Original

**Evaluation of Cathepsin D Immunostaining in
Colorectal Epithelial Tumors**

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Abstract : We evaluated the expression of cathepsin D (CD) in a series of colorectal tumor cells and stromal cells, in relation to the clinicopathological and histopathological features. Tissue samples were obtained from the archival surgical specimens of 72 primary colorectal adenocarcinomas and endoscopic mucosal resection (EMR) specimens of 53 colorectal adenomas and adenocarcinomas. The carcinomas were sited in the mucosa (M) in 23 patients, in the submucosa (SM) in 28 patients, in the muscularis propria (MP) in 16 patients, and in the subserosa (SS) in 39 patients. The adenoma samples comprised 19 severe dysplastic adenomas (AD). High CD expression in the tumor cells was observed in 1 case (5.3%) of AD, 2 cases (8.7%) of M, 10 cases (35.7%) of SM, 7 cases (43.8%) of MP, and in 15 cases (38.4%) of SS. There was a significant correlation between the CD expression of M and SM samples ($p < 0.05$). High CD expression in the stromal cells was observed in 1 case (5.3%) of AD, in 1 case (4.3%) of M, in 12 cases (42.9%) of SM, in 12 cases (75%) of MP, and in 27 cases (69.2%) of SS. There was correlation between the CD expression of M and SM carcinomas ($p < 0.01$) and between that of SM and MP ($p < 0.05$). In 72 surgical specimens, lymph nodes metastasis was proven histologically in 1 case (5.9%) of SM, 2 cases (12.5%) of MP, and in 17 cases (43.6%) of SS. In both tumor cells and stromal cells, there were no correlation between CD expression and lymph nodes metastasis. These results indicate that cathepsin D expression in carcinoma and stromal cells reflects a potential for invasive growth of colorectal tumors.

Key words : colorectal tumor, adenocarcinoma, adenoma, cathepsin D, immunohistochemistry

Introduction

Degradation of the extracellular matrix and basement membrane that surrounds tumor cells is an essential step in the processes of tumor invasion and metastasis. This degradation and passage through the membrane is regulated largely by proteolytic enzymes¹⁾. Cathepsin D (CD) is an aspartic lysosomal protease that is localized widely in tissues, and can digest extracellular matrix, including basement membrane components²⁾. A high level of CD in

carcinoma cells has been associated with a poor prognosis, regardless of the presence or absence of lymph nodes metastasis, and this association has been confirmed in breast and laryngeal carcinoma^{3,4)}. Normal stromal cells contain cathepsin D in their cytosol, and this pool of protein has been suggested to play a more important role in cancer progression than the CD in carcinoma cells⁵⁻⁷⁾.

There have been only a few reports describing the immunohistochemical expression⁸⁻¹¹⁾ and intracytoplasmic localization of CD in colorectal tumors¹²⁾. Since the role of cathepsin D expression in predicting invasive potential has not been clarified, we evaluated the expression of this enzyme in a series of colorectal adenomas, adenocarcinomas and reactive stromal cells, and investigated any correlation with the associated clinical and histopathological features.

Materials and Methods

Samples

Tissue samples were obtained from the archival surgical specimens of 72 primary colorectal adenocarcinomas and endoscopic mucosal resection (EMR) specimens of 53 colorectal adenomas and adenocarcinomas. All samples were collected between 1997 and 2002 at Showa University Hospital. The patient age ranged from 40 to 87 years, with 89 males and 36 females. The specimens were fixed in 10% formalin for 24–48 hours before embedding in paraffin. The carcinoma samples consisted of 69 well-differentiated adenocarcinomas and 37 moderately differentiated tubular adenocarcinomas, and based on a depth grading of tumor invasion, there were 23 carcinoma samples from the mucosa (M), 28 from the submucosa (SM), 16 from the muscularis propria (MP), and 39 from the subserosa (SS). Nineteen severe dysplastic adenomas (AD) were collected from patients with adenomas. The histological diagnoses were made according to the criteria of the Japanese Society for Cancer of the Colon and Rectum¹³⁾. Lymph nodes metastasis was defined as “positive” only when histologically proven.

Immunohistochemistry

Consecutive 3 μ m sections were dewaxed with xylene and rehydrated through a graded alcohol series. After quenching the endogenous peroxidase activity with absolute methanol containing 3% H₂O₂, the sections were stained using the labeled streptavidin-biotin peroxidase complex technique (K0675, DAKO, Kyoto, Japan). A primary antibody against cathepsin D (CD, H908, NICHIREI, Tokyo, Japan) was applied for 18 hours at 4°C. The sections were colored with benzidine and lightly counterstained with hematoxylin for light microscopy.

