Cathepsin L is highly expressed in gastrointestinal stromal tumors

KOHTARO MIYAMOTO[1*], MANABU IWADATE[1*], YUKA YANAGISAWA[2], EMI ITO[2], JUN-ICHI IMAI[2], MASAYA YAMAMOTO[3], NAOKI SAWADA[3], MOTONOBU SAITO[1], SATOSHI SUZUKI[1], IZUMI NAKAMURA[1], SHINJI OHKI[1], ZENICHIRO SAZE[4], MICHIHIKO KOGURE[4], MITSUKAZU GOTOH[4], KAZUTOSHI OBARA[5], HIROMASA OHIRA[6], KAZUHIRO TASAKI[7], MASAFUMI ABE[7], NAOKI GOSHIMA[8], SHINYA WATANABE[2], SATOSHI WAGURI[3] and SEIICHI TAKENOSHITA[1]

Departments of [1]Organ Regulatory Surgery, [2]Clinical Genomics, [3]Anatomy and Histology, [4]Organ Regenerative Surgery, [5]Endoscopy, [6]Gastroenterology and Rheumatology, [7]Pathology and Diagnostic Pathology, Fukushima Medical University, School of Medicine, Fukushima; [8]National Institute of Advanced Industrial Science and Technology, Tokyo, Japan

Received May 13, 2011; Accepted June 19, 2011

DOI: 10.3892/ijo.2011.1127

Abstract. Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the gastrointestinal tract that are diagnosed by c-kit staining in most cases. A lysosomal cysteine proteinase termed cathepsin L has been commonly associated with malignancy in several cancer types, but this finding has not been reported for GISTs. We analyzed the cathepsin L mRNA and protein expression in GISTs. Real-time reverse transcription polymerase chain reaction (RT-PCR) analysis revealed that cathepsin L levels were higher in GISTs than those in gastric or colorectal tumors; this finding was supported by results of the Western blot analysis. Immunohistochemistry revealed that cathepsin L was localized to the cytoplasm of GIST cells as an intense granular signal, which was not observed in the cells of leiomyoma, a mesenchymal tumor that was analyzed as a control specimen. Double immunofluorescence microscopy revealed that a portion of the granular signal colocalized with lysosome-associated membrane protein-1 (LAMP-1), which is a lysosomal marker. Moreover, immunohistochemical analysis of 43 tumor specimens revealed that 86.0% (n=37) were cathepsin-L positive, and this positivity was significantly correlated with c-kit positivity but not with other clinicopathological factors, including gender, age, region, size, mitosis and risk of recurrence. From these results, we conclude that cathepsin L is highly expressed in GISTs compared to its expression in other cancerous lesions; this identifies cathepsin-L as a new diagnostic marker for GISTs.

Correspondence to: Professor Seiichi Takenoshita, Department of Organ Regulatory Surgery, Fukushima Medical University School of Medicine, Fukushima, Japan
E-mail: takenoss@fmu.ac.jp

*Contributed equally

Key words: cathepsin L, gastrointestinal stromal tumors, cysteine proteinase, c-kit, tumor marker

Introduction

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the gastrointestinal tract (1). They presumably arise from the interstitial cells of Cajal, which control the autonomic nervous system of the intestine, followed by gain-of-function mutations in c-kit or platelet-derived growth factor receptor α (PDGFRA) genes (2-4). c-kit staining is the most reliable and widely used marker for GIST diagnosis, but the occurrence of c-kit-negative GISTs remains an issue. Although CD34 staining and/or the detection of c-kit and PDGFRA gene mutations can facilitate the diagnosis of c-kit-negative GISTs (5), more accurate diagnostic markers are required.

The primary treatment for GISTs is surgical resection. Imatinib mesylate, which selectively inhibits a group of tyrosine kinase receptors including c-kit and PDGFRA, has proven effective in the management of recurrent and unresectable GISTs (6). The risk of recurrence after surgery is predicted according to tumor size and mitotic rate, which stratify the risk into four categories: very low, low, intermediate and high (1). Because GISTs exhibit unpredictable behavior and tend to recur within the first 3-5 years, long-term follow-up using contrast computed tomography (CT) scanning should be applicable for all patients, including those fitting into the very low risk category (7). Therefore, the discovery of sensitive GIST markers would greatly enhance the effectiveness of this postoperative follow-up protocol.

