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COLORECTAL CANCER

Clinical significance of subcellular localization of KL-6 mucin in primary colorectal adenocarcinoma and metastatic tissues

Qian Guo, Wei Tang, Yoshinori Inagaki, Yutaka Midorikawa, Norihiro Kokudo, Yasuhiko Sugawara, Munehiro Nakata, Toshiro Konishi, Hirokazu Nagawa, Masatoshi Makuuchi

Qian Guo, Wei Tang, Yutaka Midorikawa, Norihiro Kokudo, Yasuhiko Sugawara, Masatoshi Makuuchi, Hepatobiliary-Pancreatic Surgery Division, Department of Surgery, Graduate School of Medicine, the University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan
Yoshinori Inagaki, Munehiro Nakata, Department of Applied Biochemistry and Institute of Glycotechnology, Tokai University, Hiratsuka, Kanagawa 259-1292, Japan
Toshiro Konishi, Kanto Medical Center, NTT EC, 5-9-22 Higashigotanda, Shinagawa-ku, Tokyo 141-8625, Japan
Hirokazu Nagawa, Surgical Oncology Division, Department of Surgery, Graduate School of Medicine, the University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan
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Correspondence to: Dr Wei Tang, Hepatobiliary-Pancreatic Surgery Division, Department of Surgery, Graduate School of Medicine, the University of Tokyo, 7-3-1 Hongo, Bunkyoku, Tokyo 113-8655, Japan. tang-sur@h.u-tokyo.ac.jp
Telephone: +81-3-5800-8654 Fax: +81-3-5684-3989
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Abstract

AIM: To assess subcellular localization of KL-6 mucin and its clinicopathological significance in colorectal carcinoma as well as metastatic lymph node and liver tissues.

METHODS: Colorectal carcinoma tissues as well as metastatic lymph node and liver tissues were collected from 82 patients who underwent colorectomy or hepatectomy. Tissues were subjected to immunohistochemical analysis using KL-6 antibody.

RESULTS: Of the 82 colorectal carcinoma patients, 6 showed no staining, 29 showed positive staining only in the apical membrane, and 47 showed positive staining in the circumferential membrane and/or cytoplasm. Positive staining was not observed in non-cancerous colorectal epithelial cells surrounding the tumor tissues. The five-year survival rate was significantly lower in cases showing positive staining in the circumferential membrane and/or cytoplasm (63.0%) than those showing positive staining only in the apical membrane (85.7%) and those showing no staining (100%). Statistical analysis between clinicopathological factors and subcellular localization of KL-6 mucin showed that KL-6 localization in the circumferential membrane and/or cytoplasm was significantly associated with the presence of venous invasion (P = 0.0003), lymphatic invasion (P < 0.0001), lymph node metastasis (P < 0.0001), liver metastasis (P = 0.058), and advanced histological stage (P < 0.0001). Positive staining was observed in all metastatic lesions tested as well as in the primary colorectal carcinoma tissues.

CONCLUSION: The subcellular staining pattern of KL-6 in colorectal adenocarcinoma may be an important indicator for unfavorable behaviors such as lymph node and liver metastasis, as well as for the prognosis of patients.

Key words: KL-6 mucin; Colorectal carcinoma; Metastasis; Prognosis; Immunohistochemistry

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INTRODUCTION

MUC1, a transmembrane glycoprotein[1, 2], has been detected in various cancer cell lines and secretory epithelial cells lining the respiratory, reproductive, and gastrointestinal tracts[3-6]. It has been suggested that MUC1 may influence cell-to-cell adhesion, diminish the immune response, and be involved in intracellular signaling[7,8]. In carcinoma cells, it has been reported that high levels of MUC1 expression correlate with the invasive characteristic of tumors[9-13]. Our latest study has also shown that aberrant expression of MUC1, which was detected by KL-6 antibody, is associated with cancer progression in the carcinoma of the ampulla of Vater[14].

