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ARTICLE

Expression of P-glycoprotein and Metallothionein in Gastrointestinal Stromal Tumor and Leiomyosarcomas. Clinical Implications

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We investigated the expression of P-glycoprotein (P-GP) and metallothionein (MT) in a series of 92 GIST and 14 gastrointestinal leiomyosarcomas (GI-LMS) with the purpose to expand our knowledge on the biological bases of GIST chemo-resistance and to ascertain their significance in patients' prognosis. P-GP expression was more frequent in GIST than in GI-LMS (83.7% vs. 21.4%, p<0.001), with no difference between low- and high-risk GIST (p=1.000) or low- and high-grade GI-LMS (p=0.538). P-GP expression was unrelated to anatomic location (gastric vs. intestinal) in GIST (39/45 vs. 35/43, p=0.770) and in GI-LMS (0/2 vs. 2/6, p=1.000). MT expression was non-significantly higher in GI-LMS than in GIST (35.7% vs. 14.1%, p=0.060), with no difference between low- and high-risk GIST (p=1.000) or low- and high-grade GI-LMS (p=1.000). MT expression was unrelated to the anatomic location (gastric vs. intestinal) in GIST (7/45 vs. 6/43) and GI-LMS (0/2 vs. 1/6) (p=1.000 and p=0.1000, respectively). Overall tumor-specific survival (p< 0.001) and disease-free survival (p<0.001) were different in GIST as compared with GI-LMS, and the number of events was higher in GI-LMS. When the survival analysis took into consideration P-GP or MT expression, the overall survival in GIST was influenced by the expression of MT (p=0.021) but not by that of P-GP (p=0.638). However, in GI-LMS, P-GP expression influenced disease-free survival (p=0.050); in addition, it is important to recognize the limited value of these results because of the low number of cases involved in the study. Differential expression of P-GP and MT might explain the known variability in response to systemic chemotherapy in these tumors. Detection of P-GP and MT seems to add certain prognostic value in GIST (MT) or GI-LMS (P-GP). (Pathology Oncology Research Vol 13, No 3, 203–208)

Key words: gastrointestinal stromal tumors, GIST, leiomyosarcomas, P-glycoprotein, metallothionein

Introduction

Gastrointestinal stromal tumors (GIST) are neoplasms originating from interstitial cells of Cajal (ICC) or their precursor,[9] and account for 1-3% of malignancies in the gastrointestinal tract (GI).[1] Until 1998, when Hirota et al[10] and Kindblom et al[13] demonstrated that GIST are a special group of GI mesenchymal tumors characterized by CD117 expression and frequent KIT mutation, most of these neoplasms were considered leiomyosarcomas (LMS), were treated with chemotherapy and had low response rate. This chemo-response contrasted with those reported in LMS in other locations where the response rate was significantly higher.[3] The biological bases of chemo-sensitivity of these neoplasms remain uncertain, but it is well known that anthracyclines and cisplatin, drugs used in

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Table 1. **Primary antibodies used in the assessment of GIST and GI-LMS**

Primary antibodies	*Clone*	*Working dilution*	*Source*	*Controls*
CD117 (c-kit)	104D2	1:50	Dako	mast cells
CD34	AM236-5M	prediluted	Biogenex	endothelial cells
Smooth muscle actin	1A4	prediluted	Dako	muscle layer from intestinal wall
Desmin	D33	prediluted	Dako	muscle layer from intestinal wall
S-100 protein	AM058-5M	prediluted	Biogenex	Schwann cells from normal intestinal wall
Metallothionein	E9	1:200	Dako	mammary gland
P170/P-glycoprotein MDR Ab-5	C494	prediluted	LabVision Co	cortical adrenal gland

advanced LMS and GIST, are the substrate for multi-drug resistance (MDR) proteins[15] and metallothionein (MT),[12] respectively.

