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Cathepsin D expression as a possible predictor of lymph node metastasis in submucosal colorectal cancer

H. Oh-e [a], S. Tanaka [b,*], Y. Kitadai [b], F. Shimamoto [c], M. Yoshihara [d], K. Haruma [a]

[a] *First Department of Internal Medicine, Hiroshima University School of Medicine, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan*
[b] *Department of Endoscopy, Hiroshima University School of Medicine, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan*
[c] *Department of Pathology, Hiroshima University School of Medicine, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan*
[d] *Health Service Center, Hiroshima University, Hiroshima 734-8551, Japan*

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Abstract

The aim of this study was to clarify the usefulness of cathepsin D expression as a predictor of lymph node metastasis in submucosal colorectal cancer (CRC). Cathepsin D expression was examined immunohistochemically in cancer and stromal cells located at the deepest portion of 254 invasive tumours that had been resected from patients with submucosal CRC. In cancer cells, the expression was classified according to differences in intracellular localisation: polarity positive, apical type (PA); polarity positive, basal type (PB); polarity negative (PN); or no expression (NE). Lesions with PN or NE expression showed a significantly higher incidence of lymph node metastasis than those with PA or PB expression. Alternatively, lesions with positive expression in stromal cells showed a significantly higher incidence of lymph node metastasis than that of those with negative expression. None of the lesions with PA or PB expression and negative expression in stromal cells had metastasised to the lymph node. In conclusion, analysis combining cathepsin D expression in cancer and stromal cells may be a quite useful predictor for lymph node metastasis and may broaden the indications for curative endoscopic treatment of submucosal CRC.

Keywords: Cathepsin D; Submucosal colorectal cancer; Lymph node metastasis; Immunohistochemistry; Endoscopic mucosal resection

1. Introduction

Endoscopic mucosal resection (EMR; the so-called strip biopsy) allows for the complete resection of not only polypoid lesions but also flat or depressed lesions, and relatively large elevated lesions in the colorectum [1,2]. Previous reports indicate that, among cases of early colorectal cancer (CRC), intramucosal lesions show no lymph node metastasis [3]. Submucosally invasive lesions, however, show lymph node metastasis in 3.6–16.2% of patients with this condition [4–9]. The success of curative EMR of early CRC depends on there being no or very limited lymph node metastasis. We previously reported that histological grade at the deepest invasive portion was the most important risk factor for lymph node metastasis in submucosal CRC [7–9]. This is because the deepest invasive portion is the invasive tumour region with the highest malignant potential, being the part that ultimately will invade, spread locally, and metastasise. Our previous results indicate that scanty submucosal invasion (sm-s) and a histological grade of well or moderately-to-well differentiated adenocarcinoma at the deepest invasive portion are the appropriate indicators for curative EMR of submucosal CRC because of the very low incidence of lymph node metastasis [7–10]. At present, however, CRCs with massive submucosal invasion (sm-m) or histological grade of moderately-to-poorly differentiated, poorly differentiated or mucinous adenocarcinomas usually have undergone additional surgical resection, even after complete EMR, because there are few definite predictors

* Corresponding author. Tel.: +81-82-257-5537; fax: +81-82-257-5538.

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of negative lymph node metastases for these lesions [8,10]. If more useful and strict predictors for lymph node metastases are established, we can broaden the curative conditions after EMR and provide a better quality of life for patients with submucosal CRC.

Cathepsin D expression has been reported as a useful prognostic factor in patients with cancer of various organs [11–14]. As for CRC, several studies have demonstrated the significance of cathepsin D expression [15–17]. There has been, however, no definite evaluation concerning the relationship of cathepsin D expression and lymph node metastasis of submucosal CRC. In this study, we examined cathepsin D expression immunohistochemically in order to clarify its usefulness as a predictor of lymph node metastasis after EMR of submucosal CRC.

