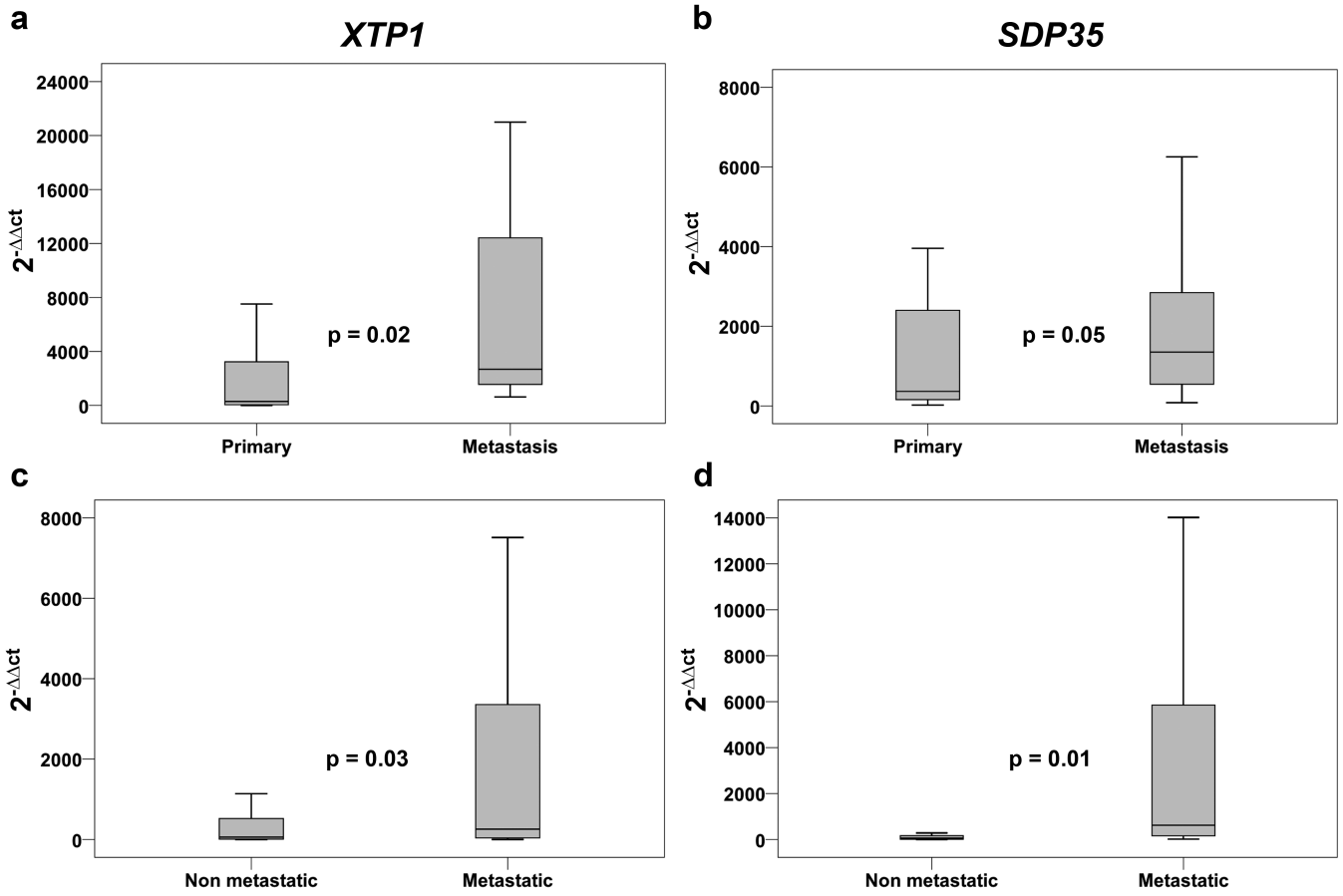


**Fig. 2.** Relative levels of *XTP1/DEPDC1B* **(a)** and *SDP35/DEPDC1A* **(b)** mRNA expression in 22 primary STS and paired lung metastasis. Wilcoxon analysis revealed statistically significant differences ($p<0.05$). Patients who developed metastases exhibited higher levels of *XTP1/DEPDC1B* **(c)** and *SDP35/DEPDC1A* **(d)** transcripts when compared to primary tumours from metastasis-free patients. Mann Whitney analysis revealed statistically significant differences ($p<0.05$).

in more than 50% of the tumour cells, scores 4-5 of SDP35/DEPDC1A was seen in 78 (59%) cases and of XTP1/DEPDC1B in 51 (40%). Generally, XTP1/DEPDC1B was diffusely distributed throughout the cytoplasm, whereas SDP35/DEPDC1A displayed a predominant nuclear localization, although focal cytoplasmic immunostaining was also observed (Fig. 4). Coherently with what was observed for the relative expression levels of the transcripts, distribution and intensity of RhoGAP proteins did not show significant differences among the different STS histotypes.

