MRP1 Overexpression Determines Poor Prognosis in Prospectively Treated Patients with Localized High-Risk Soft Tissue Sarcoma of Limbs and Trunk Wall: An ISG/GEIS Study

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Abstract

Patients with localized high-risk soft tissue sarcomas (STS) of the limbs and trunk wall still have a considerable metastatic recurrence rate of more than 50%, in spite of adjuvant chemotherapy. This drug-ceiling effect of chemotherapy in sarcoma setting could be explained, at least partially, by multidrug resistance (MDR) mechanisms. The aim of this study was to ascertain whether mRNA and protein expression of ABCB1 (P-glycoprotein), ABCC1 (MRP1), and GSTA1 (glutathione S-transferase pi) was prognostic in localized high-risk STS. Immunohistochemistry and reverse transcriptase-PCR studies were performed from biopsies at the time of diagnosis. Patients of this series were prospectively enrolled into a phase III trial that compared three versus five cycles of epirubicin plus ifosfamide. The series of 102 patients found 41 events of recurrence and 37 of death with a median follow-up of 68 months. In univariate analysis, variables with a statistically significant relationship with relapse-free survival (RFS) were: MRP1 expression (5-year RFS rate of 23% in positive cases and 63% in negative cases, \(P = 0.029\)), histology (5-year RFS rate of 74% in undifferentiated pleomorphic sarcoma and 43% in synovial sarcoma, \(P = 0.005\)), and ABCC1 expression (5-year RFS rate of 33% in overexpression and 65% in downregulation, \(P = 0.012\)). Combined ABCC1/MRP1 was the only independent prognostic factor for both RFS (HR = 2.704, \(P = 0.005\)) and overall survival (HR = 2.208, \(P = 0.029\)). ABCC1/MRP1 expression shows robust prognostic relevance in patients with localized high-risk STS treated with anthracycline-based chemotherapy, which is the standard front line treatment in STS. This finding deserves attention as it points to a new targetable protein in STS. Mol Cancer Ther; 13(1); 249–59. ©2013 AACR.
explained at least in part by the existence of multidrug resistance (MDR) mechanisms. This phenomenon is defined as an intrinsic or acquired resistance to multiple anticancer drugs that seem to be structurally and functionally unrelated.

One crucial cellular mechanism related to MDR involves the efflux pumps of drugs from the cancer cells by specific transmembrane transporters. These ATP-binding cassette (ABC superfamily) protein pumps include 49 genes, distributed in 7 families (from A to G). The “C” branch contains the largest number of transporters known at the present time, with \( \text{ABCC1/MRP1} \) being the first to be characterized (5). P-glycoprotein (P-gp), encoded by the \( \text{MDR1} \) gene, also called the \( \text{ABCB1} \) gene, belongs to branch “B.” P-gp was the first member of the ABC transporter family, and its overexpression is probably the most frequent molecular event related to MDR (6, 7). Even though there is a significant overlapping between both pumps as regards the substrate specificity, MRP1 transports a broader range of xenobiotics, including anthracyclines and glucuronide conjugates (8).

As with the previous transporters, glutathione S-transferases (GST) may play a relevant role in the cell’s defense against drugs. GSTs are isozymes encoded by several genes and have been implicated in catalyzing the conjugation of glutathione to several chemotherapeutic agents, thus detoxifying these compounds (9, 10).

Expression of P-gp, MRP1, or GSTs may be inherent to cancer cells, as well as being induced after exposure to chemotherapy. It is estimated that 1 of 10^6 cancer cells is likely to have inherent resistance to a particular drug; a clinically detectable tumor contains about 10^9 cells. Taking this into account, it could be expected that the larger the tumor size, the greater the probability of its containing inherently resistant tumor cells.

There are scarce and at the same time controversial publications analyzing the prognostic or predictive impact of MDR in sarcomas. A significant correlation has been found between protein expression of P-gp and poor pathologic response in high-grade sarcomas (11). Positive P-gp immunohistochemistry was observed more frequently than immunostaining of MRP1, and both expressions varied according to histological type (12). These published studies were based on retrospective series of STS cases and therefore focus on correlations between expression and clinicopathologic parameters, but not on survival (13). The most robust prognostic information of ABC transporters in sarcoma stems from analyses carried out on high-grade osteosarcoma. P-glycoprotein overexpression at diagnosis was significantly associated with an unfavorable outcome, to such an extent that it is now considered to be a stratification factor in high-grade osteosarcoma (14, 15) by some groups. In the pediatric population, there was also a correlation between MDR expression and the higher risk rhabdomyosarcoma population and it was proven that chemotherapeutic treatment was followed by an increased expression of MDR proteins (16).