2. Patients and methods

2.1. Patients and specimens

The specimens studied comprised submucosal CRC lesions that had been surgically resected from 254 patients treated at the Hiroshima University Medical Hospital and affiliated hospitals between 1988 and 1998. All regional lymph nodes at the para-colorectal artery and along the main artery supplying the primary tumour were resected and cut into two or three sections. The entire tumour was cut into parallel 2–3 mm thick sections, and the deepest portion of submucosal invasion was selected for microscopic examination. Pathological microscopic examination of the primary tumours and lymph nodes was performed with haematoxylin-and-eosin staining. Tumour size, macroscopic type, depth of submucosal invasion, histologic subclassification, degree of desmoplastic reaction, presence of adenoma, vessel involvement and histological evidence of lymph node metastasis were recorded. The macroscopic classification used was similar to that previously described for gastric carcinoma [7,9]. Histological subclassification was performed as previously described [7–10,18,19]. In brief, well differentiated and moderately differentiated adenocarcinomas were classified initially according to the World Health Organization classification [20]. The moderately differentiated tumours were then subclassified into moderately-to-well differentiated and moderately-to-poorly differentiated adenocarcinomas. The depth of submucosal invasion was classified as scanty submucosal invasion (down to 400 μm from the muscularis mucosae) or massive submucosal invasion (deeper than 400 μm). The degree of desmoplastic reaction was graded as (−) when there was no desmoplastic reaction, (+) when weak desmoplastic reaction was observed, and (++) when strong desmoplastic reaction was observed.

2.2. Immunohistochemistry

Immunohistochemical studies were performed on 4-μm thick formalin-fixed, paraffin-embedded sections from primary tumours and metastatic lymph nodes using the labelled streptavidin–biotin method (Dako LSAB kit; Dako Japan Co., Kyoto, Japan). After deparaffinisation and rehydration, pronase pretreatment (Trypsin 1:250, Difco Laboratories, Detroit, MI, USA) was carried out for 30 min at 37°C. Endogenous peroxidase activity was quenched with hydrogen peroxide, and sections were incubated with goat serum for 20 min at room temperature to block non-specific binding. Anti-cathepsin-D monoclonal antibody (NCL-CDm, 1:200, Novocastra Laboratories, Newcastle, UK) was applied for 1 h at room temperature. This was followed by an incubation with a biotinylated secondary antibody for 20 min and then a streptavidin–biotin complex reagent for 20 min at room temperature. Sections were washed thoroughly with phosphate-buffered saline (PBS) between incubation steps. Diaminobenzidine tetrahydrochloride was used as the chromogen, and sections were counterstained with haematoxylin. Positive controls were obtained from breast cancer sections previously positive for cathepsin D. Negative controls were prepared in the absence of primary antibody, as described above.

2.3. Interpretation of immunostaining

Immunoreactions were evaluated microscopically (×400) for cancer cells at the superficial portion and the deepest invasive portion of the primary tumours, and in the metastatic lymph nodes. Cathepsin D expression in the cancer cells was classified as follows into four types according to differences in the intracellular localisation or its total absence: polarity positive, apical type (PA) in which immunoreactive cathepsin D was detected on the apical side of cytoplasm; polarity positive, basal type (PB) in which immunoreactive cathepsin D was detected on the basal side of cytoplasm; polarity negative (PN) in which immunoreactive cathepsin D was detected in the cytoplasm without polarity; and no expression (NE) in which no expression was detected (Fig. 1a–d). As the staining patterns often varied within the same tumour, particularly when the degree of differentiation varied, cathepsin D expression in the cancer cells was based on the predominant pattern. Cathepsin D expression in the stromal cells was also examined at the border of the invasive front and was defined as the mean percentage of stromal cells showing cathepsin D-positive staining per 1000 stromal cells counted in three randomly chosen microscopic fields. Cathepsin D expression in stromal cells was considered positive when $>15\%$ of stromal cells were stained (Fig. 1e and f). All sections were examined by two investigators who were blinded to the

patient's status, and a high level of concordance (90%) was achieved. In cases of disagreement, the slides were reviewed and a consensus view was achieved.

2.4. In situ *hybridisation*

In situ hybridisation was performed as previously described [21] with minor modifications. Briefly, a cathepsin D-specific oligonucleotide probe was designed complementary to the 5′ end of the human *cathepsin D* mRNA transcript [22]. The DNA oligonucleotide sequence 5′-GTG-CAG-CGG-GAT-CCT-GAC-GAG-CGC-GGA-G-3′ was of the antisense orientation and hence complementary to *cathepsin D* mRNA. A d(T)20 oligonucleotide was used to verify the integrity and lack of degradation of mRNA in each sample. All DNA probes were synthesised with six biotin molecules (hyperbiotinylated) at the 3′ end via direct coupling using standard phosphoramidite chemistry (Research Genetics, Huntsville, AL, USA). *In situ* hybridisation