Negative and focal expression of XTP1/DEPDC1B was observed in normal lung and kidney tissues (score 0 and 1-2 respectively), while SDP35/DEPDC1A immunostaining was weakly but uniformly expressed in prostate tissue (score 3) (Fig. 4). In agreement with mRNA expression pattern, the 70 metastatic patients showed more frequent up-regulation of XTP1/DEPDC1B (43.4%) and SDP/DEPDC1A (72%) than the 61 metastasis-free patients (33.8%, p>0.05; 45%, p=0.001 respectively). 5-year MFS was significantly higher in patients with SDP35/DEPDC1A increased expression (Table 3) and Kaplan Meier analyses revealed that the up-regulation significantly decreased MFS probability (log Rank=12.108; p=0.001), while no significant differences were seen for XTP1/DEPDC1B (p>0.05; Fig. 5a,b).

In all 22 paired lung metastases examined, XTP1/DEPDC1B and SDP35/DEPDC1A were uniformly expressed in tumour cells with variable staining intensity that ranged from moderate to strong according to the adopted arbitrary scoring (scores 4-5).

Supportive WB analysis confirmed high levels of both proteins in the STS specimens and similarly indicated a stronger expression of SDP35/DEPDC1A in metastases when compared to primary tumours (Fig. 6a). In STS cell lines used as reference, SDP35/DEPDC1A showed a double band, suggesting that it was present as alternatively spliced isoforms (Fig. 6b). Protein expression had no effect on OS.

Finally, Cox's regression multivariate analyses with variables significant to univariate analysis showed that the risk of developing metastases significantly increased with the grade (p=0.04), lack of radiation therapy (p=0.001) and high *SDP35/DEPDC1A* mRNA and

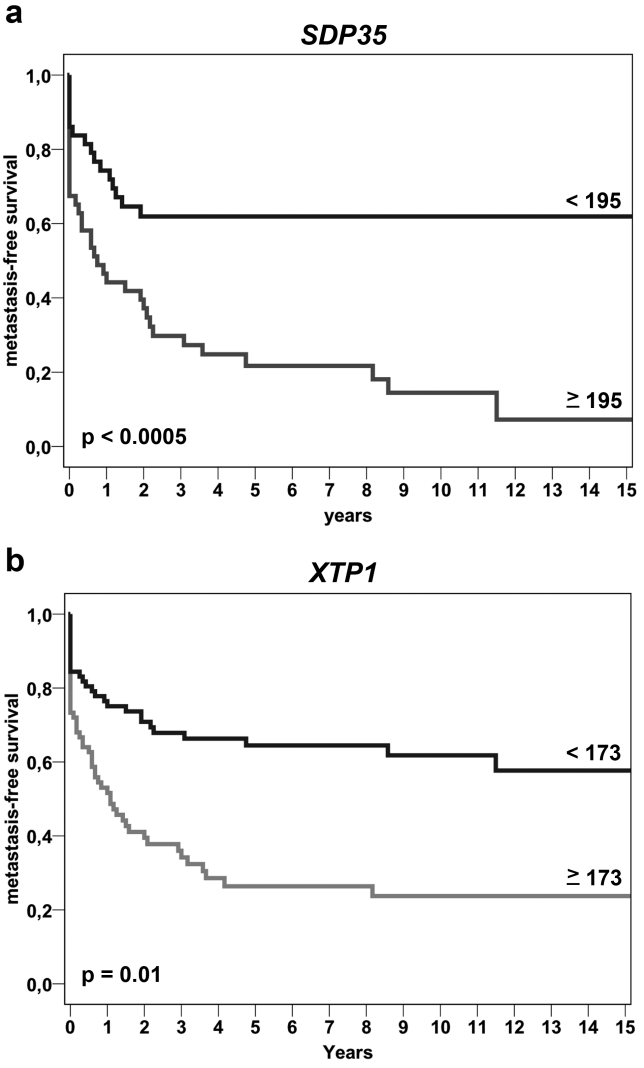


**Fig. 3.** Kaplan-Meier curves for MFS in relation to *SDP35/DEPDC1A* **(a)** and *XTP1/DEPDC1B* mRNA **(b)** relative levels expressed as $2^{-\Delta\Delta}$CT. Cut-off levels correspond to the median values calculated on the 86 primary tumours included in this analysis. The differences were statistically significant (p<0.05).