Cathepsin L is a lysosomal enzyme that exhibits strong endopeptidase activity. Over-expression of cathepsin L and abnormality of the protein sorting system are known etiologies of the extracellular secretion of cathepsin L, which is capable of degrading extracellular matrix proteins such as collagen and elastin (8,9). Therefore, cathepsin L has often been reported to be associated with cellular invasion and metastasis in several types of cancer (10). To our knowledge, however, no report has documented the role of cathepsin L in the progression of GISTs, probably because GISTs are known to have a lower incidence of invasiveness or metastasis than that of gastric and colon cancers. We analyzed the cathepsin L mRNA and

protein expression in GIST patients and found that this lysosomal proteinase had unexpectedly high expression in GISTs.

Materials and methods

Patients and tissue samples. Tumors were surgically resected from patients with GISTs, gastric leiomyoma, gastric cancer and colorectal cancer at Fukushima Medical University Hospital, Fukushima, Japan, between 2004 and 2010. A portion of each tissue specimen was snap-frozen immediately after resection and stored at -80˚C until use. Paraffin-embedded blocks for pathological diagnosis were later used for immunohistochemistry.

A total of 43 patients had primary or recurrent operable GISTs (23 males and 20 females), whose mean age was 63.5 years (range, 14-85 years). The location and number of tumors were as follows: stomach (n=24), duodenum (n=3), ileum (n=6), esophagus (n=2), rectum (n=2), small omentum (n=1), peritoneum (n=3) and liver (n=2). The mean tumor size was 44.2 mm (range, 3-130 mm). The risk of recurrence was judged on the basis of the National Institutes of Health classification. This study was approved by the Ethics Committee of Fukushima Medical University.

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was extracted from specimens of GIST (n=15), gastric cancer (n=11) and colorectal cancer (n=9) using TRIZOL (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Non-cancerous gastric and colorectal regions were also excised for this analysis. Total RNA (2 μg) was used for the synthesis of first-strand cDNA using the Superscript III first Strand cDNA Synthesis Kit (Invitrogen). Real-time RT-PCR analysis was performed using the ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with TaqMan Gene Expression Assays. Primers for cathepsin L (ID Hs00377632) and β-actin (ID Hs9999903) were purchased from Applied Biosystems. Expression was normalized to that of β-actin, and then the cathepsin L/β-actin ratio in tumor tissues was further normalized to the mean value of that of non-cancerous gastric regions.

Immunohistochemistry and immunofluorescence. Tissue specimens were fixed in formalin and embedded in paraffin. Antigens were retrieved by autoclaving the specimens in 0.01 M citrate buffer (pH 6.0) followed by immunolabeling procedures. Antibodies against cathepsin L (sc-6500; Santa Cruz Biotechnology, CA, USA) and c-kit were both used at a dilution of 1:100. Their reaction with secondary antibodies and detection by diaminobenzidine were performed using the EnVision™ plus HRP system (Dako, Glostrup, Denmark), and were observed under a microscope (BX50, Olympus). Positivity was assessed according to the percentage area of the granular signal, and was classified into three grades as follows: negative (<10%), weakly positive (10-50%) and strongly positive (>50%).

For immunofluorescence, sections were blocked with 2% donkey serum and 0.1% Tween-20 in phosphate-buffered saline, followed by overnight incubation at 4˚C with primary antibodies for anti-cathepsin L and anti-lysosome-associated membrane protein-1 (LAMP-1) (Cell Signaling Technology, Inc.). They were then incubated with AlexaFluor488-conjugated donkey anti-goat immunoglobulin G (IgG) (1:400; Invitrogen) and AlexaFluor594-conjugated donkey anti-mouse IgG (1:400; Invitrogen) antibodies at room temperature for 30 min. Sections were examined by a confocal laser scanning microscope (FV1000; Olympus).

Western blot test. Tissues were homogenized in a cold 0.5 M Tris-HCl (pH 7.6) buffer containing 0.15 M NaCl, 0.1% sodium dodecyl sulfate, 1% deoxycholate Nonidet P-40 (NP40), and complete protease inhibitors (Roche, Indianapolis, IN). Protein samples were run on SDS-polyacrylamide gels (5-15% gradient; Invitrogen) and blotted onto 0.45-μm nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked with 5% skim milk in Tris-buffered saline before incubation with the primary antibodies; i.e., anti-cathepsin L (sc-6500; Santa Cruz Biotechnology) and β-actin (ac-15: Santa Cruz Biotechnology), followed by incubation with horseradish peroxidase-conjugated anti-goat IgG. Signals were detected by ImageQuant LAS4000 (GE Life Science Companies) using SuperSignal West Pico Chemiluminescent Substrate (Thermo).