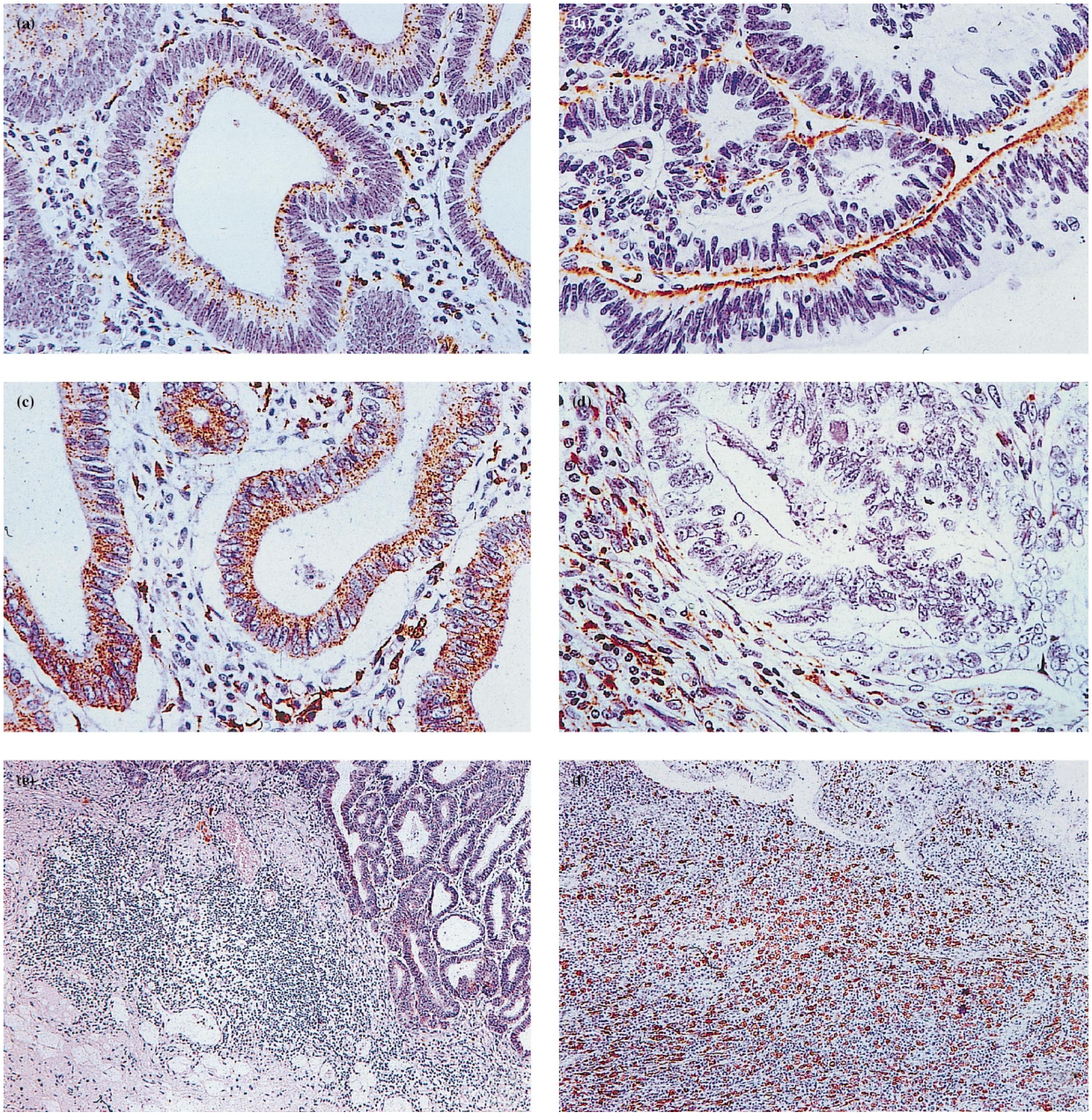

Fig. 1. Cathepsin D expression in cancer (a–d) and stromal (e, f) cells. Cathepsin D expression patterns in cancer cells could be classified into four types: PA (polarity positive, apical type), cathepsin D expression was detected on the apical side of cytoplasm (a); PB (polarity positive, basal type), cathepsin D expression was detected on the basal side of cytoplasm (b); PN (polarity negative), cathepsin D expression was detected in the cytoplasm without polarity (c); NE (no expression), no cathepsin D expression (d); ×400. Cathepsin D expression in stromal cells: negative for cathepsin D