**Table 4.** Multivariate analysis for 5-year OS and MFS.

| Variable | HR for OS (95% CI) | HR for MFS (95% CI) |
|---|---|---|
| SDP35 mRNA ≥195 $^{2-\Delta\Delta ct}$ | 1.27 (0.58-2.8) p=0.5 | 2.75 (1.31-5.8) p=0.008 |
| XTP1 mRNA ≥173 $^{2-\Delta\Delta ct}$ | 1.4 (0.65-2.96) p=0.4 | 1.31 (0.64-2.67) p=0.4 |
| SDP protein up-regulation | 1.64 (0.94-2.93) p=0.07 | 2.53 (1.38-4.61) p=0.002 |
| Grade III | 2.21 (0.99-5.28) p=0.07 | 2.55 (1.03-6.36) p=0.04 |
| Radiation therapy | 0.72 (0.43-1.21) p=0.2 | 0.37 (0.21-0.65) p=0.001 |
| Age ≥60 | 1.63 (0.99-2.66) p=0.04 | 1.24 (0.75-2.07) p=0.4 |

HR, hazard ratio; CI, confidence Interval.

protein expression levels (p=0.008 and p=0.002 respectively) (Table 4).

No clinical and biological parameter analysed resulted to be significant prognostic independent factors for OS.

## Discussion

Efficient clinical management of STS patients requires a more profound knowledge about the factors that may predict development of post-surgery metastases. Although radiation therapy associated with chemotherapy has improved patient survival, about 50% of STS patients invariably develop metastases, so more effective therapeutic approaches are needed.

In a previous differential mRNA expression study (Gazziola et al., 2003) we identified the unique RhoGAP family member *XTP1/DEPDC1B* as one of the transcripts differentially expressed by STS cells when compared to their putative counterpart healthy cells.