In normal epithelium, MUC1 is predominantly present on the apical surface of the epithelial cells[15,16]. Recently, it has been reported that, in breast carcinoma, MUC1 is expressed not only on the apical surface but also on

circumferential and basal membranes and in the cytoplasm of the carcinoma cells[15,16]. Furthermore, this aberrant localization of MUC1 has been reported to be associated with worse prognosis for the patient[17]. These findings suggest that subcellular observation of MUC1 expression in carcinoma cells is likely important for understanding the function of MUC1 and improving the prediction of prognosis. However, little is known about the clinical significance of subcellular localization of KL-6 mucin in other carcinomas.

In this study, we focused on subcellular localization of MUC1 in colorectal carcinoma as well as in the metastatic lymph nodes and liver tissue. MUC1 expression was immunohistochemically detected using KL-6 antibody, which recognizes the sialylated oligosaccharide moiety of MUC1 as a part of an epitope[18]. In colorectal carcinoma tissues, it has been suggested that the expression of KL-6 mucin is associated with tumor aggressiveness[10,19]. However, the subcellular localization and physiological function of KL-6 mucin in colorectal carcinoma have remained unknown. In this paper, we report that aberrant subcellular expression of KL-6 mucin in the circumferential membrane and/or cytoplasm is associated with lymph node and liver metastases and worse prognosis in colorectal carcinoma.

MATERIALS AND METHODS

Patients

Colorectal carcinoma tissues were collected from 82 consecutive patients (55 males and 27 females; 64.5 ± 11.4 years, mean ± SD) with a single primary colorectal adenocarcinoma who underwent surgical resection at the Department of Surgery, Graduate School of Medicine, the University of Tokyo, between January 1991 and December 1992. For all cases with lymph node and liver metastasis, whole specimens of resected lymph nodes and metastatic liver tissues were collected from 36 and 7 patients, respectively, in the study group. All specimens were classified according to Japanese Classification of Colorectal Carcinoma by the Japanese Society for Cancer of the Colon and Rectum[20], including the status of lymph node and liver metastasis at the time of surgical intervention and the depth of invasion (m, invasion of mucosa; sm, invasion of submucosa; mp, invasion of muscularis propria; ss, invasion of subserosa or subadventitia; se, invasion of serosa or adventitia; and si, invasion of adjacent structures).

Immunohistochemical staining

The immunohistochemical staining approach matched that of previous studies[14]. Briefly, 4 μm-thick sections were cut from archival formalin-fixed paraffin-embedded tissue blocks, deparaffinized, and dehydrated using a graded series of ethanol solutions. Endogenous peroxidase activity was halted through administration of 3 mL/L hydrogen peroxide/methanol for 30 min. The slides were rinsed with phosphate-buffered saline and then blocked with normal goat serum for 30 min at room temperature. The sections were then incubated with a KL-6 monoclonal antibody solution (1:200 dilution; Eisai, Tokyo, Japan) for 60 min at room temperature. After the sections were incubated with biotinylated secondary antibody for 60 min, bound biotinylated antibody was then tested by the biotin-streptavidin-peroxidase complex method following the manufacturer's instructions (Histofine SAB-PO kit; Nichirei, Tokyo, Japan). 3,3'-Diaminobenzidine was used as the chromogen, and hematoxylin was used as a counterstain. The negative control sections were treated by omitting the primary antibody to monitor background staining.

Evaluation of immunohistochemically stained carcinomas

Overall staining was evaluated in carcinoma cells observed in 10 random microscopic fields, or in the entire area if the tissue sample comprised less than 10 fields. Subcellular staining patterns were recorded by judging the apical membrane, circumferential membrane, and cytoplasm as described elsewhere[17]. Three investigators (Q.G., W.T., and N.K.) separately judged the staining characteristics, and the discrepancies were resolved through mutual observation and discussion of the microscopic fields.

Statistical analysis

The χ^2-test was used to evaluate the relationship between staining pattern and clinicopathological parameters. Survival curves were calculated using the Kaplan-Meier method and compared with the results of the log-rank test. Two patients (one in the no-staining group, another in the apical membrane staining group) were excluded from the data analysis for survival because the cause of death for these patients was not colorectal cancer. $P < 0.05$ was considered statistically significant. Statview 5.0J (Abacus Concepts, Berkeley, CA, USA) statistical software was used for data analyses.