was carried out using the microprobe manual staining system (Fisher Scientific, Pittsburgh, PA, USA) [21]. A positive reaction in this assay stained red. Control for endogenous alkaline phosphatase included treatment of samples in the absence of the biotinylated probe and the use of chromogen alone. To check the specificity of the hybridisation signal, the following controls were used: RNase pretreatment of tissue sections, substitution of the antisense probe with a biotin-labelled sense probe, and a competition assay with unlabelled antisense probes. No or markedly decreased signals were obtained after all these treatments.

2.5. Statistical analysis

Data were evaluated by the Chi-square test. Additionally, to clarify the relative importance of each risk factor for the presence of lymph node metastasis, multivariate analysis with logistic regression was performed. The stepwise method was used for selecting the variables. All statistical analyses were performed by two-tailed test with the SAS statistical package (SAS Institute Japan, Tokyo, Japan). P values of <0.05 were considered significant.

3. Results

3.1. Cathepsin D expression patterns in cancer cells of each specimen

Lymph node metastases were detected in 35 (14%) of 254 patients. The difference in cathepsin D expression patterns in the cancer cells of each specimen is shown in Table 1. In both the non-metastatic and metastatic groups, cancer cells at the superficial portion showed a significantly higher incidence of PA expression than those at the deepest invasive portion ($P<0.01$). In the metastatic group, cancer cells at the deepest invasive portion of primary tumour and metastatic lymph node showed a significantly higher incidence of PN or NE expression than those in the superficial portion of the primary tumour ($P<0.01$). Out of 35 metastatic lymph nodes, 28 (80%) showed the same expression patterns as those of cancer cells at the deepest invasive portion of their primary tumours.

3.2. Relationship between clinicopathological findings and cathepsin D expression

The relationships between clinicopathological findings and cathepsin D expression are shown in Table 2. Cathepsin D expression patterns in the cancer cells at the deepest invasive portion correlated significantly with macroscopic type ($P<0.05$), depth of submucosal invasion ($P<0.01$), histological grade ($P<0.05$), presence of adenoma ($P<0.01$), vessel involvement ($P<0.05$–0.01) and lymph node metastasis ($P<0.05$). Cathepsin D expression in the stromal cells correlated significantly with depth of submucosal invasion ($P<0.01$), desmoplastic reaction ($P<0.01$), vessel involvement ($P<0.05$), and lymph node metastasis ($P<0.01$). No relationship between cathepsin D expression in the cancer and stromal cells was observed.

3.3. In situ hybridisation of cathepsin D

In situ hybridisation of cathepsin D was performed using 32 samples of submucosal CRC for each expression pattern (8PA, 8PB, 8PN and 8NE). For the cancer cells, lesions with PA, PB or PN expression demonstrated an intense histochemical reaction in their cytoplasm with a cathepsin D-specific antisense probe (Fig. 2), but lesions with NE expression did not show

Table 1
Cathepsin D expression patterns in cancer cells of each specimen

	Cathepsin D expression patterns in cancer cells			
	PA *n* (%)	PB *n* (%)	PN *n* (%)	NE *n* (%)
Non-metastatic group ($n=219$; 86%)				
Superficial portion	29 (42)[a]	29 (13)	53 (24)	44 (20)
The deepest invasive portion	41 (19)	71 (32)	47 (21)	60 (27)
Metastatic group ($n=35$; 14%)				
Primary tumour				
Superficial portion	13 (37)[b]	5 (14)	9 (26)	8 (23)
The deepest invasive portion	1 (3)	5 (14)	14 (40)[c]	15 (43)[c]
Metastatic lymph node	1 (3)	3 (9)	13 (37)[d]	18 (51)[d]

[z] PA, polarity positive, apical type; PB, polarity positive, basal type; PN, polarity negative; NE, no expression.
[a] $P<0.01$, versus the deepest invasive portion of the non-metastatic group.
[b] $P<0.01$, versus the deepest invasive portion of the metastatic group or metastatic lymph node.
[c] $P<0.01$, versus the superficial portion of the metastatic group.
[d] $P<0.01$, versus the superficial portion of the metastatic group.

any reaction. Moreover, expression of *cathepsin D* mRNA in the stromal cells was consistent with that of immunohistochemical cathepsin D expression.