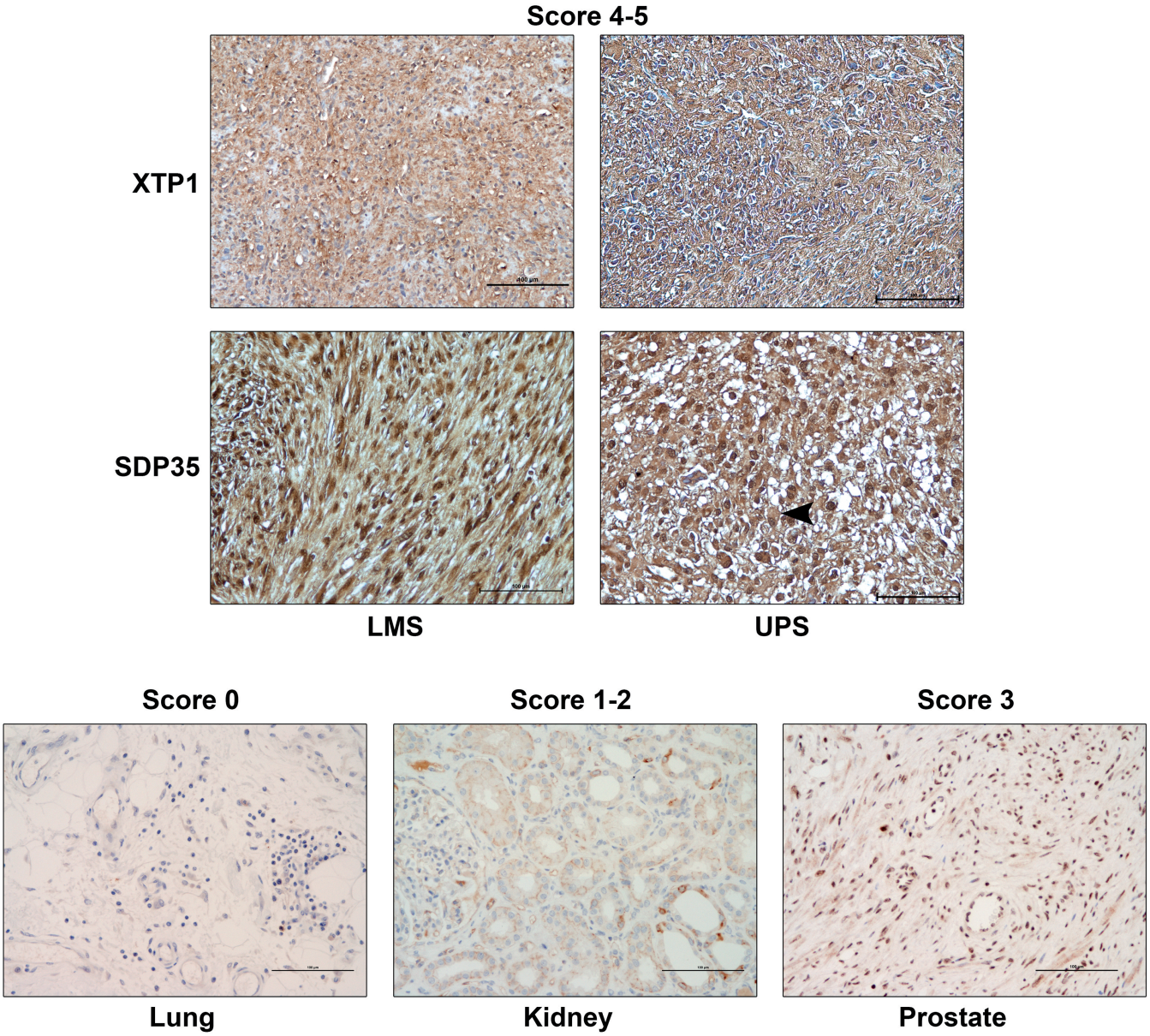


**Fig. 4.** Expression of SDP35/DEPDC1A and XTP1/DEPDC1B proteins determined by IHC on TMA sections. XTP1/DEPDC1B appeared diffusely distributed through the cytoplasm in leiomyosarcoma (LMS) and undifferentiated pleomorphic sarcoma (UPS), while SDP35/DEPDC1A showed a preferential nuclear accumulation. Lung, kidney and prostate normal tissues showed a variable IHC grading from 0 to 3. Scale Bar: 100 µm.

Subsequent independent investigations implicated *XTP1/DEPDC1B* in neoplastic transformation (Nicassio et al., 2005) and as regulator of cancer cell migration and invasion (Lin et al., 2011), also suggesting that it may additionally act as a cell-cycle regulator (Johannsdottir et al., 2006; Boudreau et al., 2007) by inhibiting RhoA/Rho-Associate Protein Kinase activity during G2/M transition to promoting mitotic entry (Marchesi et al., 2014).

In the human genome *XTP1/DEPDC1B* has a homologue denoted *SDP35/DEPDC1A*, which is reported to be up-regulated in bladder cancer (Harada et al., 2010) and its enhanced expression may discriminate malignant from non-malignant forms of breast carcinoma (Kretschmer et al., 2011). Publicly accessible global gene mappings infer that *XTP1/DEPDC1B* and *SDP35/DEPDC1A* are poorly transcribed in most healthy human tissues, but are selectively up-regulated in certain tumour types. SDP35/DEPDC1A is not expressed in human heart, liver, kidney, lung, but it was seen in testis (Kanehira et al., 2007), prostate (Huang et al., 2017; Ramalho-Carvalho et al., 2017), bone marrow, lymphoid organs and in human embryonic pluripotent stem cells (Kassambara et al., 2013). Structure, clinical relevance and function of *SDP35/DEPDC1A* and *XPT1/DEPDC1B* are not clearly understood as to date only few studies have been performed. Some data report that these molecules are involved in progression of various tumours, but their role in STS is still to be investigated. SDP35/DEPDC1A is recognized to be an important gene involved in cancer cell proliferation, migration and invasion as well as in some types of carcinogenesis (Kanehira et al., 2007; Harada et al., 2010; Kretschmer et al., 2011; Obara et al., 2012; Okayama et al., 2012; Kassambara et al., 2013; Mi et al., 2015). SDP35/DEPDC1A expression is associated with poor prognosis in many tumours (Kanehira et al., 2007; Okayama et al., 2012; Yuan et al., 2014). Indeed, in multiple myeloma SDP35/DEPDC1A promotes cell