Patients with localized high-risk STS, with a recurrence risk ranging from 50% to 65%, offer a suitable scenario for which complementary chemotherapy could be potentially curative. The aims of this study were to find out prospectively whether the mRNA and protein expression of \( \text{ABCB1 (P-gp)} \), \( \text{ABCC1 (MRP1)} \), and \( \text{GSTA1} \) [glutathione S-transferase pi (GST-p)] at time of diagnosis had any prognostic role in a subset of patients with localized high-risk STS who were enrolled prospectively into a randomized phase III trial (17).

Patients and Methods

As mentioned earlier, the patients of this series were enrolled prospectively into a phase III trial conducted by Italian Sarcoma Group (ISG) with the collaboration of Spanish Sarcoma Group (GEIS) in the ISG-GEIS 0101 trial. This trial randomized 328 high-risk localized (>5 cm, deep, grade 3 by FNNLC) resectable STS of the extremities or of the trunk wall patients to receive either 3 or 5 cycles of epirubicin 120 mg/m^2 plus ifosfamide 9,000 mg/m^2 per cycle (17). The first 3 cycles were given as neoadjuvant in both arms and 2 additional cycles were given as adjuvant in one arm. RECIST response was evaluated after the first 3 cycles in both arms. An appropriate surgical excision had to be performed after neoadjuvant treatment; and this was defined as wide resection, or a more radical procedure (i.e., amputation) if, with limb sparing surgery, the function of the salvaged limb was predicted to be poor. Follow-up imaging tests were scheduled every 3 months during the first 3 years, then every 6 months for the next 2 years, and thereafter once a year, in order to assess metastatic sites. For local control, an MRI was usually done every 6 months in the first 3 years and thereafter once a year. All patients signed the informed consent. We selected a subgroup of patients among those who entered that study whose adequate tumor tissue was available for mRNA and protein expression analysis.

Immunohistochemistry

Most tissue material of this series stemmed from core-biopsy and some cases from surgical specimens; all of them were collected at the time of diagnosis before the administration of any neoadjuvant treatment. All the cases were reviewed by expert pathologists in the field of sarcoma: 2 pathologists (PDT, ALLB) for diagnostic confirmation and another pathologist (RR) carried out the current analysis without any knowledge of clinical data.

Immunohistochemistry studies were performed on formalin-fixed, paraffin-embedded (FFPE) tissues, 3–4 μm sections on all our cases. The following monoclonal antibody markers were used in this study: \( \text{P-gp1 (C494 Ab Purified; Covance), MRP1 (QCRL-1; Santa Cruz biotechnology), and glutathione S-transferase pi (GST-p; LW29; Novostra)} \). Working dilutions were as follows: \( \text{P-gp1 1:20, MRP1 1:100, and GST-p 1:200} \). Positive controls were tested according to the manufacturer’s
instructions: human liver for P-GP-1, kidney for MRP1, and for GST-π liver and bile ducts. In each batch, positive and negative controls were done. Primary antibodies were incubated for 40 minutes at room temperature with pH 6. The detection was based on the Envision system (Dako cytometry) with diaminobenzidine as the chromogen. Immunohistochemical positive cases were considered if they displayed staining in at least 10% of cells. All proteins were evaluated qualitatively, as positive or negative. In P-gp in particular, a quantitative scoring was considered as 0 (negative), 1 (moderate), and 2 (strong).

RT-PCR for the quantification of ABCB1 (MDR1 or P-gp), ABCC1 (MRP1), and GSTA1

One representative FFPE tumor block was identified and three 20-μm-thick sections were obtained for total RNA extraction using the Recover All Total Nucleic Acid Isolation kit (Ambion) as per the manufacturer’s instructions. RNA concentration was measured at 260 and 280 nm using the Nanodrop 1000 spectrophotometer (Thermo Scientific). After this, 1 μg of the RNA obtained was used to test RNA integrity, by checking the 28S and 18S ribosomal bands in a 1% agarose gel electrophoresis stained with ethidium bromide and visualized with ultraviolet light.