As a positive control for the three forms of cathepsin L, an expression vector containing human cathepsin L cDNA was transfected into HeLa cells using FuGENE (Roche). The cells were then lysed with NP40 lysis buffer as described above.

Statistical analysis. The Mann-Whitney test was used for the analysis of real-time RT-PCR, and the Fisher's exact test was used for determining the relation between clinical parameters and cathepsin L positivity. These tests were performed using SPSS Ver.17 software (SPSS Inc., Chicago, IL, USA). $P<0.05$ was considered statistically significant.

Results

Expression of cathepsin L in GISTs. We analyzed the levels of cathepsin L expressed in GISTs and other intestinal tumors including gastric and colorectal tumors. First, we evaluated cathepsin L mRNA expression in GIST as well as gastric and colorectal tumor specimens by real-time RT-PCR. The levels of cathepsin L mRNA in GISTs were significantly higher than those in gastric ($P<0.001$) and colorectal ($P<0.05$) tumors, but there was no significant difference between cathepsin L mRNA levels in colorectal and gastric tumor specimens (Fig. 1). Moreover, there were no significant differences between the cancerous and non-cancerous regions of both gastric and colorectal tumor specimens (Fig. 1).

Next, we analyzed cathepsin L protein expression in the three types of tumor specimens by Western blotting. We also analyzed HeLa cells with or without the overexpression of cathepsin L as positive controls, in which the pro-form (~41 kDa), single-chain form (~33 kDa), and heavy-chain form (~23 kDa) of cathepsin L were identified (Fig. 2). The heavy-chain form was consistently detected in all cases, and at least two GIST specimens (middle two lanes of GIST) exhibited higher cathepsin L protein expression than the gastric and colorectal tumor specimens. Both the pro-form and single-chain form of cathepsin L were variably detected in the examined tumor specimens; both forms were detected in four GIST but not in two gastric

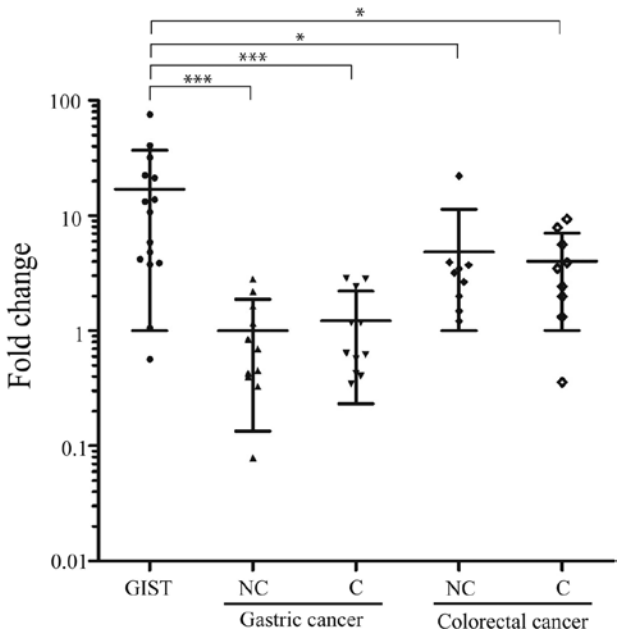

Figure 1. Cathepsin L mRNA expression in gastrointestinal stromal tumors (GISTs). Real-time polymerase chain reaction analysis of cathepsin L mRNA expression in GISTs (n=15) and in the non-cancerous (NC) and cancerous (C) regions of both gastric (n=11) and colorectal (n=9) tumor specimens. Each expression level was calculated relative to the mean of the non-cancerous region of gastric tumor specimens. Each value is plotted, and the mean ± standard deviation (SD) for each group is expressed as bars. The differences in mean values were statistically assessed using the Mann-Whitney test. *P<0.05, ***P<0.001.