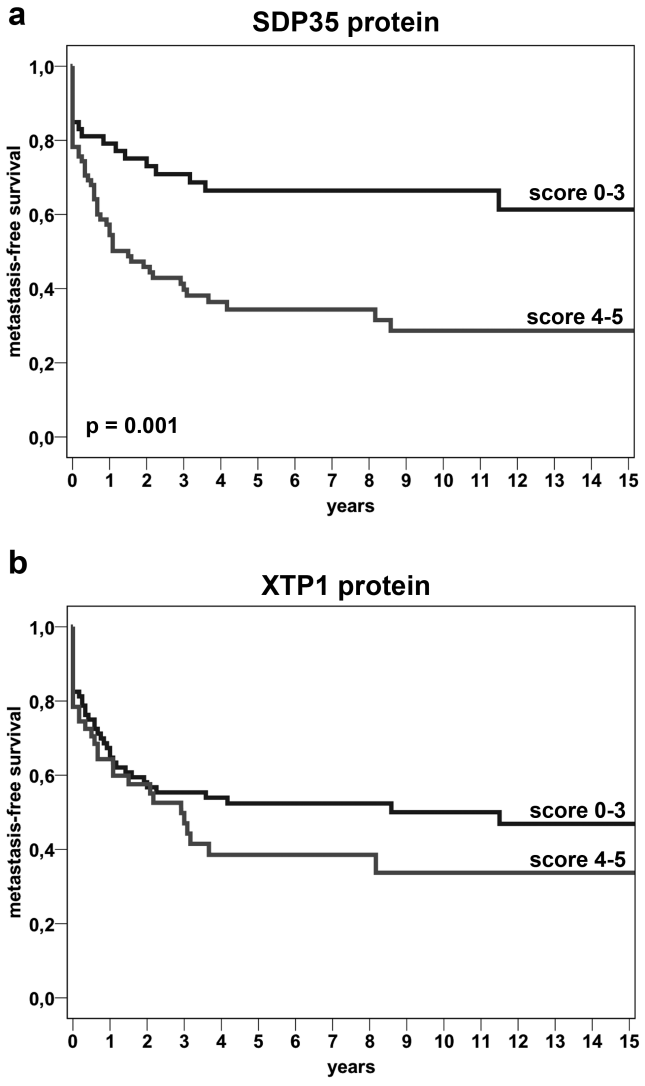


**Fig. 5.** Kaplan-Meier curves for MFS according to XTP1/DEPDC1B and SDP35/DEPDC1A protein expression. Reported IHC score is: 0-3 = no or poor expression and 4-5 = moderate to strong expression.