Reverse transcription was performed from 200 ng of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). In brief, the cDNA synthesis was carried out in a 20 μL final volume reaction containing 2 μL of RT buffer (10×), 2 μL of random hexamers (10×), 0.8 μL of dNTP Mix (25×), 1 μL of RNase inhibitor (50 U/μL), and 1 μL of MultiScribe reverse transcriptase (50 U/μL). The thermo cycler program consisted of: 10 minutes at 25°C, 120 minutes at 37°C, and a final step of 5 minutes at 85°C.

ABCB1 (MDR1 o P-gp), ABCC1 (MRP1), and GSTA1 expression levels were determined in triplicate by qRT-PCR in 10 μL reactions containing 2 μL of the synthesized cDNA, 5 μL of Taq Man Fast Universal PCR Master Mix (Applied Biosystems), and 1 μL of the corresponding expression assay (GSTA1: Hs00275575_m1; ABCC1: Hs00219905_ml; ABCB1: Hs01067802_m1; Applied Biosystems). All PCR reactions were carried out on a 7500 Fast Real Time PCR system (Applied Biosystems) with the following PCR parameters: an initial hold of 95°C for 10 seconds, followed by 95°C for 30 seconds and 30 seconds at 60°C, repeated for 40 cycles. The expression of these genes was normalized with the β2-microglobulin (Hs99999907_m1; Applied Biosystems) and GAPDH (Hs00266705_m1; Applied Biosystems). Expression was then calibrated using a universal human RNA pool (Catalog No. 740000 Stratagene) to normalize the relative expression of the genes analyzed following the 2−ΔΔCt method (18).

For the purpose of this analysis, the values of these expression genes were grouped either above/below the median value or by quartiles.

Statistics

For variables that follow binomial distributions (i.e., response rate) and categorical variables, frequency and percentages were calculated. A χ2 test and Fisher exact test were used to compare categorical variables. For time-to-event variables [i.e., relapse-free survival (RFS) or overall survival (OS)], Kaplan–Meier estimations were used and the log-rank test was used to compare groups. To analyze the reduction of risk and the influence of other variables on time-to-event variables, Cox regression was applied.

Results

A subset of 102 patients was considered for this analysis. The median age was 51 years and the median of tumor size was 11 cm (5–22 cm). There were more cases located in lower limbs than in upper limbs or trunk wall (Table 1).

Immunohistochemistry

Immunostaining was positive in 64%, 19%, and 18% of cases for P-gp, GST-π, and MRP1, respectively. From 102 cases, tumor tissue of paraffin-embedded core biopsies was not sufficient to allow an appropriate immunohistochemical evaluation in 2 cases for P-gp, 11 cases for GST-π, and 26 for MRP1. For all proteins,
immunohistochemical staining for positive and negative controls were relevant. MRP1 staining was membranous and occasionally also cytoplasmic, whereas for P-gp only membranous staining was considered positive and GST-π was cytoplasmic and nuclear (Fig. 1). Histologic types were grouped as undifferentiated pleomorphic sarcoma (n = 43), synovial sarcoma (n = 23), and others (n = 36). This latter subset included 11 leiomyosarcomas, 10 spindle cell sarcomas (NOS), 5 pleomorphic liposarcomas, 3 fibrosarcomas, 3 malignant peripheral nerve sheath tumors (MPNST), 2 pleomorphic rhabdomyosarcomas, and 2 myxoid/small round cell liposarcomas. Intraobserver reproducibility for qualitative immunohistochemistry was at least 91%.