RESULTS

Subcellular localization of KL-6 mucin

Among the 82 cases of colorectal carcinoma, 76 cases showed positive staining of KL-6 mucin. As shown in Figure 1, there was a considerable heterogeneity in the subcellular localization of KL-6 mucin. Staining was observed in either the apical or circumferential membrane (Figures 1A and 1B). Some cases showed positive staining in the cytoplasm in addition to the membranous region (Figures 1C and 1D). The number of cases showing the respective subcellular staining patterns are summarized in Table 1. It is notable that cytoplasmic staining tended to be accompanied by positive staining in the circumferential membrane (37/45, 82%) rather than in the apical membrane (8/45, 18%). Positive staining was not observed in non-cancerous colorectal epithelial cells in any case of this study (data not shown).

Relationship between survival and subcellular localization of KL-6 mucin

The five-year survival rate was 85.7% for cases showing positive staining only in the apical membrane (n = 28), 61.5% for cases showing positive staining in the circumferential membrane (n = 39), and 64.4% for cases showing positive staining in cytoplasm (n = 45) (data

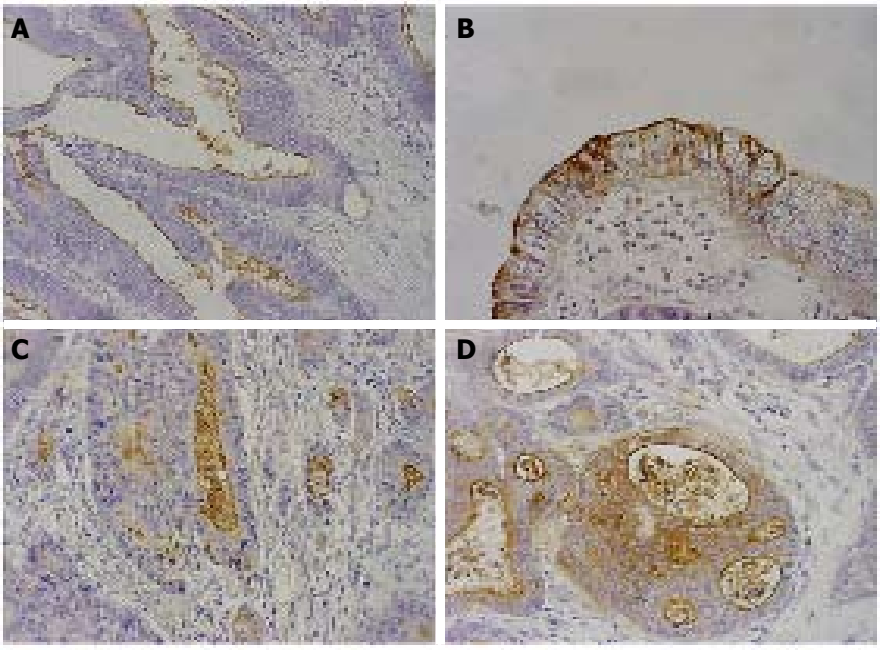

Figure 1 Immunohistochemical staining of colorectal adenocarcinoma tissues using KL-6 antibody (× 200). **A**: Positive staining in the apical membrane; **B**: positive staining in the circumferential membrane; **C:** positive staining in the apical membrane and cytoplasm; **D**: positive staining in the circumferential membrane and cytoplasm.

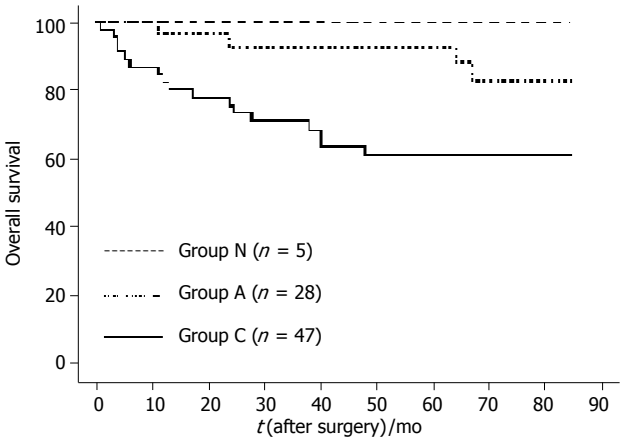

Figure 2 Kaplan-Meier curves for overall survival rates of patients with colorectal adenocarcinoma. Patients with KL-6 expression in the circumferential membrane and/or cytoplasm (solid line, group C, *n* = 46), in the apical membrane (dashed line, group A, *n* = 29) and without KL-6 staining (dotted line, group N, *n* = 5) were followed up for more than 70 mo. Two of 82 patients were excluded from the data analysis as described in Materials and Methods.