On the other hand, since the introduction of therapeutic targeting with tyrosine kinase inhibitor imatinib (Gleevec, Novartis Pharma AG, Basel, Switzerland) for GIST treatment,[11] special effort has been made to separate this tumors from their mimicry, and specially from LMS, which has very low-to-null response to imatinib,[14] which is *in vitro* also a substrate for MDR.[7]

Our purpose is to establish whether or not the expression of the MDR proteins, particularly P-glycoprotein (P-GP) and metallothionein (MT) may correlate to the patients' outcome and if additionally might assist to explain the observed differences in response to systemic chemotherapy in a series of GIST and gastrointestinal LMS (GI-LMS).

Material and methods

Patients

The study group included 106 patients with GIST (n=92) or GI-LMS (n=14) retrieved from 3 hospitals in Southern Spain. Patients' follow-up was available in 77 cases (64 GIST and 12 GI-LMS) and was calculated as the number of months from the date of the surgical procedure to the last visit or death. The overall tumor-specific survival was defined as the time between diagnosis and the patient's death. The disease-free survival was defined as the time between the diagnosis and the first relapse or the appearance of metastases. Cancer-related death was defined as that caused by the tumor.

Pathology

GIST tumors were grouped into two prognostic categories resulting from grouping the four categories originally proposed by Fletcher et al[5] with the addition of an "overtly malignant group":[16] low (65: 3 very low risk, 42 low risk, 20 intermedium risk) and high (27: 26 high risk, 1 overtly malignant with metastasis). LMS[20] were graded as low (n=8) and high (n= 6). In order to determine if the expression of P-GP or MT in GI-LMS is region-dependent, an additional group of 26 non-GI LMS from the uterus (12), retroperitoneum (8), skin and soft tissues (6) was evaluated. Additional immunohistochemical markers (CD117, CD34, smooth muscle actin, desmin and S-100) were included to confirm pathologic diagnosis in GIST and LMS.

Available hematoxylin and eosin stained slides were reassessed by three pathologists (SGP, RGC, ALB) blind to the clinical status. For immunohistochemistry, a representative paraffin block from each tumor, selected based on the amount of tumor present, was serially cut at 4 µm thick, deparaffinized in xylene, rehydrated in graded ethanol and washed for 5 min with phosphate-buffered saline. For antigen retrieval, the sections were boiled immersed in 10 mM citrate buffer (pH 6.0). To avoid non-specific CD117 immunostaining, we followed the manufacturer recommendations and the procedure included with Target Retrieval Solution (S1699, Dako, Glostrup, Denmark). Endogenous peroxidase was blocked by incubation of the slides for 30 minutes with 3% hydrogen peroxide in methanol. Sections were then incubated with the primary mouse monoclonal antibodies at room temperature (*Table 1*).

Immunohistochemistry was performed using the highly sensitive polymer-based system (EnVision, Dako) 30 min at room temperature with diaminobenzidine chromogen substrate solution (0.6 mg/ml in Tris-buffered saline, pH 7.6, with 12 ml 30% hydrogen peroxide). Sections were counterstained with Mayer's hematoxylin, dehydrated and mounted following standard procedure. Three pathologists (SGP, RGC, ALB) independently evaluated all immunostainings in a blinded fashion. The following staining patterns were considered: CD117, cytoplasmic and/or membrane; CD34, membrane; smooth muscle actin and desmin, cytoplasmic; S-100, cytoplasmic and nuclear; P-GP, membrane and sometimes also cytoplasmic; MT, cytoplasmic and nuclear. Cases with less than 10% positive cells were considered negative.

Statistical analysis

Bivariate analysis to compare prognostic categories and the expression of P-GP and MT were undertaken by Fisher test and chi-square analysis. Univariate survival analysis for P-GP and MT expressions was conducted using Kaplan-Meier method and differences among groups were tested by log-rank test. All statistical analyses were performed using the SPSS for Windows Software (SPSS Inc, Chicago, IL, USA). A p-value of less than 0.05 was considered as significant**.**

Results

Table 2 shows relevant comparative data on GIST and GI-LMS cases included in this study. Plasma membrane and sometimes cytoplasmic expression of P-GP was observed in GIST (83.7%) and GI-LMS (21.4%) (p<0.001) (*Fig. 1a,b*). No differences were found between low- and high-risk GIST (p=1.000) or low- and high-grade GI-LMS (p=0.538). In the same way, P-GP expression was unrelated to anatomic location (gastric vs. intestinal) in GIST (39/45 vs. 35/43, p=0.770), in both low- (p=0.447) and high-risk groups (p=0.355), and in GI-LMS (0/2 vs. 2/7, p=1.000).

