

Upregulation of kallikrein-related peptidase 5 is associated with the malignant behavior of colorectal cancer

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Abstract. Kallikrein-related peptidase 5 (KLK5) is a serine protease that has exhibited upregulated expression in numerous types of human cancer. The present study assessed KLK5 expression in colorectal cancer (CRC) tissues, in order to determine its association with clinicopathological data and prognosis. The mRNA and protein expression levels of KLK5 were detected using reverse transcription-quantitative polymerase chain reaction, immunohistochemistry and enzyme-linked immunosorbent assay, respectively. KLK5 expression was detected in 48 paraffin-embedded tumor tissue samples and corresponding tumor-free areas within the same specimens, 40 paired normal and CRC frozen tissues, and serum samples from 70 patients with CRC (including 38 serum samples taken pre- and post-surgery) and 53 healthy individuals. The results demonstrated that KLK5 protein was strongly expressed in CRC; however, its expression was hardly detected in the matched normal mucosal tissue. The KLK5 mRNA expression levels were significantly upregulated in CRC tissues compared with the paired normal tissues, and were higher in Dukes' stage C/D cancer than in stage A/B ($P<0.001$). Furthermore, sera from patients with CRC exhibited increased KLK5 levels, as compared with that of healthy volunteers (878.02 vs. 391.07 pg/ml; $P<0.001$). Serum KLK5 levels were also significantly higher in patients prior to surgery compared with post-surgery (909.48 ± 536.72 vs. 644.00 ± 522.87 pg/ml; $P<0.001$). In addition, increased serum KLK5 levels were associated with CRC lymph node or distant metastasis ($P=0.003$), tumor-lymph node-metastasis stage

($P=0.004$), and Dukes' stage ($P=0.005$). The results of the present study indicated that the mRNA and protein expression levels of KLK5 were significantly upregulated in CRC tissues and sera, and were associated with an advanced tumor stage. Further studies may identify KLK5 as a biomarker of CRC recurrence or treatment response.

Introduction

Colorectal cancer (CRC) is the third most common type of human malignancy, and the second leading cause of cancer-associated mortality worldwide (1). The incidence of CRC has been increasing in Asian countries, including China, Korea, Japan, and Singapore. The reason for this increasing incidence is currently unclear; however, demographic trends and adaptation to a westernized lifestyle, particularly with regards to factors such as diet, may have a role in the increased incidence of CRC in these countries (2). During the past few decades, there have been numerous advances in CRC research, including early detection, prevention by adaptation to a healthier lifestyle, and improved treatment strategies; however, a high percentage of patients with CRC still develop advanced stages of the disease and tumor metastasis (3). Therefore, the identification and evaluation of biomarkers for the prediction of disease progression, prognosis, and treatment response may help physicians effectively treat and control the spread of cancer in these patients. To date, clinicopathological characteristics, including CRC tumor-lymph node-metastasis (TNM) stage, differentiation grade, lymph node metastasis, invasion to other tissue or structures, and tumor size, have been used to evaluate treatment selection and prognosis of patients with CRC. Various biomarkers, such as carcinoembryonic antigen (CEA) and carbohydrate antigen (CA)19-9, have previously been identified and evaluated with regards to the diagnosis, and prediction of tumor recurrence or treatment response (4-6). However, these biomarkers are not without fault, due to the lack of specificity and sensitivity; therefore, more research is required to identify novel serum-based tumor markers that may be used screen the general or high-risk population for early diagnosis of CRC, or predict CRC prognosis and treatment response (4).