Figure 1. A, MRP1 immunohistochemistry. Positive mixed (membrane and cytoplasmic) immunostaining for MRP1 in undifferentiated pleomorphic sarcoma (UPS) (i), predominantly membranous in synovial sarcoma (ii), in NOS fusocellular sarcoma (iii), and in dedifferentiated liposarcoma (iv). MRP1 positive control in parietal cells of gastric mucosa (v) and negative control in tonsil tissue (vi). B, P-gp and GST-π immunohistochermistry. Immunostaining for P-gp was membranous, examples in UPS (i) and monomorphic synovial sarcoma (ii), P-gp positive control in hepatic bile duct (iii) and negative control in tonsil (iv). Immunostaining for GST-π was mixed cytoplasmic and nuclear, examples of UPS (v) and synovial sarcoma (vi). GST-π positive control in Kupffer cells (vii) and negative control in tonsil (viii).
RT-PCR
Determination of \textit{ABCB1} (\textit{MDR1} or \textit{P-gp}), \textit{ABCC1} (\textit{MRP1}), and \textit{GSTA1} expression was available for 78 cases. Only in 3 cases was no expression of the gene seen: one case of undifferentiated pleomorphic sarcoma in \textit{ABCB1}, one case of leiomyosarcoma in \textit{ABCC1}, and synovial sarcoma in \textit{GSTA1}. RNA gene expression values were as follows: \textit{ABCB1} ranged from 0.0027 to 2.0863 with a median of 0.4695, \textit{ABCC1} ranged from 0.0418 to 1.5771 with a median of 0.2532, and \textit{GSTA1} ranged from 0.0052 and 3.6790 with a median of 0.6925.

Association analyses
A trend toward a significant association between mRNA and protein expression for \textit{ABCC1}/\textit{MRP1} \( \text{P} = 0.056 \) was found. However, in the case of \textit{ABCB1} (\textit{P-gp}) and \textit{GSTA1} (\textit{GST-\text{\pi}}) there was not a positive significant association.

Forty-one events of recurrence and 37 events of death occurred in this series. There was a statistically significant relationship between protein expression of \textit{MRP1} and recurrence: 73\% versus 35\% of recurrences for \textit{MRP1} positive or negative, respectively \( \text{P} = 0.02 \). However, despite a trend toward more death events, positive immunostaining of \textit{MRP1} was not associated significantly with death events: 50\% versus 35\% \( \text{P} = 0.34 \). However, neither \textit{P-gp} nor \textit{GST-\text{\pi}} protein expression showed significant correlation with recurrence or death (Table 2). Similarly, relapses were more frequently associated with \textit{ABCC1} (MRP1) expression levels above the median value: 61\% versus 39\% \( \text{P} = 0.06 \), although no further correlations were found between \textit{ABCB1} (\textit{P-gp}) or \textit{GSTA1} (\textit{GST-\text{\pi}}) expression levels and relapse or death events (data not shown).

Interestingly, a statistically significant relationship was seen between immunostaining for \textit{P-gp} and \textit{GST-\text{\pi}} and RECIST response. Positive cases were thus more probably related to progressive disease (12\% vs. 3\% in the case of \textit{P-gp}/\textit{P-gp} and 25\% vs. 6\% in the case of \textit{GST-\text{\pi}}/\textit{GST-\text{\pi}}) and less probably related to partial response (14\% vs. 42\% in the case of \textit{P-gp}/\textit{P-gp} and 8\% vs. 33\% in the case of \textit{GST-\text{\pi}}/\textit{GST-\text{\pi}}). Regarding \textit{MRP1}, a similar tendency was seen, but it did not reach statistical significance (Table 3).

Survival analysis
The median follow-up duration for alive patients was 68 months. The 5-year RFS and OS for the 102 patients was 59\% [95\% confidence interval (CI), 49–68] and 64\% [95\% CI, 55–74], respectively. All but 3 of the recurrences were at least metastatic and 4 patients died because of second neoplasia without recurrence.

\textbf{Clinicopathologic and molecular variables.} Age, gender, location (lower limbs, upper limbs, and trunk wall), size (5–9.9 cm vs. \geq 10 cm), and RECIST response had no influence on RFS. With regard to histologic subtypes, the following distribution was associated with significantly different RFS \( \text{P} = 0.028 \) in univariate analysis: undifferentiated pleomorphic sarcoma (5-year RFS rate of 74\%), synovial sarcoma (5-year RFS rate of 43\%), and other histologies (5-year RFS rate of 49\%). Patients with positive immunostaining of \textit{MRP1} had significantly lower RFS (5-year RFS rate of 23\%) than patients with negative protein expression of \textit{MRP1} (5-year RFS rate of 63\%) with \text{P} = 0.029. Similarly, as regards gene expression, cases with \textit{ABCC1} (MRP1) expression level above the median value had worse RFS (5-year RFS rate of 47\%) compared with those below the median value (5-year RFS rate of 67\%) that came close to, but did not entirely reach, statistical significance \( \text{P} = 0.06 \). Remarkably, if RNA expression of \textit{ABCC1} (MRP1) was distributed by quartiles, cases belonging to the 4th quartile had significantly worse RFS and OS (Table 4). Hence, we built a new variable combined \textit{ABCC1}/\textit{MRP1} that included either those positive cases of \textit{MRP1} or those patients with RNA \textit{ABCC1} values within the 4th quartile. This combined variable showed a highly significant prognostic role for RFS and OS (Table 4 and Fig. 2). In addition to this, if the specific OS is taken into account, the 5-year OS rate was 40\% versus 76\% for the highest or the lowest values, respectively, of combined \textit{ABCC1}/\textit{MRP1} \( \text{P} = 0.008 \). The remaining genes: \textit{ABCB1} (\textit{P-gp}) and \textit{GSTA1} (\textit{GST-\text{\pi}}) expression levels did not have any prognostic role (data not shown).