Immunohistochemical evaluation. Arao *et al*¹²⁾ classified the immunoreactivity of colorectal epithelial cells into four types: no staining, granular staining at the supranuclear portion of the cytoplasm (Fig. 1A), granular staining at the basal portion of the cytoplasm (Fig. 1B), and diffuse staining throughout the cytoplasm (Fig. 1C). We designated the CD expressions as positive when the tumor cells showed the basal or diffuse type of staining. The immunostaining was compared by calculating the percentage of positive tumor cells in relation to the total number of cells of adenocarcinomas and adenomas. In each section, at least 10 high-power fields ($\times 400$) were examined under light microscopy. CD immunohistochemical expression in reactive stromal cells was also evaluated (Fig. 2). In most cases the adjacent

Fig. 1. Immunostaining of cathepsin D. A Granular staining at the supranuclear portion of the cytoplasm in ordinal colorectal mucosa. B. Granular staining at the basal portion of the cytoplasm in colorectal adenocarcinoma. C. Diffiti8e staining throughout the cytoplasm in colorectal adenocarcinoma. Original magnification, $\times 400$.

Fig. 2. Immunostaining of cathepsin D in stromal cells observed in colorectal adenocarcinoma. Original magnification, $\times 200$.

Table 1. High Cathepsin D Expression in correlation with clinicopathological features in colorectal tumors : Expression in Tumor and Stromal Cells

	Severe dysplastic Adenoma		Adenocarcinoma						
			Depth of invasion						
			M		SM		MP		SS
number	19		23		28		16		39
Tumor cell	1(5.3%)	NS	2(8.7%)	p<0.05	10(35.7%)	NS	7(43.8%)	NS	15(38.4%)
Stromal oell	1(5.3%)	NS	1(4.3%)	p<0.01	12(42.9%)	p<0.05	12(75%)	NS	27(69.2%)

Table 2. Relationship between High Oathepsin D Expression and Duke's Stage

	Duke's stage				
	A		B		C
number	20		30		22
Tumor cell	9(45%)	NS	13(43.3%)	NS	9(40.9%)
Stromal cell	12(60%)	NS	14(46.7%)	p<0.01	20(90.9%)

normal mucosas were also examined. We separated the cases into two groups, such as "high CD" and "low CD", according to the percentage of positive tumor cells and non-neoplastic reactive stromal cells. The cut-off line was 10% for CD expression of tumor cells and 15% for CD expression of reactive stromal cells, as suggested by previous studies in macrophages^{11, 14)}.

Statistical analysis

The association of continuous variables was assessed using the non-parametric test for two independent samples. P-values smaller than 0.05 were considered statistically significant.

Results

CD expression in the tumor cells was identified in all cases of our series. High CD expression in the tumor cells was observed in 1 case (5.3%) of AD, 2 cases (8.7%) of M, 10 cases (35.7%) of SM, 7 cases (43.8%) of MP and in 15 cases (38.4%) of SS (Table 1). There was a positive correlation between high CD expression of M and SM ($p<0.05$) samples, but there was no correlation with the depth grading of tumor invasion. According to Duke's staging, high CD expression in the tumor cells was observed in 9 cases (45%) of Duke's A, 13 cases (43.3%) of Duke's B, and in 9 cases (40.3%) of Duke's C (Table 2). There was no correlation between the different Duke's stages.

CD expression in the stromal cells was also present in all the specimens of our series. High CD expression in the stromal cells was observed in 1 case (5.3%) of AD, 1 case (4.3%) of M, 12 cases (42.9%) of SM, 12 cases (75%) of MP, and in 27 cases (69.2%) of SS (Table 1). There was a correlation between high CD in the stromal cells of M and SM ($p<0.01$) samples and also SM and MP ($p<0.05$). However, as for the tumor cells, there

Table 3. Relationship between Depth of Invasion and Lymph Node Metastasis

	Depth of invasion		
	SM	MP	SS
number	17	16	39
N(+)	1(5.9%)	2(12.5%)	17(43.6%)

Table 4. Relationship between High Cathepsin D Expression and Lymph Node Metastasis

	Lymph node metastasis	
	N (-)	N (+)
number	52	20
Tumor cell	22(42.3%)	NS 9(45%)
Stromal cell	34(65.4%)	NS 11(55%)

was no correlation with the depth grading of tumor invasion (Table 1). High CD in the stromal cells was observed in 12 cases (60%) of Duke's A, 14 cases (46.7%) of Duke's B, and in 20 cases (90.9%) of Duke's C (Table 2). There was a correlation between Duke's B and Duke's C ($p < 0.01$).