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Kallikrein-related peptidase 5 (KLK5), which is one of 15 kallikrein subfamily members, is a serine protease located on chromosome 19 (7). Functionally, KLK5 is usually expressed in the epidermis and regulates cell shedding in conjunction with KLK7 and KLK14 (7-9). KLK5 can become activated from the secreted pro-KLK5, in order to activate several other KLKs, such as KLK2, -3, -6, -7, -11, -12 and -14 (10). In addition, KLK5 proteolytic activity is able to target metalloproteases and extracellular matrix components, including collagens, fibronectin, and laminin (11,12). Previous studies have demonstrated that KLK5 is upregulated in numerous types of human cancer (7,13-19). Indeed, kallikrein-mediated extracellular proteolysis is important in several facets of cancer development, including regulation of tumor growth, invasion, metastasis, and angiogenesis (20). A previous study demonstrated that altered expression of KLKs is associated with cancer development and diagnosis, and may therefore be considered a useful prognostic marker for prostate, breast, and ovarian cancer (13). In CRC, KLK5 protease accumulates in tumor tissues and is associated with an unfavorable outcome for patients (21). The present study further assessed KLK5 expression in CRC tissue specimens and serum samples from patients with CRC using reverse transcription-quantitative polymerase chain reaction (RT-qPCR), immunohistochemistry, and enzyme-linked immunosorbent assay (ELISA). In addition, the association between KLK5 expression and clinicopathological parameters of the patients was determined.

Materials and methods

Study subjects. The present study collected 40 fresh CRC and paired normal tissues from patients who had undergone surgical resection of CRC lesions at the Department of Surgery, General Hospital of Jinan Military Command (Jinan, China) between November 2006 and May 2012. Among these patients, there were 23 men and 17 women with a median age of 58 years (range, 31-77 years). CRC was diagnosed and graded according to the revised World Health Organization grading system and was staged according to the Dukes' operative staging system. In particular, 4/40 cases of CRC (10%) were well-differentiated, 27/40 (67.5%) were moderately differentiated, and 9/40 (22.5%) were poorly differentiated; whereas 10/40 cases of CRC (25%) were at stage A, 11/40 (27.5%) were at stage B, 17/40 (42.5%) were at stage C, and 2/40 (5%) were at stage D (Dukes' stages). A total of 17 patients exhibited regional lymph node tumor metastasis, and 2 patients exhibited tumor metastasis to the liver.

In addition, 48 paraffin-embedded CRC tissue samples were obtained from 28 male and 20 female patients with a median age of 60 years and a range of 22-80 years, and serum samples were collected from an additional 70 patients with CRC (Table I), including 38 serum samples taken pre- and post-surgery, and 53 healthy individuals from the same hospital. The present study was approved by the Medical Ethics Committee of the General Hospital of Jinan Military Command (Jinan, China), and all participants provided written informed consent.

RT-qPCR. Total RNA was isolated from the 40 paired normal and tumor frozen tissues using TRIzol reagent

(Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. Frozen samples (100 mg) were homogenated in 1 ml TRIzol, RNA quality and quantity were measured using a LAMBDA Bio UV/VIS Spectrophotometer (PerkinElmer, Inc., Waltham, MA, USA), and RNA was reverse transcribed into cDNA using a real-time qPCR kit (Takara Biotechnology Co., Ltd., Dalian, China), according to the manufacturer's protocol. The SYBR Green qPCR assay was performed to amplify the cDNA samples using a Roche LightCycler 480 II (Roche Diagnostics, Basel, Switzerland). The primers used were as follows: KLK5, forward 5'-AAGGTCCTCCAGTGC TTGAA-3', reverse 5'-CCAACAGACCGGGTGTCTAC-3' (synthesized in our laboratory); and GAPDH, forward 5'-GAA GGTGAAGGTCGGAGTC-3' and reverse 5'-GAAGATGGT GATGGGATTTC-3' (Sango Biotech, Shanghai, China). The qPCR 25- μ l reaction mixture consisted of 12.5 μ l 2X SYBR *Premix Ex Taq* (Takara Biotechnology Co., Ltd.), 1 μ l each primer (100 nM), 1 μ l cDNA and RNase free H₂O up to 25 μ l. The qPCR conditions were set as follows: Initial denaturation at 95°C for 5 min, followed by 40 cycles at 95°C for 5 sec and 60°C for 10 sec, the temperature was then increased from 65 to 92°C to obtain the melting curve, which was used to distinguish specific products from non-specific products or primer dimers. The relative mRNA expression levels of KLK5 were determined by normalization to the endogenous control, GAPDH mRNA, and were calculated using the $2^{-\Delta\Delta C_q}$ method as follows: $\Delta C_q = C_q$ (mRNA of KLK5) - C_q (mRNA of GAPDH). The C_q value was the threshold cycle at which fluorescence was detected. Each sample was measured in duplicate and repeated at least once. The KLK5 and GAPDH qPCR products subsequently underwent 1.5% agarose gel electrophoresis, and were visualized by ethidium bromide staining in order to confirm product size.