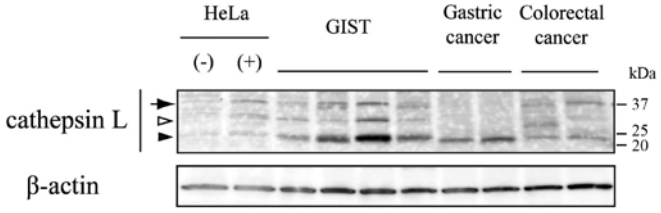

Figure 2. Cathepsin L protein expression in GIST. Western blot analysis of cathepsin L expression in GIST (n=4), gastric (n=2) and colorectal (n=2) tumor specimens. HeLa cells with (+) or without (-) transfection by cathepsin L cDNA are also included in the analysis. β-actin is shown as a loading control. The three forms of cathepsin L; i.e., pro-form (arrow), single-chain form (white arrowhead) and heavy-chain form (black arrowhead) are indicated on the left.

tumor specimens, and only the pro-form was evident in two colorectal tumor specimens. Overall, these results indicate that GISTs tend to express higher cathepsin L mRNA and protein levels than gastric or colorectal tumors.

Localization of cathepsin L in GISTs and non-cancerous regions of the gastric mucosa. Although lysosomal localization of cathepsin L has been well documented in rodents (13), its distribution in the human gastric mucosa is not well reported. Thus, we first examined the distribution of cathepsin L in non-cancerous gastric regions by immunohistochemistry. Although cathepsin L immunoreactivity was scarcely detected in the cells of the surface mucus and gastric glands, it was

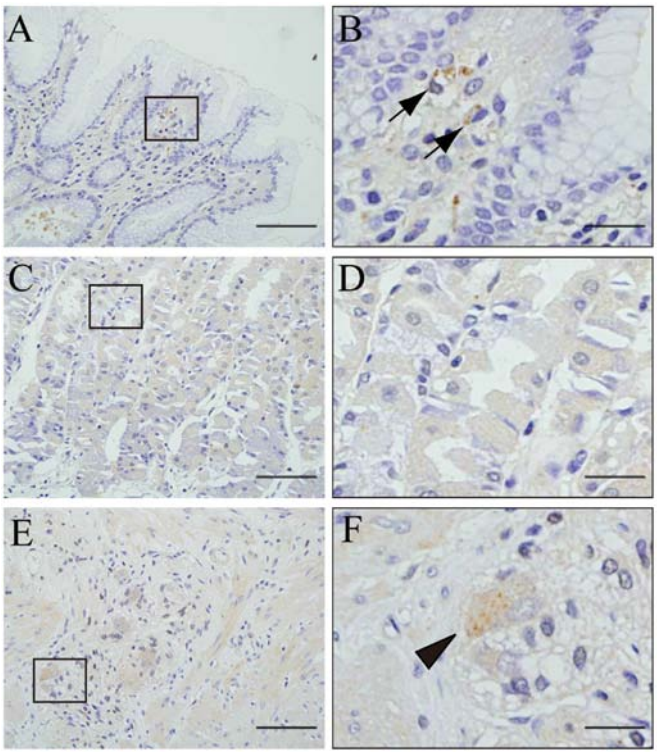

Figure 3. Immunohistochemical distribution of cathepsin L in the gastric mucosa. Cathepsin L staining in the gastric mucosa including gastric pits (A and B), gastric glands (C and D) and muscle layer (E and F). B, D and F are enlarged images of the rectangular areas in A, C and E, respectively. Positive granular staining in macrophage-like cells (B) and ganglion cells (F) are indicated by arrows and arrowheads, respectively. Scale bars: 100 μm in A, C and E; 25 μm in B, D and F.

clearly detected in the form of cytoplasmic granular staining in some macrophage-like cells in the lamina propria (Fig. 3A-D). Similar immunoreactivity was also detected in the ganglion cells of the nerve plexus, but was not evident in the smooth muscle cells of the muscularis mucosae (Fig. 3E and F).