Table 2. **Clinical, pathological data and marker expression in 92 cases of GIST as compared with 14 GI-LMS cases**

	GIST (n=92)	*GI-LMS (n=14)*
Age (years)		
Mean	61.44	70.83
Median	64.00	74.50
Sex		
Male	49 (53.3%)	4 (28.6%)
Female	43 (46.7%)	10 (71.4%)
Location		
Stomach	45 (48.9%)	2 (14.3%)
Small bowel	38 (41.3%)	5 (35.7%)
Large bowel	5 (5.4%)	2 (14.3%)
Mesentery-omentum	4 (4.3%)	5 (35.7%)
Size (cm)		
Mean	6.85	8.28
Median	5.00	9.00
Cell type		
Spindle	66 (71.7%)	14 (100%)
Epithelioid	22 (23.9%)	–
Mixed	4 (4.3%)	–
Risk/grade category		
Low	65 (70.7%)	8 (57.1%)
High	27 (29.3%)	6 (42.9%)
Maker immunoreactivity		
P-glycoprotein	77 (83.7%)	3 (21.4%)
	Low-risk: 58 (89.2%)	Low-grade: 1 (12.5%)
	High-risk: 19 (70.4%)	High-grade: 2 (33.3%)
Metallothionein	13 (14.1%)	5 (35.7%)
	Low-risk: 8 (12.3%)	Low-grade: 3 (37.5%)
	High-risk: 5 (18.5%)	High-grade: 2 (33.3%)
Clinical follow-up	n=64 (69.5%)	n=13 (86.6%)
	Low-risk: 43 (67.2%)	Low-grade: 8 (61.5%)
	High-risk: 21(32.8%)	High-grade: 5 (38.5%)
Mean (months)	66.48	32.08
Median (months)	36.00	23.50
Tumor recurrence	10 (15.6%)	4 (33.3%)
Metastases	8 (12.5%)	6 (50%)
Alive with disease	11 (17.2%)	2 (16.7%)
Alive without disease	41 (64.0%)	3 (25.0%)
Dead of disease	8 (12.5%)	7 (58.3%)
Dead of other causes	4 (6.2%)	0 (0%)

MT was detected in scattered tumor cells in the cytoplasm and occasionally in the nucleus (*Fig. 1c,d*) in GI-LMS (35.7%) and GIST (14.1%) (p=0.060). No differences were observed between low- and high-risk GIST (p=1.000) or low- and high-grade GI-LMS (p=1.000). No association was found between MT expression and anatomical location (gastric vs. intestinal) in GIST (7/45 vs. 6/43) or GI-LMS (0/2 vs.1/6) (both p=1.000). Simultaneous P-GP and MT expression was observed in 11/92 GIST and 1/14 GI-LMS cases (p=1.000).

Overall tumor-specific survival and disease-free survival were different in GIST as compared with GI-LMS (both p<0.001); the number of events was higher in GI-LMS (*Table 3*). When the study was restricted to high-risk GIST and all GI-LMS, no significant differences were observed in overall or disease-free survival (p=0.081 and p=0.373, respectively). When the survival analysis took into consideration P-GP or MT expression, the overall survival in GIST was influenced by the expression of MT (p=0.021) but not by P-GP (p=0.638). On the other hand, P-GP expression significantly influenced disease-free survival in GI-LMS (p=0.050) (*Table 3*); however, it is important to recognize the limited value of these results because of the low number of cases involved in the study. No differences were observed