Immunohistochemistry. The indirect immunoperoxidase method was used to analyze the KLK5 expression in archived formalin-fixed, paraffin-embedded samples from 48 CRC and matched tumor-free tissues. These tissue samples were used to confirm the RT-qPCR results. For the immunohistochemistry experiments, tissue sections (4 μ m) were cut from the paraffin blocks, deparaffinized twice in warm xylene (5 min each), and rehydrated through a series of graded alcohol solutions. Endogenous peroxidase activity was blocked with 3% H₂O₂ in methanol for 10 min, and with normal serum for 30 min at room temperature. Subsequently, tissue sections were incubated with 200 μ l KLK5 primary antibody at a dilution of 1:400 in phosphate-buffered saline (PBS) at 4°C overnight. This KLK5 rabbit polyclonal antibody was generated in our laboratory using recombinant KLK5 protein (22). Immunohistochemical staining of antibodies was performed using the Dako K5007 Envision Plus System (Dako, Glostrup, Denmark). The antibody binding was visualized with a 3,3'-Diaminobenzidine staining prior to a brief counterstaining with Mayer's hematoxylin. In the negative control experiments, the primary antibody was replaced with antibody diluent. The stained tissue sections were reviewed and scored under an Olympus BX53 microscope (Olympus Corporation, Tokyo, Japan) using a semi-quantitative scoring system for both the intensity of the stain and the percentage of positive neoplastic cells (23).

Table I. Association of serum KLK5 levels with the clinicopathological features of colorectal cancer.

Characteristics	Total number	Serum KLK5 level		P-value
		High	Low/negative	
Gender				0.092
Male	44	33	11	
Female	26	17	9	
Age				0.720
<60 years old	33	24	9	
≥60 years old	37	26	11	
Tumor site				0.700
Rectum	49	35	14	
Colon	21	15	6	
Differentiation				0.890
Well/moderate	51	36	15	
Poor	11	6	5	
Tumor size				0.874
<5 cm	32	24	8	
≥5cm	30	23	7	
Dukes' stage				0.005
A/B	37	23	14	
C/D	28	25	3	
TNM stage				0.004
I/II	40	26	14	
III/IV	26	23	3	
Metastasis				0.003
Absent	37	23	14	
Present	32	23	9	

TNM, tumor-lymph node-metastasis.