Table 1 Summary of subcellular staining of KL-6 mucin in colorectal adenocarcinoma

Group[1]	KL-6 mucin staining			*n*
	Apical membrane	Circumferential membrane	Cytoplasm	
N	Negative	Negative	Negative	6
A	Positive	Negative	Negative	29
C	Positive	Negative	Positive	8
C	Negative	Positive	Negative	2
C	Negative	Positive	Positive	37

[1]Patient groups N, A, and C were categorized according to the subcellular expression profile of KL-6 mucin (see text).

not shown). There were significant differences between the cases showing positive staining only in the apical membrane and the cases showing positive staining in the circumferential membrane ($P = 0.021$), and between the cases showing positive staining only in the apical membrane and the cases showing positive staining in cytoplasm ($P = 0.033$). On the other hand, the five-year survival rate was 100% for the cases showing no staining ($n = 5$). These results suggested that a subcellular KL-6 expression profile was associated with survival, and that cases showing positive staining in the circumferential membrane and/or cytoplasm showed worse prognosis.

As described above, cytoplasmic staining tended to be accompanied with positive staining of the circumferential membrane. Therefore, we classified the cases into the following three groups according to their subcellular staining profile: group N, negative ($n = 6$); group A, positive only in the apical membrane ($n = 29$); and group C, positive in the circumferential membrane and/or cytoplasm ($n = 47$) (Table 1). As shown in Figure 2, the five-year survival rate was significantly lower in group C (63.8%) than that in group A (85.7%; $P = 0.029$). On the other hand, group N showed the highest five-year survival rate (100%).

Relationship between clinicopathological factors and subcellular localization of KL-6 mucin

The relationship between clinicopathological factors and subcellular KL-6 mucin staining of the colorectal adenocarcinomas is summarized in Table 2. Positive staining in the circumferential membrane and/or cytoplasm was significantly associated with the presence of venous invasion, lymphatic invasion, and lymph node metastasis. This subcellular staining characteristic was also associated with the progression of the depth of invasion and histological stage (Table 2). Notably, all cases having lymph node ($n = 36$) or liver metastasis ($n = 7$) showed positive staining in the circumferential membrane and/or cytoplasm. This suggested that aberrant subcellular expression of KL-6 mucin in the circumferential

Table 2 Relationship between clinicopathological factors and sbcellular staining profile of KL-6 mucin in colorectal adenocarcinoma *n*(%)

Factors	*n*	Subcellular staining profile of KL-6 mucin			*P*
		No staining	Apical membrane	Circumferential membrane and/or cytoplasm	
Age (yr)					0.449
≤60	29	2 (6.9)	12 (41.4)	15 (51.7)	
>60	53	4 (7.5)	17 (32.1)	32 (60.4)	
Sex					0.469
Male	55	4 (7.3)	21 (38.2)	30 (54.5)	
Female	27	2 (7.4)	8 (29.6)	17 (63.0)	
Differentiation					0.055
Well	50	6 (12.0)	21 (42.0)	23 (46.0)	
Moderate	29	0 (0)	8 (27.6)	21 (72.4)	
Poor	3	0 (0)	0 (0)	3 (100)	
Venous invasion					0.0003
(+)	40	0 (0)	9 (22.5)	31 (77.5)	
(-)	42	6 (14.3)	20 (47.6)	16 (38.1)	
Lymphatic invasion					<0.0001
(+)	24	0 (0)	2 (8.3)	22 (91.7)	
(-)	58	6 (10.3)	27 (46.6)	25 (43.1)	
Depth of invasion					0.009
m	3	3 (100)	0 (0)	0 (0)	
sm, mp	14	2 (14.3)	8 (57.1)	4 (28.6)	
ss, se	59	1 (1.7)	20 (33.9)	38 (64.4)	
si	6	0 (0)	1 (16.7)	5 (83.3)	
Histological stage					<0.0001
0	3	3 (100)	0 (0)	0 (0)	
I	12	2 (16.7)	8 (66.6)	2 (16.7)	
II	29	1 (3.4)	20 (69.0)	8 (27.6)	
IIIa	29	0 (0)	1 (3.4)	28 (96.6)	
IIIb	6	0 (0)	0 (0)	6 (100)	
IV	3	0 (0)	0 (0)	3 (100)	
Lymph node metastasis					<0.0001
(+)	36	0 (0)	0 (0)	36 (100)	
(-)	46	6 (13.0)	29 (63.0)	11 (24.0)	
Liver metastasis					0.058
(+)	7	0 (0)	0 (0)	7 (100)	
(-)	75	6 (8.0)	29 (38.7)	40 (53.3)	