3.4. Relationship between the incidence of lymph node metastasis and cathepsin D expression

The incidence of lymph node metastasis in lesions with PA, PB, PN and NE expression was 2, 7, 23 and 20%, respectively. Lesions with PN or NE expression had a significantly higher incidence of lymph node metastasis than those with PA or PB expression ($P < 0.05$–0.01) (Fig. 3a). The incidence of lymph node metastasis in lesions with negative and positive cathepsin D expression in the stromal cells was 4% and 20%. Lesions with positive expression in stromal cells had a significantly higher incidence of lymph node metastasis than those with negative expression in the stromal cells ($P < 0.01$) (Fig. 3b).

3.5. Univariate and multivariate analyses of factors related to lymph node metastasis

In the univariate analysis, macroscopic type, depth of submucosal invasion, histological grade, the presence of adenoma, vessel involvement, and cathepsin D expression in the cancer and stromal cells correlated significantly with lymph node metastasis ($P = 0.0191$–< 0.0001). In the multivariate analysis, the significant risk factors were macroscopic type histological grade,

Table 2
Relationship between clinicopathological findings and cathepsin D expression in submucosal colorectal cancer

Clinicopathological findings	No. of cases ($n = 254$)	Cancer cells					Stromal cells		
		PA	PB	PN	NE	*P* value	Negative	Positive	*P* value
	n (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)		*n* (%)	*n* (%)	
Size									
⩽20 mm	159 (63)	25 (16)	54 (34)	30 (19)	50 (31)	NS	68 (43)	91 (57)	NS
>20 mm	95 (37)	17 (18)	22 (23)	31 (33)	25 (26)		31 (33)	64 (67)	
Location									
Rectum	92 (36)	5 (5)	33 (36)	23 (25)	31 (34)	NS	27 (29)	65 (71)	NS
Left	109 (43)	25 (23)	33 (30)	27 (25)	24 (22)		47 (43)	62 (57)	
Right	53 (21)	12 (23)	10 (19)	11 (21)	20 (38)		25 (47)	28 (53)	
Macroscopic type									
I	177 (70)	36 (20)	54 (31)	49 (28)	38 (21)	<0.05	64 (36)	113 (64)	NS
IIa	36 (14)	5 (14)	11 (31)	5 (14)	15 (42)		18 (50)	18 (50)	
IIa + IIc	29 (11)	1 (3)	6 (21)	7 (24)	15 (52)		10 (34)	19 (66)	
IIc	12 (4)	0	5 (42)	0	7 (58)		7 (58)	5 (42)	
Depth of submucosal invasion									
sm-s	67 (26)	17 (25)	25 (37)	8 (12)	17 (25)	<0.01	43 (64)	24 (36)	<0.01
sm-m	187 (74)	25 (13)	51 (27)	53 (28)	58 (31)		56 (30)	131 (70)	
Histological grade									
W	129 (51)	30 (23)	44 (34)	26 (20)	29 (22)	<0.05	57 (44)	72 (56)	NS
Mw	68 (27)	6 (9)	27 (40)	15 (22)	20 (29)		25 (37)	43 (63)	
Mp	41 (16)	3 (7)	4 (10)	14 (34)	20 (49)		11 (27)	30 (73)	
Por	5 (2)	0	0	3 (60)	2 (40)		1 (20)	4 (80)	
Muc	11 (4)	3 (27)	1 (9)	3 (27)	4 (36)		5 (45)	6 (55)	
Desmoplastic reaction									
(−)	21 (8)	4 (19)	6 (29)	4 (19)	7 (33)	NS	13 (62)	8 (38)	<0.01
(+)	94 (37)	17 (18)	32 (34)	20 (21)	25 (27)		49 (52)	45 (48)	
(++)	139 (55)	21 (15)	38 (27)	37 (27)	43 (31)		37 (27)	102 (73)	
Adenoma									
(−)	141 (56)	15 (11)	38 (27)	35 (25)	53 (38)	<0.01	52 (37)	89 (63)	NS
(+)	113 (44)	27 (24)	38 (34)	26 (23)	22 (19)		47 (42)	66 (58)	
Lymphatic invasion									
(−)	122 (48)	26 (21)	42 (34)	20 (16)	34 (28)	<0.01	56 (46)	66 (54)	<0.05
(+)	132 (52)	16 (12)	34 (26)	41 (31)	41 (31)		43 (33)	89 (67)	
Venous invasion									
(−)	214 (84)	38 (18)	67 (31)	45 (21)	64 (30)	<0.05	89 (42)	125 (58)	<0.05
(+)	40 (16)	4 (10)	9 (23)	16 (40)	11 (28)	<0.05	10 (25)	30 (75)	
Lymph node metastasis									
(−)	219 (86)	41 (19)	71 (32)	47 (21)	60 (27)	<0.05	95 (43)	124 (57)	<0.01
(+)	35 (14)	1 (3)	5 (14)	14 (40)	15 (43)		4 (11)	31 (89)	