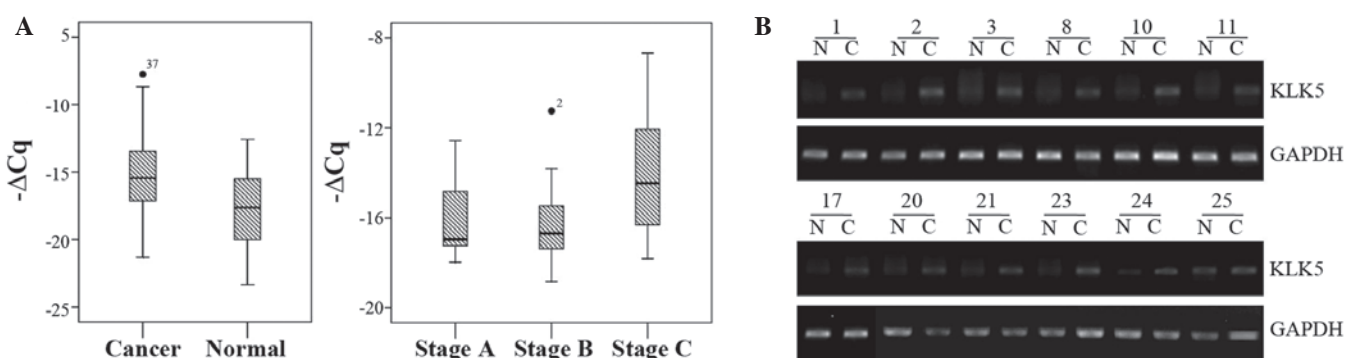


Figure 1. Upregulation of kallikrein-related peptidase 5 (KLK5) mRNA in colorectal cancer (CRC) tissues. (A) Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis of KLK5 mRNA expression levels in paired cancerous (n=40) and normal (n=40) tissues (P<0.001), and stages A-C (n=10, 11, 17, respectively) of the disease (P<0.001 vs. the normal samples) (stars indicate the extreme value and the numbers represent the corresponding sample number). (B) Agarose gel of the KLK5 and GAPDH RT-qPCR products from representative colorectal cancer tissue samples and the corresponding normal colorectal mucosa samples (N) (numbers above the gel indicate the initial subsequence of the specimen). GAPDH exhibited a consistent pattern of expression in all samples, indicating the integrity of the RNA and equal loading.

ELISA. A monoclonal anti-KLK5 antibody generated in our laboratory (22) was used to coat 96-well white polystyrene plates in PBS (750 ng/well) overnight at 4°C. Subsequently, the plates were washed three times with PBS plus 0.5%

Tween 20 (PBS-T), and diluted serum samples or calibrators (100 μl/well; Sino Biological, Inc., Beijing, China) were added to each well and incubated at 37°C for 1 h. The plate was then washed a further six times with PBS-T. Subsequently,

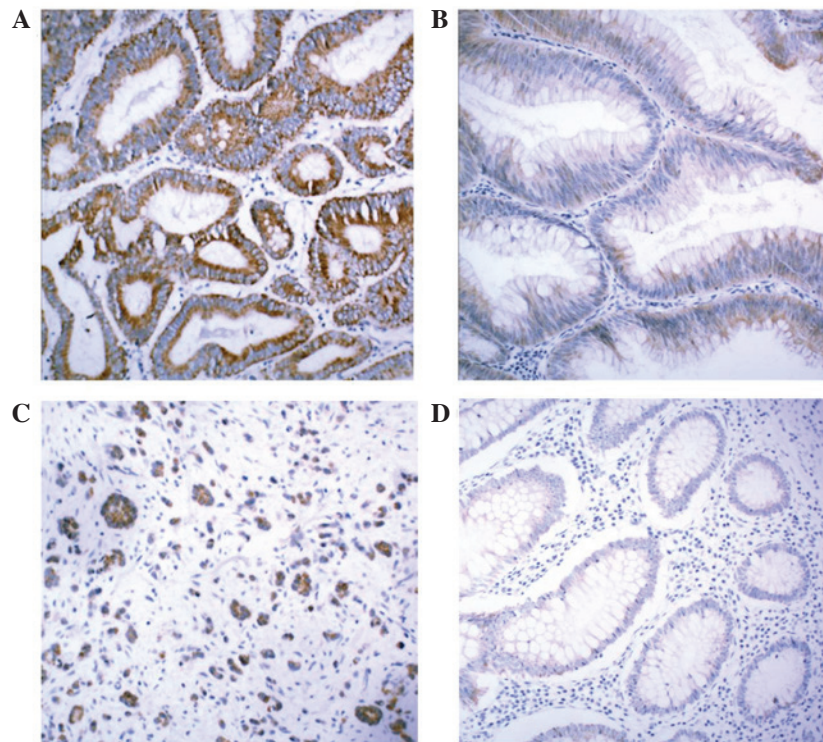


Figure 2. Immunohistochemical analysis of kallikrein-related peptidase 5 (KLK5) protein expression in colorectal cancer (CRC) and normal tissues. (A) Well-differentiated CRC; (B) adenoma; (C) poorly differentiated CRC; (D) normal colonic mucosa. KLK5 protein was upregulated in well-differentiated and poorly differentiated CRC tissues, and KLK5 staining was weak or negative in adenoma and normal colonic mucosa tissues. Magnification, x200.