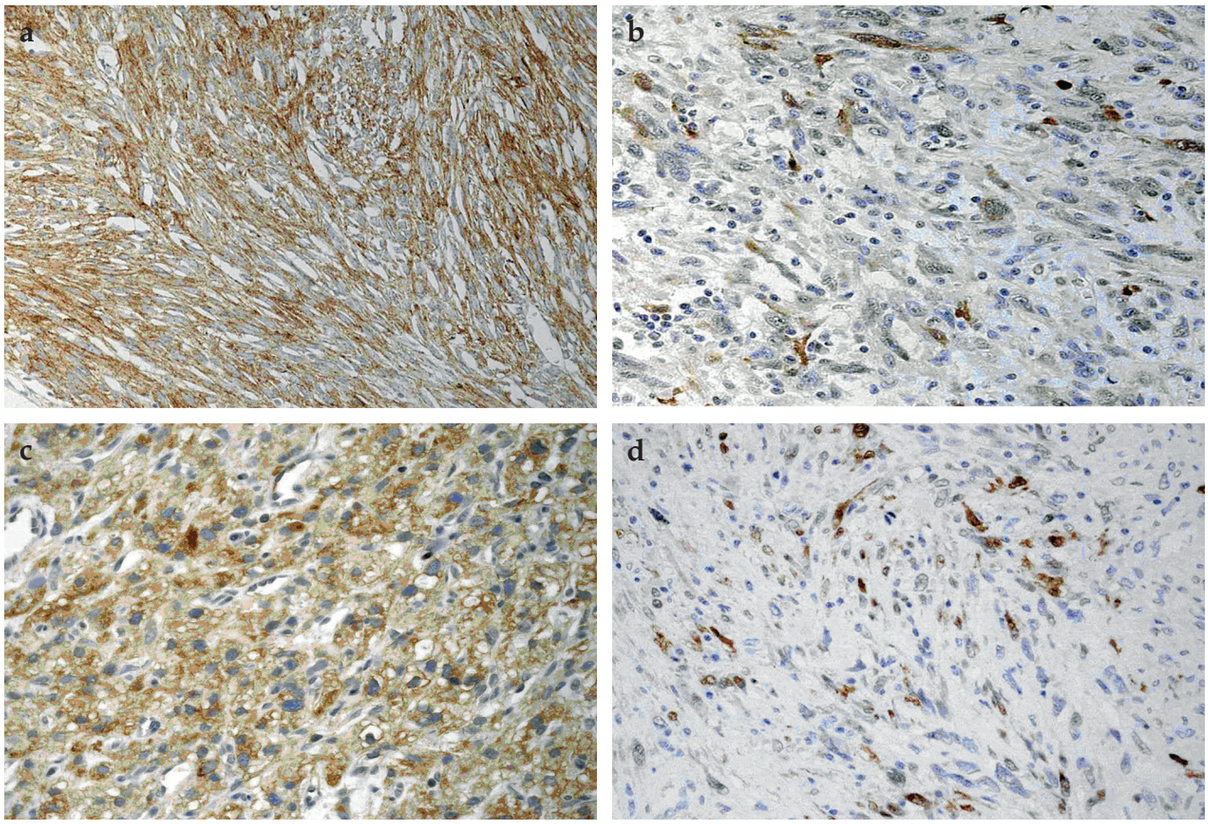

Figure 1. *Gastrointestinal stromal tumor. Positive immunoreaction to P-GP, 20x* ***(a)*** *and MT, 40x* ***(b)****. Gastrointestinal leiomyosarcoma. Positive immunoreaction to P-GP, 40x* ***(c)*** *and MT, 20x* ***(d)***

Table 3. **Overall and disease-free survival of patients with GIST and GI-LMS according to P-glycoprotein and metallothionein expression**