sm-s, scanty submucosal invasion; sm-m, massive submucosal invasion; W, well differentiated adenocarcinoma; Mw, moderately-to-well differentiated adenocarcinoma; Mp, moderately-to-poorly differentiated adenocarcinoma; Por, poorly differentiated adenocarcinoma; Muc, mucinous adenocarcinoma; PA, polarity positive, apical type; PB, polarity positive, basal type; PN, polarity negative; NE, no expression; NS, non significant.

and cathepsin D expression in the stromal cells (P values range from 0.0329 to <0.0001) (Table 3).

3.6. Incidence of lymph node metastasis by combining cathepsin D expression in cancer and stromal cells

In relation to lesions with PA or PB expression and negative expression in stromal cells, which showed no lymph node metastasis (0/22; 0%, 0/37; 0%), lesions with PN or NE expression and positive expression in stromal cells had statistically significant rates of metastasis ($P<0.01$; Table 4).

4. Discussion

Cathepsin D, a member of the aspartic protease family, is an acid lysosomal enzyme that directly correlates with the prognosis of patients with cancer of various organs [11–14]. It has been suggested that this is because the production of cathepsin D increases the invasive potential of the tumour cells, thus increasing the probability of metastasis. In this context, cathepsin D has been proven to be capable of degrading the extracellular matrix and to activate other proteases, such as cathepsin B and L and collagenases, which are also thought to be correlated with tumour progression [23]. Furthermore, cathepsin D is also considered to promote tumour cell proliferation by interacting with the mannose-6-phosphate/insulin growth factor (IGF)II-receptor (M6P/IGFII-receptor), which could explain the mitogenic IGFII-like effect of this enzyme [24]. Except for cancer cells, stromal cells contain cathepsin D in their cytosol, and several investigators have suggested that the cathepsins derived from stromal cells play a more important role in cancer progression than those from cancer cells [25–27]. Immunohistochemical analysis has the advantage of precise and selective tissue localisation of the antigen and can easily be used to discriminate cathepsin D expression in stromal cells from that in cancer cells. As for CRC, several immunohistochemical studies of cathepsin D expression have been previously reported [15–17], however, no conclusive evidence exists regarding the usefulness of cathepsin D expression in predicting lymph node metastasis in submucosal CRC. Most of these studies dealt only with advanced CRCs and evaluated immunohistochemical cathepsin D expression using the positivity rate or the staining intensity method without considering the difference in the intracellular localisation of cathepsin D.

To the best of our knowledge, this is the largest series of submucosal CRCs in which the role of cathepsin D in lymph node metastasis has been studied. The novel findings in this study were the polar distribution of cathepsin D in cancer cells and the heterogeneity of its expression patterns in identical tumours. The incidence of PN or NE expression in the cancer cells tended to be higher in the deepest invasive portion than in the

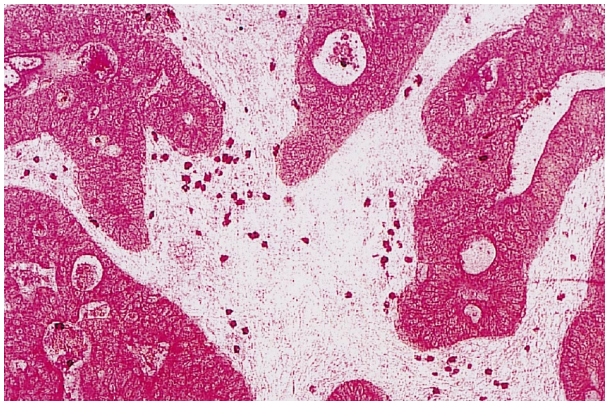

Fig. 2. *In situ* hybridisation for cathepsin D. This is a case with polarity negative (PN) type in cancer cells and positive in the stromal cells immunohistochemically. Expression of *cathepsin D* mRNA was observed in both the cancer and stromal cells. The cells that expressed *cathepsin D* mRNA were stained red ×200.