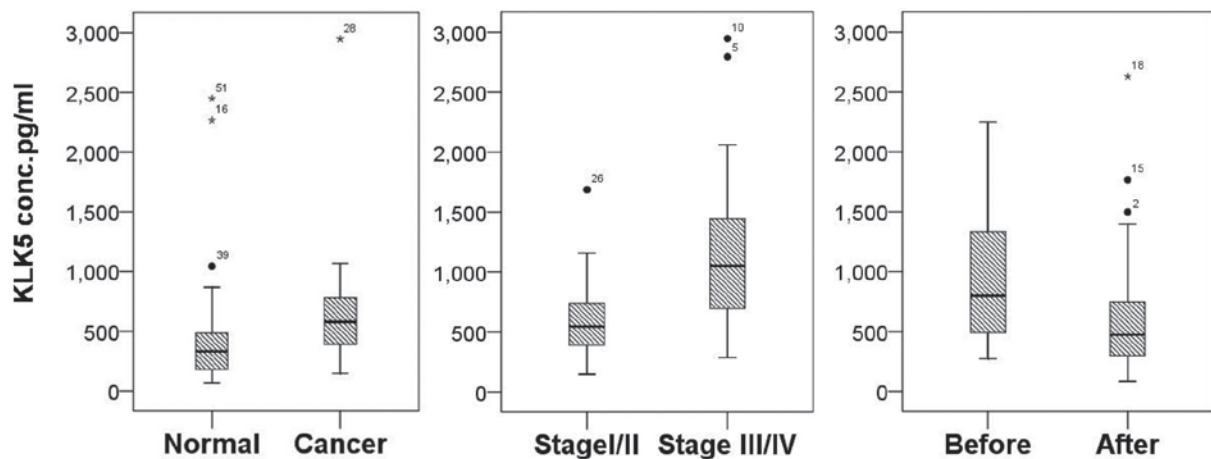


Figure 3. Enzyme-linked immunosorbent assay (ELISA) analysis of serum kallikrein-related peptidase 5 (KLK5) levels in patients with colorectal cancer (CRC) and healthy controls ($P < 0.001$). KLK5 protein concentrations were determined in sera from patients with CRC (cancer; stages I/II and III/IV; $P = 0.004$) and healthy controls (normal), including pre-surgery (before) and post-surgery (after) samples from patients with CRC ($P < 0.001$) (stars indicate the extreme value and the numbers represent the corresponding sample number).

100 μ l horseradish peroxidase-conjugated rabbit anti-KLK5 antibody (1 mg/ml, diluted 1,000-fold in PBS; generated in our laboratory) was added to each well and incubated at 37°C for 40 min. After washing six times with PBST, 100 μ l chromogen (Tetramethylbenzidine) was added to each well and the plates were incubated at 37°C for 10 min. After quenching the reaction with 50 μ l 2 M H_2SO_4 , the absorbance (optical density) was measured at 450 nm using a SpectraMax M2 Multi-Mode microplate reader (Molecular Devices LLC, Sunnyvale, CA, USA).

Statistical analysis. All statistical analyses were performed using the SPSS software package, version 17.0 (SPSS Inc., Chicago, IL, USA). The serum KLK5 levels in healthy individuals compared with patients with CRC and the differences in various stages were analyzed with the Mann-Whitney U test. The serum KLK5 levels were analyzed by Wilcoxon test in patients prior to surgery and were compared with results following the surgery. The differences in expression of KLK5 mRNA in CRC and normal tissues were analyzed by Wilcoxon test. Receiver operating characteristic (ROC)

curves were performed to analyze the data of CEA and serum KLK5 levels. The ROC curves were delineated using Sigmaplot 11.0 (Systat Software Inc., Chicago, IL, USA). Data are expressed as the mean \pm standard deviation. $P < 0.05$ (two-sided) was considered to indicate a statistically significant difference.

Results

KLK5 mRNA expression is upregulated in CRC tissues. The present study initially assessed the mRNA expression levels of KLK5 in 40 tumor and paired normal tissue samples from patients with CRC. The mRNA expression levels of KLK5 were upregulated in CRC tissues, as compared with in the paired normal controls (Fig. 1), and 32/40 CRC tissue samples (80%) exhibited increased KLK5 expression. In addition, the upregulated KLK5 levels were associated with advanced tumor stages (stage C/D vs. stage A/B; $P < 0.001$; Fig. 1).