	Overall survival	*p-value*	*Disease-free survival*	*p-value*
GIST	P-glycoprotein – + 1/12 7/52 Metallothionein – + 5/56 3/8	 p=0.638 p=0.021	P-glycoprotein – + 4/12 15/52 Metallothionein – + 16/56 3/8	 p=0.692 p=0.571
GI-LMS	P-glycoprotein – + 7/10 0/2 Metallothionein – + 3/7 4/5	 p=0.066 p=0.258	P-glycoprotein – + 8/10 1/2 Metallothionein – + 4/7 5/5	 p=0.050 p=0.210
GIST vs. GI-LMS	8/64 vs.7/12	p<0.001	19/64 vs. 9/12	p<0.001

Data are presented as number of events / case number in the groups with negative or positive expression of the markers

between GI-LMS and non-GI LMS for P-GP (p=0.199) or MT expression (p=0.979).

Discussion

Several drug resistance proteins have been considered as the source of explaining chemo-sensitivity in malignant tumors. Two of the most extensively studied proteins are P-GP and MT. P-GP is a transmembrane protein, encoded by the MDR1 gene, which acts as an efflux transporter system for a variety of organic substances, including cytotoxic drugs, like anthracyclines, *Vinca* alkaloids and epipodophyllotoxins.[15] MT is a low-molecular-weight cystein-rich enzyme that binds divalent

metal ions and plays a protective role against anticancer drugs, such as cisplatin.[4,12] It is well known that GIST is more chemo-resistant than LMS,[3,14,18] but little is known on the subjacent mechanism. Plaat et al[18] studied the expression of P-GP, MRP1 and LRP in 29 patients with soft tissue LMS and 26 patients with a primary malignant GIST, and found that P-GP and MRP1 expressions were significantly higher in GIST than in LMS, and that patients with LMS had a better overall survival and a metastatic pattern different from GIST. More recently, Theou et al[19] investigated the expression of MDR proteins (P-GP, MRP1 and BCRP) by Western blotting in 21 GIST cases, showing P-GP and MRP1 in 86% and 62%, respectively, and negativity for BCRP. These authors found significant differences in P-GP expression between gastric and non-gastric tumors. In addition, they suggested that MDR proteins do not impair the initial response of the tumor to imatinib, since none of the six patients treated with imatinib was resistant.

In the present series we have found similar results regarding P-GP, showing significant differences between GIST and GI-LMS. On the other hand, GI-LMS had a worse overall ($p<0.001$) and disease-free ($p=0.001$) survival than GIST, but when only high-risk GIST entered the analysis, no differences were found. This finding, which differs from that reported by Plaat et al,[18] may be related to Plaat's selection criteria since they included high-risk GIST only, 68% of which expressed c-kit. In this series we could not confirm the different P-GP expression between gastric and intestinal cases, since 39/45 of gastric and 35/43 of non-gastric neoplasms expressed P-GP. None of our patients were treated with imatinib.

MT expression is known as an adverse prognostic factor in some tumors, including sarcomas[2] but, as far as we know, this is the first report relating its expression with survival in GIST. We have found no significant differences between GI-LMS (35.7%) and GIST (14.1%) ($p=0.060$), but in GIST, MT expression was associated with overall cancer-specific survival ($p=0.021$). Our results in GI-LMS agree with those of Gaumann et al[6] where MT expression in LMS was unrelated to the patients' survival.

We did not find any differences in the P-GP and MT expression between GI-LMS and non-GI LMS, a finding that may help to explain that the lower response rate to conventional chemotherapy, observed in previous GI-LMS series,[3] could be related to misclassification of tumors rather than their location. Nonetheless, chemo-resistance is a complex phenomenon where other drug resistance[21] and/or anti-apoptotic mechanisms related to KIT or PDGFRA overexpression are implicated.[8,17] In GIST, imatinib resistance seems to be related to KIT or PDGFRA mutations rather than to MDR proteins systems, since some specific mutations cause a low to null response.[8]

In conclusion, differences in P-GP and MT expression could help to explain the observed response to systemic chemotherapy in GIST and LMS. Immunoexpression of P-GP and MT may assist in differentiating GIST and GI-LMS in selected cases, and seems to and certain prognostic value in GIST (MT) and GI-LMS (P-GP).

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