Lymph node metastasis was assessed for the 72 cases of surgical specimens, and it was observed in 1 case (5.9%) of SM, 2 cases (12.5%) of MP, and in 17 cases (43.6%) of SS (Table 3). There was no correlation between high CD expression and lymph node metastasis in either tumor cells or stromal cells (Table 4).

Discussion

Carcinoma cells must penetrate the epithelial basement membrane to initiate invasive growth. In this study, we demonstrated a correlation between cathepsin D expression in colorectal tumors and both the tumor progression and depth of invasion. High CD expression in the tumor cells was less frequent in severe dysplastic adenoma (AD) and intramucosal adenocarcinomas (M), but it was enhanced in invasive carcinomas (SM, MP, SS). There was a strong correlation between high CD expression in carcinomas of the M and SM. These results support previous studies in gastric adenocarcinomas that found a significant association between CD expression and the depth grading of tumor invasion^{15, 16}. In addition, a correlation between CD expression and prognosis was reported in oesophageal squamous cell carcinoma¹⁷ and breast carcinoma^{6, 7, 18}. However, until now a similar association in colorectal tumors has not been confirmed⁸⁻¹¹. Our findings suggest that CD is highly expressed in the early stages of tumor invasion when carcinoma cells have invaded beyond the muscularis mucosa and into the submucosal stroma. We hypothesized therefore that CD plays an important role in degrading the basement membrane, and that CD expression may correlate with the invasive potential of tumor cells.

Similar results were found for CD expression in the stromal cells. There was correlation between high CD expression in the stromal cells of M and SM, and also between SM and

Table 5. Relationship between High Oathepsin D Expression and Grading of Lymph Node Metastasis

	Grading of lymph node metastasis		
	N1 (+)		N2(+) or N3(+)
number	15		5
Tumor Gell	7(46.7%)	NS	2(40%)
Stromal cell	8(53.3%)	NS	3(60%)

MP. Furthermore, there was correlation between Duke's B and Duke's C stages. There are no previous reports concerning the depth grading correlation in colorectal tumors, but Theodoropoulos *et al*⁽¹¹⁾ also reported a correlation between CD expression in stromal cells and the Duke's stage. A relationship between CD expression in stromal cells and prognosis has also been reported in breast cancer^{7,16)}, and further studies are needed to ascertain whether the same association exists in colorectal adenocarcinoma. Tetu *et al*⁽⁷⁾ showed that expression of CD in cancer cells may not be involved in the invasive phenotype of breast cancer, instead that the enzyme derived from the reactive stromal cells may play an important role. In vitro assays by Johnson *et al*⁽⁶⁾ and some other clinical studies^{19,20)} demonstrated that an increase in CD activity in stromal components, such as infiltrative inflammatory cells, and not the cancer-related synthesis of the enzyme, may have a more significant biological role. Although it is known that inflammatory cell infiltration at the border of the invasive front acts as a defense mechanism against tumor invasion, inflammatory cells of the stroma inside and adjacent to the tumor mass contain various enzymes including cathepsin D that could degrade extramatrixes and destroy the tissue architecture thereby facilitating tumor spread⁽¹¹⁾. Graf *et al*⁽⁵⁾ reported that cathepsins discharged from inflammatory or carcinoma cells may activate other proteases. Subsequently, the degradation of extracellular matrix by such proteases may provide an effective microenvironment for proliferation and invasion of the carcinoma cells.

Lymph node metastasis was proven histologically in 72 archival surgical specimens of primary colorectal adenocarcinomas. There was no correlation between CD expression and lymph node metastasis for either tumor cells or stromal cells. Furthermore, no correlation was found when the cases that had lymph node metastasis were separated into two groups according to the grading of the metastasis, such as "N1 (+)" and "N2 (+) or N3 (+)" (Table 5). In a study of breast cancer, Xu *et al*⁽²¹⁾ reported a significant difference in the rate of the lymphatic metastasis between CD-positive and CD-negative cancer cells, however, a study by Aziz *et al*⁽²²⁾ found no significant correlation between axillary lymph node metastasis and CD positivity. As for colorectal tumors, although Arao *et al*⁽¹²⁾ found a correlation between CD expression and lymphatic invasion, there was no correlation with lymph node metastasis. The findings from our study suggest a poor correlation between CD expression and lymph node metastasis in both tumor cells and stromal cells.

In conclusion, cathepsin D expression in carcinoma cells and stromal cells may reflect a potential for invasive growth of colorectal tumors. Our study findings could be useful for predicting the malignant potential of colorectal tumors obtained by endoscopic mucosal resection.

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