KLK5 protein expression is upregulated in CRC tissues and sera. The present study also assessed the protein expression levels of KLK5, since mRNA expression does not necessarily correlate with protein abundance. Immunohistochemical staining of KLK5 was performed in 48 CRC samples. KLK5 protein was highly expressed in cancerous lesions but not in normal tissues (Fig. 2A-D).

The present study also analyzed the KLK5 levels in serum samples from 70 patients with CRC and 53 healthy individuals. Among the 70 CRC serum samples, there were 38 paired pre- and post-surgery serum samples taken from patients with CRC. The serum levels of KLK5 were upregulated in patients with CRC, as compared with in the healthy controls (878.02 ± 602.02 vs. 391.07 ± 331.13 pg/ml; $P < 0.001$; Fig. 3). The serum KLK5 levels were also significantly higher in patients prior to surgery compared with after surgery (909.48 ± 536.72 vs. 644.00 ± 522.87 pg/ml; $P < 0.001$). Furthermore, the serum levels of KLK5 were higher in stage III/IV CRC than in stage I/II CRC (1153.56 ± 679.97 vs. $737.91.23 \pm 500.53$ pg/ml; $P = 0.004$).

Finally, ROC curve analysis revealed the significant and the independent value of the KLK5 expression, for the discrimination of the CRC from the normal individuals. The curve demonstrated that serum KLK5 levels [area under the curve (AUC), 0.81; 95% confidence interval (CI), 0.7375-0.8919; $P < 0.001$] exhibited a significant discriminatory value in the whole population (Fig. 4), compared to that of CEA (AUC, 0.77; 95% CI, 0.6901-0.8533; $P < 0.001$). No significant difference was observed between the KLK5 and CEA curves ($P = 0.462$).

Association of KLK5 expression with clinicopathological features of patients with CRC. The association between KLK5 expression and clinicopathological characteristics is summarized in Tables I and II. There were significant associations between serum KLK5 levels and CRC lymph node or distant metastasis ($P = 0.003$), TNM stage ($P = 0.004$), and Dukes' stage ($P = 0.005$) (Table I); whereas the mRNA expression levels of KLK5 in CRC tissues were associated with a high Dukes' stage ($P < 0.001$; Table II).

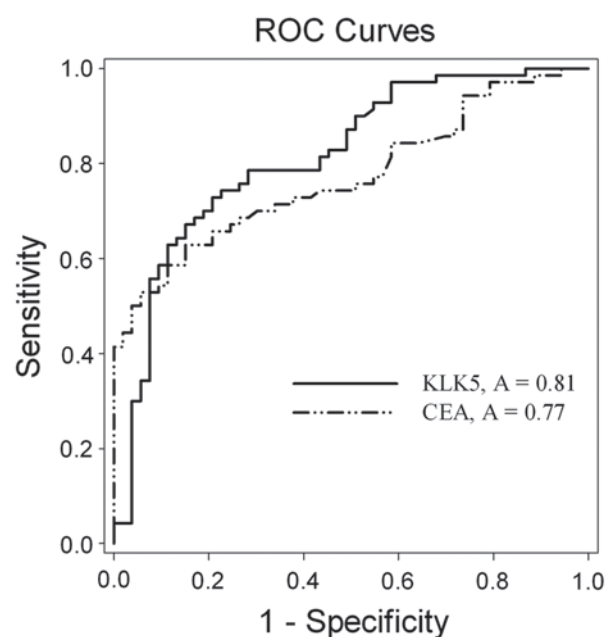


Figure 4. Receiver operating characteristic (ROC) curve analysis of serum kallikrein-related peptidase 5 (KLK5) and carcinoembryonic antigen (CEA) levels. KLK5, 95% confidence interval (CI), 0.7375-0.8919; CEA, 95% CI, 0.6901-0.8533 ($P = 0.462$).

Table II. Association of KLK5 mRNA expression with the clinicopathological features of colorectal cancer.