Next, we analyzed the GIST tumor specimens. Several granular signals for cathepsin L were detected in the tumor cell cytoplasm of many c-kit-positive specimens (Fig. 4A, B, D and E). The intensity of cathepsin L staining and the number of cathepsin L-positive cells varied depending on the cases and tumor regions examined. For example, in specimens that exhibited relatively weak staining for cathepsin L, the positive cells appeared to gather in distinct small clusters in the cancerous region (Fig. 4D). Cathepsin L immunoreactivity was absent in two specimens of c-kit-negative GIST (Fig. 4G, H and I; Table I). We examined a specimen of gastric leiomyoma as a control stromal tumor; no cathepsin L positivity was detected (Fig. 4J, K and L). To confirm that the cathepsin L-positive granules in the cytoplasm were lysosomes, we performed double immunofluorescence microscopy using an antibody against LAMP-1, a marker for lysosomes (Fig. 5). A majority of cathepsin L-positive granules were found to contain a signal for LAMP-1, indicating that cathepsin L-positive granules were lysosomal structures. Conversely, several granular structures that were positive for cathepsin L but negative for LAMP-1 were present in the cytoplasm of the tumor cells. They were presumed to be endosomes or intracellular transport vesicles.

Table I. Immunohistochemical staining and clinicopathological data for 43 gastrointestinal stromal tumor (GIST) cases.

	Gender	Age	Region	Size (mm)	Mitosis (/50HPF)	Risk	KIT	CD34	Cathepsin L
1	F	80	S	3	0	Very low	+	+	++
2	M	76	S	3	3	Very low	+	+	++
3	M	68	S	10	0	Very low	+	+	++
4	F	77	S	12	0	Very low	+	+	++
5	M	60	I	20	0	Low	+	+	++
6	F	62	S	28	2	Low	+	+	++
7	M	69	S	30	1	Low	+	+	++
8	M	66	S	30	1	Low	+	+	++
9	M	68	S	32	0	Low	+	+	++
10	F	82	S	33	4	Low	+	+	++
11	F	56	D	37	3	Low	+	+	++
12	F	69	S	40	2	Low	+	+	++
13	F	61	S	45	0	Low	+	+	++
14	M	56	S	45	1	Low	+	+	++
15	M	80	S	50	1	Low	+	+	++
16	M	70	I	60	4	Int.	+	+	++
17	M	38	I	65	4	Int.	+	+	++
18	M	62	S	95	38	High	+	+	++
19	F	63	S	108	1	High	+	+	++
20	M	60	I	110	3	High	+	+	++
21	F	65	O	110	3	High	+	+	++
22	F	55	D	15	1	Very low	+	-	++
23	M	64	S	25	8	Low	+	-	++
24	F	85	R	50	62	High	+	-	++
25	M	57	E	4	0	Very low	+	+	+
26	F	69	E	5	0	Very low	+	+	+
27	F	74	S	10	0	Very low	+	+	+
28	M	57	S	25	5	Low	+	+	+
29	F	49	D	80	0	Int.	+	+	+
30	F	70	I	80	6	Int.	+	+	+
31	M	70	S	130	2	High	+	+	+
32	F	38	S	30	1	Low	+	-	+
33	M	59	I	51	1	Int.	+	-	+
34	F	57	S	33	1	Low	+	+	-
35	F	14	S	65	14	Int.	+	+	-
36	F	72	S	97	5	Int.	+	+	-
37	M	58	S	30	0	Low	-	+	-
38	F	78	R	40	0	Low	-	+	-
39	M	58	L[a]	70	7	-	+	+	++
40	M	62	P[a]	24	45	-	+	-	++
41	M	63	P[a]	20	55	-	+	+	++
42	M	64	L[a]	40	25	-	+	+	++
43	M	70	P[a]	10	45	-	+	+	-

++, strongly positive; +, positive; -, negative; M, male; F, female; E, esophagus; S, stomach; D, duodenum; I, ileum; R, rectum; O, small omentum; [a]L, recurrence of the liver; [a]P, recurrence of the peritoneum; HPF, high power field; Int., intermediate.

Relationship between cathepsin L positivity and other clinicopathological factors of GISTs. We evaluated the intensity of cathepsin L staining in 43 GIST specimens according to the percentage area occupied by cathepsin L-positive staining.