\begin{table}
\centering
\begin{tabular}{|l|c|c|c|c|}
\hline
\textbf{Protein} & \textbf{n} & \textbf{Recurrence (\%)} & \textbf{P} & \textbf{Death (\%)} & \textbf{P} \\
\hline
\textbf{P-gp (n = 100)} & & & & & \\
Positive & 64 & 39 & 0.59 & \\
Negative & 36 & 44 & 0.34 & \\
\hline
\textbf{MRP1 (n = 76)} & & & & & \\
Positive & 11 & 73 & 0.02 & \\
Negative & 65 & 35 & 0.34 & \\
\hline
\textbf{GST-\text{\pi} (n = 91)} & & & & & \\
Positive & 17 & 35 & 0.55 & \\
Negative & 74 & 43 & 0.93 & \\
\hline
\end{tabular}
\caption{Crosstab of protein expression and events of recurrence or death}
\end{table}
Only those variables showing a statistically significant relationship to RFS or to OS in the univariate analysis were entered in the Cox’s proportional hazard model. Thus, histologic types according to intended grouping, immunostaining for MRP1, RNA ABCC1 expression quartiles (4th vs. < 4th), and combined ABCC1/MRP1 were examined in the multivariate model, using a forward and backward stepwise method. Combined ABCC1/MRP1 was shown to be the only independent predictor for both RFS with HR \(= 2.704\) (95% CI, 1.361–5.374), \(P = 0.005\), and OS with HR \(= 2.208\) (95% CI, 1.086–4.492), \(P = 0.029\). If we consider the combination of positive MRP1 and mRNA ABCC1 above the median values, similar results are obtained (data not shown). Similarly, our study was unable to demonstrate a significant prognostic role for P-gp for survival after analyzing this protein by quantitative or qualitative methods; neither was there seen to be a prognostic role for GST-\(\pi\).

**Discussion**

This study demonstrates the prognostic relevance for RFS and OS of MRP1 expression in a series of patients with localized high-risk STS, treated homogeneously, in a prospective phase III trial in which all patients received a chemotherapeutic regimen consisting of epirubicin plus ifosfamide every 3 weeks followed by complete surgical resection. To the best of our knowledge, this study is the first one demonstrating a significant prognostic relationship between one MDR factor, MRP1, and survival in patients with sarcoma. Moreover, the statistical association was not only significant for RFS and independent in Cox’s multivariate model, it also significantly correlated with OS, if we take into account the combined ABCC1/MRP1 expression. As mentioned earlier, a remarkably wider variety of transport substrates have been identified for MRP1, in comparison with P-gp. Thus, whereas epirubicin, an anthracycline compound, is a substrate for both efflux pumps P-gp and MRP1, the oxazaphosphorine ifosfamide is extruded from the cell through MRP1 (5, 19). More specifically, GSTs coparticipate in the detoxification of ifosfamide with GSH conjugation reactions (20) and it has been established that the active cellular efflux of GSH conjugates is mediated primarily by MRP1 (21, 22). Together, these data suggest a prominent role of MRP1 as the main drug resistance mechanism in the series of patients treated with anthracyclines and ifosfamide as our results confirm.