Characteristics	N	KLK5 mRNA level (Δ Cq)	P-value
Gender			0.436
Male	23	-15.2 ± 2.48	
Female	17	-14.4 ± 3.08	
Age			0.224
<60 years old	20	-14.40 ± 2.82	
≥ 60 years old	20	-15.30 ± 2.64	
Tumor site			0.913
Rectum	22	-14.79 ± 3.02	
Colon	18	-14.94 ± 2.43	
Differentiation			0.295
Well/moderate	32	-15.00 ± 2.84	
Poor	8	-14.27 ± 2.39	
Tumor size			0.699
<5 cm	16	-14.81 ± 3.34	
≥ 5 cm	24	-14.88 ± 2.33	
Dukes' stage			<0.001
A/B	21	-16.23 ± 1.94	
C/D	19	-13.34 ± 2.73	

Cq, cycle quantification.

Discussion

Early clinical detection of CRC typically relies on endoscopy

and pathological analysis of tissue samples, which is expensive and invasive. Studies regarding CRC biomarker discovery have made little progress, possibly due to a lack of sensitive and specific tumor markers (3,4,6). To date, CEA and CA19-9 remain the only widely used serum tumor markers in the early detection or prediction of treatment response in CRC (24). The present study assessed KLK5 expression in CRC tissue and serum samples using RT-qPCR, immunohistochemistry, and ELISA using our own anti-KLK5 antibody. The results demonstrated that the mRNA and protein expression levels of KLK5 were significantly upregulated in independent CRC tissue and serum samples, as compared with in normal samples. As a biomarker, KLK5 exhibited a better ROC curve than CEA. In addition, KLK5 expression was associated with malignant CRC behavior. The results of the present study indicated that KLK5 may be considered a useful biomarker for CRC. However, further studies are required to confirm these findings.

Dysregulated expression of various KLKs has been detected in a large number of human malignancies during development and progression of the disease. Therefore, the detection of KLK levels may serve as a novel and useful tumor biomarker for the early detection and monitoring of progression in patients with cancer (25,26). Among these KLKs, KLK5 is important since it can activate other KLKs. A previous study reported that upregulation of KLK5 in ovarian cancer tissues was associated with advanced stages and grades of the disease, and shorter disease-free and overall survival rates of patients (27). In addition, another previous study identified a novel variant of the KLK5 5'-untranslated region due to alternative splicing, which was able to contribute to differential KLK5 expression in prostate and ovarian cancer (28). This finding indicated that KLK5-SV1 may have clinical use in various malignancies, and should be further explored as a potential novel biomarker for prostate and ovarian cancer (28). It has previously been shown that KLK6 and KLK10 are significantly upregulated in patients with CRC, and that their upregulation is associated with a poor prognosis (29,30). The present study detected the upregulation of KLK5 mRNA and protein in CRC tissues and in sera from patients with CRC, thus indicating that KLK5 may be a useful biomarker for the early diagnosis of CRC.

However, the potential mechanism underlying KLK5 activity in CRC development and progression remains to be elucidated. KLK5 was originally discovered in the epidermis and has functions related to keratinocyte turnover (desquamation), during which this serine protease can digest the extracellular matrix and increase cell mobility (8). Nevertheless, it is currently unknown why and how KLK5 is upregulated in CRC tissues and in sera from patients with CRC. Previous studies have indicated that altered microRNA expression may lead to the upregulation of KLK5 (31,32), and our unpublished data support this notion.

The present study demonstrated that upregulated KLK5 levels may promote CRC development and progression. In addition, after surgery, serum KLK5 levels were downregulated, thus suggesting that KLK5 may be used to monitor CRC recurrence, metastasis, and treatment response. However, the present study does have some limitations; for example, survival or treatment data was not collected from all of the patients with CRC, in order to determine the association with prognosis or treatment response. Furthermore, the sample size was relatively small and the conclusion needs to be veri-

fied in a study with a larger sample size. Future studies will focus on the following: Function of KLK5 in CRC cells and the underlying molecular mechanism; evaluation of KLK5 as a novel biomarker for early detection, tumor progression, and treatment response of patients with CRC; and the mechanisms underlying KLK5 upregulation in patients with CRC.

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