membrane and/or cytoplasm might participate in the metastasis of tumor.

Expression of KL-6 mucin in metastatic lesions

We next examined the expression of KL-6 mucin in lymph node and liver metastatic lesions (Figure 3). For all the 36 cases with lymph node metastasis and all seven cases with liver metastasis, positive staining was observed in metastatic lesions as well as in the primary colorectal carcinoma tissues.

DISCUSSION

MUC1 is abundantly expressed at the surface of epithelial cells in many tissues[3-6]. MUC1 expression is also observed in carcinomas that arise in several parts of the body, such as the colon, breast, lung, pancreas, papillary thyroid, and gallbladder[9-13]. The deduced amino acid sequence of MUC1 mucin reveals four distinct domains: the NH_2-terminal domain consisting of a hydrophobic signal sequence; a highly *O*-glycosylated tandem-repeat domain; a transmembrane domain; and a cytoplasmic domain[1,2,6]. Previous studies on MUC1 expression in colorectal carcinoma have primarily focused on the tandem-repeat domain, and suggested that tumor cells expressing high levels of MUC1 may have increased invasive and metastatic potential[10]. However, little is known about the detailed clinicopathological relationship among expression profile of MUC1, metastatic potentiality, and the prognosis for colorectal adenocarcinoma. On the other hand, although the processing of the full length MUC1 core proteins is similar in both normal and tumor cells, they have a remarkable diversity in oligosaccharide moieties[21,22]. Therefore, we targeted KL-6 mucin, a type of MUC1 bearing sialylated oligosaccharide recognized by KL-6 antibody[18]. Since sialylation of tumor cell glycoconjugates is thought to contribute to tumor progression and metastasis[23-26], targeting KL-6 mucin bearing sialylated oligosaccharide seems to be a reasonable strategy.

In our preliminary study, KL-6 mucin was observed in carcinoma cells but not in the surrounding normal cells. However, classification of KL-6 staining evaluated by overall expression level did not show significant relationships between the expression level of KL-6 mucin, metastasis, and patient's prognosis (data not shown). Recently, some reports on breast carcinoma have suggested a significant relationship between metastasis and subcellular location of MUC1 rather than its overall expression level, which led us to focus on the subcellular location of KL-6 mucin in colorectal carcinoma.

In the present study, the circumferential and/or cytoplasmic expression of KL-6 mucin was significantly correlated with lymph node metastasis in colorectal adenocarcinomas (Table 2). In addition, this aberrant localization of KL-6 mucin was likely to participate also in liver metastasis, since all cases having liver metastasis (n = 7) showed positive staining in the circumferential membrane and/or cytoplasm (Table 2). It is known that normal epithelial cells release a tailless, soluble form of MUC1 which targets exclusively the apical membrane in tissues[21]. However, in carcinoma cells with aberrant overexpression of MUC1, this apical polarization is lost, resulting in aberrant localization of MUC1 over the entire cell membrane and in the cytoplasm[15,16]. It has been proposed that MUC1 mediates anti-adhesion activity by interfering with cell-to-cell and/or cell-to-extracellular matrix interactions, thereby facilitating detachment of tumor cells from the primary growth[6, 27-29]. This is likely true of KL-6 mucin in colorectal adenocarcinoma, since a high frequency of metastasis was observed in cases showing any aberrant localization of KL-6 mucin (Table 2). This aberrant subcellular expression of KL-6 mucin might facilitate detachment of tumor cells from the primary growth, resulting in an increased ability of the tumor cells to metastasize.