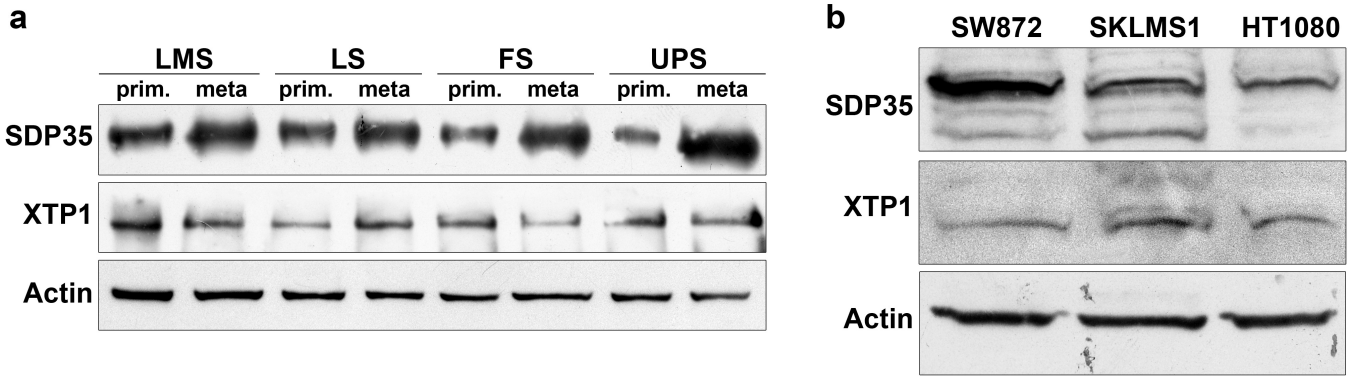


**Fig. 6.** Representative detection of SDP35/DEPDC1A (61.5kDa) and XTP1/DEPDC1B (62kDa) protein expression in paired STS primary and metastatic lesions. A higher level of SDP35/DEPDC1A was seen in STS metastases when compared to primary tumours, whereas no significant differences were seen for XTP1/DEPDC1B **(a)**. STS cell lines SW872, SKLMS1 and HT1080 known to constitutively express both RhoGAPs were used as positive control. SDP35/DEPDC1 showed two migration bands (93.1 kDa and 61.5 kDa isoforms) **(b)**. Actin was used as calibrator.

cycle, blocks cell differentiation and induces markers of mature plasma cells, suggesting its role as indicator of poor prognosis (Kassambara et al., 2013). XTP1/DEPDC1B is involved in promotion of tumour cell migration and invasion by activating Wnt/β-catenin signalling and cell proliferation (Boudreau et al., 2007; Su et al., 2014; Yang et al., 2014) and it might represent a poor prognostic marker for non-small cell lung cancer (Yang et al., 2014) and nasopharyngeal carcinoma (Tu et al., 2015).

The importance of these molecules in cancer formation/progression is largely confirmed by this study on a series of specimens from STS patients. Our findings corroborate the low frequency of *XTP1/DEPDC1B-SDP35/DEPDC1A* expression in healthy tissues and reveal a significantly higher mRNA expression of both genes in STS lesions. Thus, neoplastic up-regulation of *SDP35/DEPDC1A* and *XTP1/DEPDC1B* does not seem to be restricted to epithelial tumours, but may occur also in tumours of mesenchymal origin.

When median mRNA levels were assessed, we observed that individuals with *XTP1/DEPDC1B* and *SDP35/DEPDC1A* expression above median values had a significantly higher probability of metastatic event than patients falling below this cut-off.

Immunostaining expression in STS lesions showed that XTP1/DEPDC1B protein was predominant in cytoplasm, SDP35/DEPDC1A in nucleus. It has been shown that the levels of XTP1/DEPDC1B and SDP35/DEPDC1A proteins oscillate during the cell-cycle. SDP35/DEPDC1A is highly expressed during mitotic phase, distributed in nucleus during prophase and within cytoplasm during metaphase and anaphase (Mi et al., 2015). XTP1/DEPDC1B protein augments during the G2 phase (Marchesi et al., 2014) and its activity seems to be concentrated within the cytoplasm corroborating our on-going findings that may highlight a direct effect of these RhoGAP on actin cytoskeletal dynamics.

Concerning our STS patients clinical follow-up Kaplan Meier analysis consolidated a high prognostic value of the abundant expression of SDP35/DEPDC1A also at the protein level.

Multivariate analysis showed that *SDP35/DEPDC1A* up-regulation, grade III and lack of radiation therapy were considered independent prognostic factors for metastasis development.

Furthermore, when compared to the corresponding primary tumours, the expression of both genes was further enhanced in the 22 paired metastatic lesions, suggesting that up-regulation of the molecules could be associated with the expansion of the original tumour to form distant secondary lesions.

### *Conclusions*

Our results demonstrate that increased expression of the unique RhoGAPs, *SDP35/DEPDC1A* and *XPT1/DEPDC1B*, tightly correlated with STS metastatic progression, also providing novel evidence for an important role of the aberrant expression of *SDP35/DEPDC1A* as prognostic independent risk factor. Given the rarity of STS, multicentre studies are needed to confirm this predictive aspect and future *in vitro* and *in vivo* studies will define their possible role as candidate targets for more selective and personalized therapies.

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*Financial competing interest.* The Authors state that there is no potential conflict of interest both financial and personal

*Authors' contributions:* Serena Pollino (SP), performed the experiments and wrote the paper. Maria Serena Benassi (MSB), conceived the experiments, analysed data and wrote the paper. Laura Pazzaglia (LP) performed experiments and analysed data. Amalia Conti (AC), performed experiments. Nicoletta Bertani (NB), performed experiments. Martina Piccinni-Leopardi (MPL), patient follow-up. Piero Picci (PP), conceived the study and approved the final version to be published. Roberto Perris (RP), conceived the study and approved the final version to be published

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