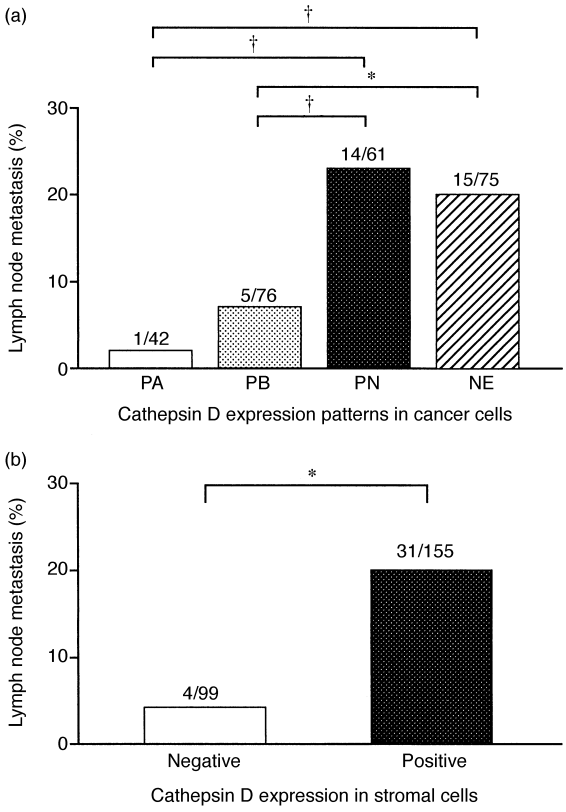

Fig. 3. Relationship between the incidence of lymph node metastasis and cathepsin D expression. (a) Lesions with polarity negative (PN) or no expression (NE) in cancer cells showed a significantly higher incidence of lymph node metastasis than those with polarity positive, apical type (PA) or polarity positive, basal type (PB) expression (*$P<0.05$, †$P<0.01$). (b) Lesions with positive expression in stromal cells showed a significantly higher incidence of lymph node metastasis than those with negative expression (*$P<0.01$).

Table 3
Univariate and multivariate analyses of the factors related to lymph node metastasis

Factors	Univariate analysis		Multivariate analysis	
	P value	Odds ratio (95% CI)	*P* value	Odds ratio (95% CI)
Macroscopic type (I, IIa versus IIa + IIc, IIc)	0.0001	4.72 (2.16–10.42)	0.0007	6.00 (2.14–16.86)
Depth of submucosal invasion (sm-s versus sm-m)	0.009	6.97 (1.62–29.88)	NS	
Histological grade (W, Mw versus Mp, Por, Muc)	< 0.0001	10.43 (4.74–22.94)	< 0.0001	7.35 (2.92–18.51)
Desmoplastic reaction (−, + versus + +)	NS		NS	
Adenoma (+ versus −)	0.0191	2.61 (1.17–5.83)	NS	
Lymphatic invasion (− versus +)	0.0008	4.42 (1.85–10.56)	NS	
Venous invasion (− versus +)	0.0004	4.20 (1.90–932)	NS	
Cathepsin D expression in the cancer cells (PA, PB versus PN, NE)	0.0005	5.06 (2.02–12.66)	NS	
Cathepsin D expression in the stromal cells (− versus +)	0.001	6.05 (2.07–17.71)	0.0329	3.92 (1.12–13.73)

CI, confidence interval.
For abbreviations see Table 2.

superficial portion of primary tumours, and the cathepsin D expression patterns in the deepest invasive portion were homologous to those in each metastatic lymph node. These findings provide some supporting evidence that the deepest invasive portion shows the highest metastatic potential, and the expression of metastasis-related molecules/factors should be examined here.