Published data addressing the prognostic impact of MRP1 expression in localized tumors, in terms of RFS or OS, are very limited. Thus, overexpression of MRP1 has been related to poor prognosis in patients with localized non–small cell lung carcinoma receiving cisplatin-based chemotherapy, a substrate of MRP1 (23–25). Similarly, the expression of MRP1 was associated with an increased risk of failure in cases with small tumors, in node-negative tumors and in node-positive patients in a retrospective series of patients with localized breast carcinoma receiving cisplatin-based chemotherapy, a substrate of MRP1 (23–25). Similarly, the expression of MRP1 was associated with an increased risk of failure in cases with small tumors, in node-negative tumors and in node-positive patients in a retrospective series of patients with localized breast cancer, who received adjuvant systemic chemotherapy based on MRP1 substrates such as cyclophosphamide and methotrexate (26). Nevertheless, another study found a statistically significant relationship of GST, but not MRP1, with disease-free survival in patients with localized breast cancer treated with adjuvant chemotherapy (27). Few retrospective studies analyze the prognostic impact of MDR factors in STS; in most of these, a correlation with clinicopathologic variables was performed, rather than survival analyses. Komdeur and colleagues found that MRP1 and lung resistance–related protein expression by immunohistochemistry was significantly higher in grades 2 and 3 STS, when compared with grade 1.

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**Table 3. Relationship between clinical and pathological variables with protein expression**

<table>
<thead>
<tr>
<th>Variable</th>
<th>MRP1+</th>
<th>MRP1−</th>
<th>PR</th>
<th>SD</th>
<th>PD</th>
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<tbody>
<tr>
<td>Histotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UPS</td>
<td>7%</td>
<td>93%</td>
<td></td>
<td></td>
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<tr>
<td>Synovial S</td>
<td>22%</td>
<td>78%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>11%</td>
<td>89%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lower limbs</td>
<td>13%</td>
<td>87%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper limbs</td>
<td>13%</td>
<td>87%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trunk wall</td>
<td>0%</td>
<td>100%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Size</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5–9.9 cm</td>
<td>14%</td>
<td>86%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥10 cm</td>
<td>14%</td>
<td>86%</td>
<td></td>
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<tr>
<td>Response</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>PR</td>
<td>5%</td>
<td>95%</td>
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<tr>
<td>SD</td>
<td>10%</td>
<td>90%</td>
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<tr>
<td>PD</td>
<td>33%</td>
<td>67%</td>
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</table>

**Abbreviations:** UPS, undifferentiated pleomorphic sarcoma; PR, partial response; SD, stable disease; PD, progressive disease.
(12) whereas Oda and colleagues detected a significant correlation between P-gp expression and the largest tumors (≥5 cm) or high AJCC-staged tumors (13). In similar vein, Nakanishi and colleagues also documented that tumors expressing P-gp had a less favorable prognosis among high- or intermediate-grade STS in a series of 55 cases, some of which were treated with adjuvant chemotherapy (28). In synovial sarcoma, P-gp, and to a lesser extent MRP1, were not prognostic factors in a series of 54 cases (29). In another subset of 44 patients with STS, moderate to high expression of P-gp showed a trend toward worse prognosis, yet not reaching statistical significance (30). The most plausible mechanism underlying the poor prognosis of ABCC1/MRP1 is related to its ability to remove diverse xenobiotics from the cell, including anticancer drugs. Besides this, some authors have found that MDR expression, at protein and/or RNA level, correlated with high grade (12, 13, 28) or stage III/IV (13) in STS. This is something that is not possible to test in our series, because only highest grade (G3 according FNCLCC) and stage III were included. Substantial evidence of regulation of ABCB1 by p53 has been reported (13), possibly by a direct interaction with the ABCB1 promoter. This interaction could also modulate the results, even though most of STS cases have p53 mutated.

Considerable differences have been described in the percentage of P-gp positivity, according to previous immunohistochemical studies in patients with STS. The results in the largest series of 140 and 141 patients have ranged considerably from 18% to 79%, respectively