It is notable that all the cases tested showed positive staining in metastatic lesions as well as the primary colorectal carcinoma tissues. Interestingly, in some cases presenting lymph node or liver metastasis, aberrant subcellular localization of KL-6 was observed in only

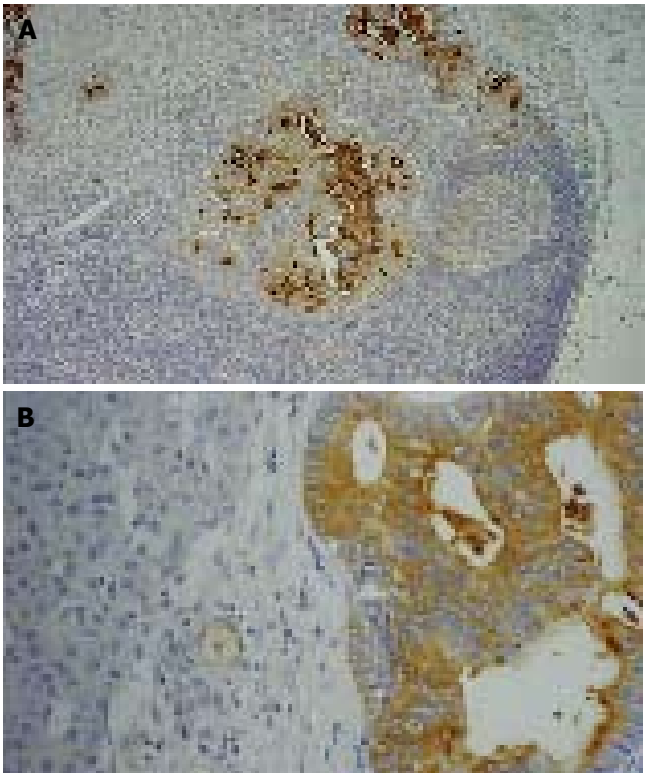

Figure 3 Immunohistochemical staining of metastatic lymph node and liver tissues using KL-6 antibody (×200). **A:**Positive KL-6 expression in a metastatic lymph node; **B:** positive KL-6 expression in metastatic liver tissues.

a few tumor cells (less than 5%) in the primary lesions (data not shown). This suggests that the significant factor is the subcellular location rather than the level of KL-6 expression, and is coincident with a report that breast carcinomas with the cytoplasmic staining pattern, even in a minor focus, are associated with a higher incidence of lymph node metastasis[17]. However, a considerable number of cases showing positive membranous and/or cytoplasmic staining did not present with metastases. Further investigation is needed to understand the role of KL-6 expression in metastatic events of colorectal adenocarcinoma.

Some studies have reported that, in breast carcinoma, patients expressing MUC1 in the non-apical membranes show worse prognosis than those expressing MUC1 in the apical membrane[17,30]. Our observation also showed that there was a significant relationship between subcellular location of KL-6 and prognosis in colorectal adenocarcinoma (Figure 2; $P = 0.029$). The five-year survival rates for the cases with apical membrane staining and those with circumferential and/or cytoplasmic staining of KL-6 mucin were 85.7% and 63.0%, respectively (Figure 2). Moreover, our data also showed that cases without KL-6 staining had a better prognosis than those with the apical staining of KL-6 mucin. These data suggest that the expression characteristics of KL-6 mucin would be useful for the prediction of a patient's prognosis in colorectal adenocarcinoma.

In conclusion, subcellular localization of KL-6 mucin plays a crucial role in determining disease outcome and expression of KL-6 mucin in the circumferential membrane and/or cytoplasm is an important indicator for lymph node and liver metastases as well as the prognosis of patients with colorectal adenocarcinoma.

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