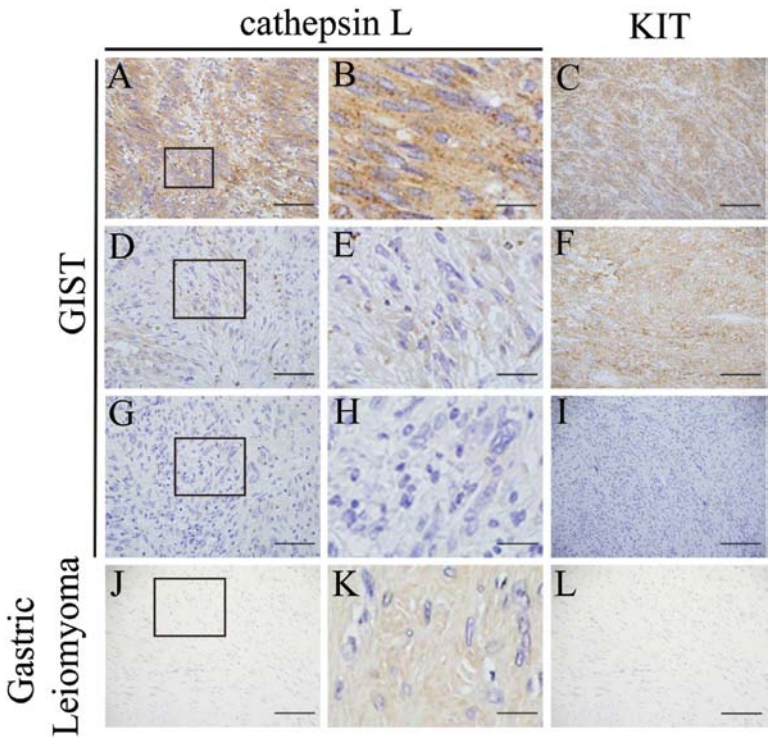

Figure 4. Immunohistochemical analysis of cathepsin L in GIST tissues. Cathepsin L and c-kit (KIT) staining in gastric GIST (A-I) and leiomyoma tissues (J-L). Three representative specimens each of strongly positive (A-C), weakly positive (D-F) and negative cathepsin L staining (G-I) are shown. B, E, H and K are enlarged images of the rectangular areas in A, D, G and J, respectively. Scale bars: 50 μm in A, D, G and J; 100 μm in B, E, H and K; and 25 μm in C, F, I and L.

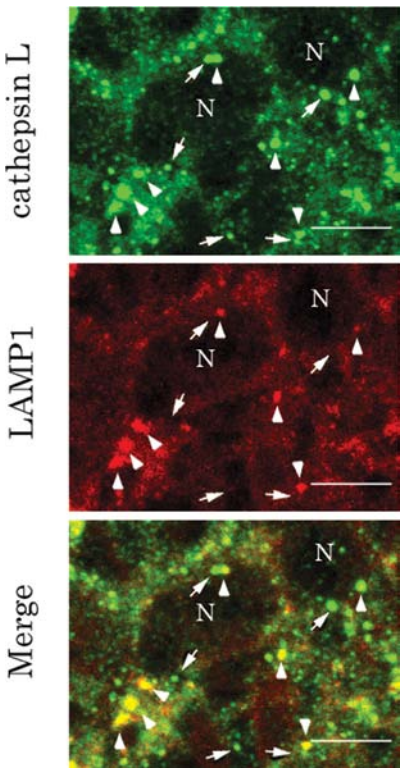

Figure 5. Double immunofluorescence microscopy for cathepsin L and lysosome-associated membrane protein-1 (LAMP-1) in GIST tissues. Paraffin sections of GIST tissues were immunolabeled with anti-cathepsin L (green in upper panel) and anti-LAMP-1 (red in middle panel) antibodies, followed by incubation with appropriate secondary antibodies. The bottom panel shows a merged image (merge). Arrowheads indicate the signals containing both cathepsin L and LAMP-1, whereas arrows indicate those containing only cathepsin L. N, nucleus. Bars, 10 μm.

As summarized in Table I, 28 specimens exhibited strongly positive staining (65.1%), 9 exhibited weakly positive staining (20.9%) and 6 exhibited negative staining (14.0%). Thus, 37 specimens (86.0%) in our cohort, which comprised weakly and strongly positive cases, were cathepsin L-positive. Moreover, in the same cohort, the rates of c-kit and CD34 positivity were 95.3 and 86.0%, respectively. Interestingly, both c-kit-negative specimens were also cathepsin L-negative, whereas all four CD34-negative specimens were cathepsin L-positive. Statistical analysis revealed a significant association between cathepsin L and c-kit staining (P=0.017), but not between cathepsin L and CD34 staining (P=0.381; Table II). We also analyzed the association of cathepsin L staining with other factors, including gender, age, region, size, mitosis and risk of recurrence; none of these factors were significantly associated with cathepsin L staining (Table II).