In the present study, the difference in the intracellular localisation of cathepsin D expression in the cancer cells at the deepest invasive portion significantly correlated with histological differentiation and with the metastatic potential of submucosal CRC. The incidence of lesions with PA or PB expression was significantly higher in well differentiated or moderately-to-well differentiated adenocarcinoma-grade lesions than in moderately-to-poorly differentiated, poorly differentiated or mucinous adenocarcinoma-grade lesions. This finding may suggest that the highly differentiated carcinomas, showing a better biological behaviour and phenotype, preserve the functional integrity of the biochemical pathways and synthesising systems that are responsible for the intracellular polarity of cathepsin D expression. *In situ* hybridisation revealed that the absence of staining for cathepsin D protein in the cancer cells (NE) indicates a defect in the transcription of the enzyme. This result indicated that negative cathepsin D expression in cancer cells corresponds to the loss of cathepsin D synthesis or to the presence of a mutant form of cathepsin D not recognised by this antibody. Lesions with PN or NE expression showed a significantly higher incidence of lymph node metastasis than did those with PA or PB expression. On the other hand, when we evaluated immunohistochemical cathepsin D expression in the cancer cells using the positivity rate or the staining intensity method as most of the previous studies have used, no association of cathepsin D expression could be found with lymph node metastasis in the submucosal CRC (data not shown). This classification of cathepsin D expression patterns, considering intracellular polarity, may thus be quite useful for understanding the metastatic potential and biological behaviour of submucosal CRC.

The present results also indicated that there was a significant relationship between cathepsin D expression in the stromal cells and the malignant or metastatic potential in submucosal CRC. Although it is known that inflammatory cell infiltration at the border of the invasive front acts as a defence mechanism against tumour invasion [28], inflammatory cells of the stroma inside and adjacent to the tumour mass contain various enzymes, including cathepsin D, that could destroy the tissue architecture and thereby promote tumour spread [29]. Our results showed that cathepsin D expression in the stromal cells is closely related to the degree of desmoplastic reaction. It is still uncertain whether this reaction serves to defend the host or to facilitate tumour growth [30]. However, cathepsin D expression in the stromal cells may be involved in the processes of degradation and remodelling of the extracellular matrix leading to a marked desmoplastic reaction. Thus, cathepsin D in the stromal cells seems to have a dual function. It

Table 4
Incidence of lymph node metastasis by combining cathepsin D expression in cancer and stromal cells

Cancer cells	Stromal cells	
	Negative (%)	Positive (%)
PA	0/22[a]	1/20 (5)[c]
PB	0/37[b]	5/39 (13)
PN	1/10 (10)	13/51 (25)[d]
NE	3/30 (10)	12/45 (27)[e]

PA, polarity positive, apical type; PB, polarity positive, basal type; PN, polarity negative; NE, no expression.
[a] $P < 0.01$, versus [d] and [e].
[b] $P < 0.01$, versus [d] and [e].
[c] $P < 0.05$, versus [d] and [e].

facilitates cancer cell invasion and metastasis and may also be involved in the stromal reactions occurring in the cancer tissue, including the desmoplastic reaction.

Multivariate analysis revealed that histological grade at the deepest invasive portion is the most important significant risk factor for lymph node metastasis and cathepsin D expression in the stromal cells and macroscopic type are also independent risk factors for lymph node metastasis. In an analysis combining cathepsin D expression in the cancer and stromal cells, lesions with PA or PB expression and negative expression in stromal cells had no lymph node metastasis, regardless of the depth of submucosal invasion or histological grade. Thus, our results raised the possibility to extend the indication of curative EMR for lesions with massive submucosal invasion or histological grade of moderately-to-poorly differentiated, poorly differentiated or mucinous adenocarcinoma that are usually excluded from those indicated for curative EMR. Further investigations using a larger number of cases, including long-term follow-up studies are needed to validate our results.

In conclusion, this study has demonstrated that cathepsin D expression in the cancer and stromal cells at the deepest invasive portion significantly correlates with lymph node metastasis in submucosal CRC. Intracellular polarity should be taken into account for immunohistochemical evaluation of cathepsin D expression in cancer cells at the deepest invasive portion. Analysis combining cathepsin D expression in the cancer and stromal cells may be one of the quite useful predictors for lymph node metastasis and a prospective study to validate our results will be able to broaden the conditions of curability of patients with submucosal CRCs treated by complete EMR.

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