### Table 4. Univariate analysis of clinicopathologic and molecular factors

<table>
<thead>
<tr>
<th>Variable</th>
<th>5y-RFS (95% CI)</th>
<th>P</th>
<th>5y-OS (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histotype</td>
<td></td>
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</tr>
<tr>
<td>UPS</td>
<td>74% (61–87)</td>
<td>0.028</td>
<td>86% (76–96)</td>
<td>0.001</td>
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<tr>
<td>Synovial sarcoma</td>
<td>43% (23–64)</td>
<td></td>
<td>55% (35–76)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>49% (32–67)</td>
<td></td>
<td>45% (28–62)</td>
<td></td>
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<tr>
<td>Location</td>
<td></td>
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<tr>
<td>Lower limbs</td>
<td>61% (49–73)</td>
<td>0.11</td>
<td>67% (56–78)</td>
<td>0.21</td>
</tr>
<tr>
<td>Upper limbs</td>
<td>43% (23–64)</td>
<td></td>
<td>50% (29–71)</td>
<td></td>
</tr>
<tr>
<td>Trunk wall</td>
<td>86% (60–100)</td>
<td></td>
<td>86% (60–100)</td>
<td></td>
</tr>
<tr>
<td>Size</td>
<td></td>
<td>0.36</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>5–9.9 cm</td>
<td>65% (46–83)</td>
<td></td>
<td>72% (54–90)</td>
<td></td>
</tr>
<tr>
<td>≥10 cm</td>
<td>53% (38–68)</td>
<td></td>
<td>61% (47–76)</td>
<td></td>
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<tr>
<td>P-gp</td>
<td></td>
<td>0.75</td>
<td>0.29</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>60% (48–72)</td>
<td></td>
<td>61% (49–73)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>55% (38–71)</td>
<td></td>
<td>70% (52–87)</td>
<td></td>
</tr>
<tr>
<td>GSTπi</td>
<td></td>
<td>0.61</td>
<td>0.74</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>65% (42–87)</td>
<td></td>
<td>61% (38–86)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>56% (45–68)</td>
<td></td>
<td>64% (53–75)</td>
<td></td>
</tr>
<tr>
<td>MRP1</td>
<td></td>
<td>0.029</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>23% (0–49)</td>
<td></td>
<td>44% (11–76)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>64% (52–73)</td>
<td></td>
<td>67% (57–77)</td>
<td></td>
</tr>
<tr>
<td>Response (RECIST)</td>
<td></td>
<td>0.57</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>58% (43–73)</td>
<td></td>
<td>72% (59–85)</td>
<td></td>
</tr>
<tr>
<td>PR</td>
<td>50% (28–72)</td>
<td></td>
<td>54% (31–76)</td>
<td></td>
</tr>
<tr>
<td>PD</td>
<td>60% (17–100)</td>
<td></td>
<td>50% (11–89)</td>
<td></td>
</tr>
<tr>
<td>MRP1 quartiles:</td>
<td></td>
<td>0.052</td>
<td>0.065</td>
<td></td>
</tr>
<tr>
<td>1st quartile</td>
<td>59% (37–82)</td>
<td></td>
<td>55% (32–77)</td>
<td></td>
</tr>
<tr>
<td>2nd quartile</td>
<td>75% (56–94)</td>
<td></td>
<td>83% (54–100)</td>
<td></td>
</tr>
<tr>
<td>3rd quartile</td>
<td>60% (38–82)</td>
<td></td>
<td>65% (45–86)</td>
<td></td>
</tr>
<tr>
<td>4th quartile</td>
<td>33% (11–55)</td>
<td></td>
<td>41% (18–63)</td>
<td></td>
</tr>
<tr>
<td>MRP1:</td>
<td></td>
<td>0.012</td>
<td>0.032</td>
<td></td>
</tr>
<tr>
<td>1st to 3rd quartile</td>
<td>65% (53–77)</td>
<td></td>
<td>68% (55–80)</td>
<td></td>
</tr>
<tr>
<td>4th quartile</td>
<td>33% (12–55)</td>
<td></td>
<td>41% (18–63)</td>
<td></td>
</tr>
<tr>
<td>Combined ABCC1/MRP1+</td>
<td></td>
<td>0.003</td>
<td>0.025</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>69% (56–81)</td>
<td></td>
<td>70% (58–82)</td>
<td></td>
</tr>
<tr>
<td>Positive/highest</td>
<td>33% (14–52)</td>
<td></td>
<td>40% (19–61)</td>
<td></td>
</tr>
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</table>

Abbreviations: UPS, undifferentiated pleomorphic sarcoma; PR, partial response; SD, stable disease; PD, progressive disease. *Positive/highest includes cases with positive immunostaining MRP1 or ABCC1 expression of the 4th quartile.
As far as MRP1 is concerned, the available data about immunostaining in STS is by far lesser in quantity and positive cases have ranged from 43% to 52%, this latter value found in a synovial sarcoma series (12, 13, 29). Published reports on GST-p immunostaining in STS are even scarcer, with positivity in around half of MRP1 negative: 63% (52–73)

UPS: 74% (61–87)

Other: 49% (32–67)

Synovial sarcoma: 43% (23–64)

A B

C D

E F

Figure 2. Kaplan–Meier curves for RFS (A–E) according to: A, histologic type grouping; B, protein MRP1 expression; C and D, RNA ABCC1 gen (MRP1) expression distributed by quartiles; E, combined ABCC1/MRP1 information and Kaplan–Meier curve for OS (F) according to combined ABCC1/MRP1 information.