Discussion

The expression of cathepsin L is increased in various malignant tumors, such as gastric, colorectal, kidney, non-small cell lung, breast, ovary, adrenal, bladder, prostate and thyroid tumors (10). Importantly, because cathepsin L can digest extracellular matrix proteins (11,12), increased expression of this enzyme is often associated with malignancy, resulting in a poor prognosis (13-17). In this study, cathepsin L mRNA and protein levels were elevated in many specimens of GIST when compared to those in gastric and colorectal tumor specimens. Cathepsin L was previously listed as a signature gene of GIST in the Nielsen database (18). More recently, Chi *et al* reported

Table II. Correlation between clinicopathological factors and cathepsin L staining for GIST cases.

	Cathepsin L		
	++ and +	-	P-value
Gender			
M	21	2	0.266
F	16	4	
Age			
<60	10	3	0.248
≥60	27	3	
Region			
Stomach	20	4	0.453
Others	17	2	
Size (mm)			
<50	24	4	0.656
≥50	13	2	
KIT			
Positive	37	4	0.017[a]
Negative	0	2	
CD34			
Positive	31	6	0.381
Negative	6	0	
Mitosis (/50HPF)			
<5	29	3	0.204
≥5	8	3	
Risk			
High	6	0	0.625
Others	27	5	
Recurrence	4	1	

[a]P<0.05.

that the ETS family member ETV1 is highly expressed in GISTs; moreover, it promotes tumorigenesis along with c-kit (19). Interestingly, they identified cathepsin L as a target gene of ETV1, because short hairpin-mediated ETV1 suppression in GIST cell lines caused a decrease in cathepsin L mRNA expression. In addition, chromatin immunoprecipitation and deep sequencing revealed that ETV1 binding sites were present in the promoter and enhancer regions of cathepsin L. Therefore, our study supports their results by providing clinical evidence that cathepsin L mRNA and protein are highly expressed in GISTs, which can be explained by the elevated levels of ETV1.

We also concluded that cathepsin L immunoreactivity was highly associated with that of c-kit but not with that of CD34. This result is also reasonably supported by the previous study described above (19). In our study, the rates of cathepsin L and c-kit positivity were 86.0 and 95.3%, respectively, and both c-kit-negative specimens were also cathepsin L-negative. These results therefore suggest that cathepsin L may not be superior to nor complement c-kit as a diagnostic marker in immunohistochemistry. However, unlike c-kit, the cathepsin L signal is detected as cytoplasmic dots, discriminating it from the diffuse background signal. We believe that this property of cathepsin L may prove this technique as more efficient for diagnosis than c-kit staining, particularly in tumors that demonstrate weak c-kit staining.

Cathepsin L is known to degrade basement membrane proteins, including collagen IV, laminin and fibronectin (20). Therefore, increased cathepsin L expression is often associated with tumor invasion and metastasis in gastric, colorectal and oral squamous cell carcinomas (15-17,21,22). In the case of GIST, however, lymph node metastasis is very rare (23), and invasiveness is not usually assessed in the pathological diagnosis (24). In concordance with this notion, our analysis revealed that the cathepsin L immunoreactivity was not correlated to the risk of recurrence after surgery (Table II). Immunofluorescence analysis revealed that in GISTs, cathepsin L was localized not only in LAMP1-positive lysosomes but also in other membrane-bound organelles, which may account for the secreted fraction of cathepsin L. However, considering the lack of evidence supporting the link between cathepsin L expression and cancer malignancy, GIST cells may have a mechanism that prevents the extracellular secretion or activation of cathepsin L. Another interesting explanation would be the influence of cathepsin L on angiogenesis. Felbor *et al* (25) reported that secreted cathepsin L generates endostatin from collagen XVIII, which could suppress angiogenesis and subsequently counteract distant metastasis. This may predominantly occur in GISTs. More precise examinations and longer follow-up studies with increased number of patients are required to explain the impact of high cathepsin L expression on the clinicopathological course of GIST.

Although it is not linked to prognosis, secreted cathepsin L could potentially be a serum marker to monitor GIST recurrence. In fact, cathepsin L is known to be secreted into the blood circulation in gastric, breast, and colorectal cancer patients, and previous studies have demonstrated its utility as a serum marker (26). We therefore plan to examine the serum levels of cathepsin L in GIST patients in future studies.

Acknowledgements

We are extremely grateful to K. Saito and M. Mashiko for their technical assistance.

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