(11–13, 30, 31). As far as MRP1 is concerned, the available data about immunostaining in STS is by far lesser in quantity and positive cases have ranged from 43% to 52%, this latter value found in a synovial sarcoma series (12, 13, 29). Published reports on GST-p immunostaining in STS are even scarcer, with positivity in around half of
the patients (32, 33). In our study, the rate of positive P-gp was 64%, which is in consonance with previous studies in STS using the same antibody (34). The differences we found as regards the positive rate for MRPI and GST-π compared with previous studies could be explained by the use of different monoclonal antibodies. In fact, specific epitopes that react to distinct reagents have been recognized in MRPI; these provide real insights into the topology of MRPI. It has thus been recognized that MAb QCRL-1, the anti-MRP1 we used in the present study, shows itself to be highly specific for the human protein MRPI, and reacts against an epitope of cytoplasmic location (35).

Few studies were performed on mRNA expression of MDR genes in STS. They mostly focus on the correlation with clinicopathologic factors instead of on survival (13, 23). Protein expression of MRPI showed a significant prognostic correlation with RFS and RNA expression of MRPI displayed a similar prognostic behavior in univariate analysis. Nevertheless, none of these expressions reached statistical significance for OS even though a clear trend was seen. Therefore, a combination of both factors was considered. Thus, even though the protein and RNA expressions exhibit a significant prognostic role in localized high-risk STS, the combined ABC1/MRPI expression is a more robust prognostic variable that also independently affects overall survival.

Interestingly, factors significantly associated with RECIST response, such as P-gp and GST-π, did not show any significant prognostic relationship with either RFS nor OS. This finding could be explained with reference to another substudy derived from the same trial. In this substudy, partial response was not able to anticipate better clinical outcome, RFS or OS, compared with stable disease according to RECIST criteria (36). Moreover, some progressive disease cases have been reported as complete pathologic response in the examination of surgical specimens.

Limitations derived from technical aspects, such as cross-reactivity of C494 (reagent of P-gp) with pyruvate carboxylase (37, 38), or the scarcity of tumor sampling are acknowledged by the authors of this article. Even though MRPI, as transporter of both anthracyclines and ifosfamide, was expected to be the most relevant prognostic factor in our series as ultimately did occur, we have to admit that significant controversy does exist in the literature around the prognostic role of MDR genes in STS, especially related to P-gp expression.

Finally, a renewed interest in targeting drug resistance mechanisms has recently emerged (39, 40), this being especially relevant in STS, where only a few active drugs are available (41–43). Thus, blocking MRPI expression by nilotinib resulted in intracellular doxorubicin retention, reverting MDR activity and showing synergistic antiproliferative effects in in vitro analysis (44). Consequently, because ABC1/MRPI expression shows prognostic relevance in the high-risk STS population and MRPI protein is in addition suitable for targeting, new therapeutic approaches deserve to be designed in this patient population. Thus, in a practical setting, a molecular targeting therapeutic strategy would be administering an MRPI inhibitor as co-adjuvant of doxorubicin in STS. This implies administering MRPI inhibitor a few days before doxorubicin and over a few additional days concurrently to doxorubicin, mimicking in vitro synergistic sequence. This strategy is being tested in an ongoing phase I/II clinical trial in STS and opens an innovative way to explore these MRPI inhibitor compounds with the most upfront active drugs in STS.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Study supervision: J. Martin-Broto, A.P. Dei Tos, A. Lopez-Pousa, A. Gronchi

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MRP1 Overexpression Determines Poor Prognosis in Prospectively Treated Patients with Localized High-Risk Soft Tissue Sarcoma of Limbs and Trunk Wall: An ISG/GEIS Study

Javier Martin-Broto, Antonio M. Gutierrez, Rafael F. Ramos, et al.


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