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BRIEF ARTICLE

Overexpression of kallikrein gene 10 is a biomarker for predicting poor prognosis in gastric cancer

Xin Jiao, Hong-Jun Lu, Mi-Mi Zhai, Zhi-Jun Tan, Hai-Ning Zhi, Xiao-Man Liu, Chen-Hao Liu, Da-Peng Zhang

Xin Jiao, Hai-Ning Zhi, Xiao-Man Liu, Chen-Hao Liu, Department of General Surgery, the First Central Clinical School, Tianjin Medical University, Tianjin 300192, China
Hong-Jun Lu, Da-Peng Zhang, Department of Hepatobiliary and Pancreatic Surgery, Tianjin Nankai Hospital, Naikai Clinical School, Tianjin Medical University, Tianjin 300100, China
Mi-Mi Zhai, Department of Gastroenterology, Weifang People's Hospital, Weifang 261041, Shandong Province, China
Zhi-Jun Tan, Department of General Surgery, Tianjin First Central Hospital, Tianjin 300192, China
Author contributions: Jiao X performed the majority of experiments and wrote the manuscript; Lu HJ, Zhai MM, Zhi HN, Liu XM and Liu CH designed the research and revised the manuscript; Zhang DP instructed the preparation of the manuscript; Tan ZJ provided experimental guidance.
Correspondence to: Da-Peng Zhang, PhD, MD, Department of Hepatobiliary and Pancreatic Surgery, Tianjin Nankai Hospital, Naikai Clinical School, Tianjin Medical University, No. 6, Changjiang Road, Nankai District, Tianjin 300100, China. dapeng721115@126.com
Telephone: +86-22-26326537 Fax: +86-22-27435552
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Abstract

AIM: To analyze the expression of kallikrein gene 10 (*KLK10*) in gastric cancer and to determine whether *KLK10* has independent prognostic value in gastric cancer.

METHODS: We studied *KLK10* expression in 80 histologically confirmed gastric cancer samples using real-time quantitative reverse transcription-PCR and hK10 expression using immunohistochemistry. Correlations with clinicopathological variables (lymph node metastasis, depth of invasion and histology) and with outcomes (disease-free survival and overall survival) during a median follow-up period of 31 mo were assessed. Gastric cancer tissues were then classified as *KLK10* positive or negative.

RESULTS: *KLK10* was found to be highly expressed in 57/80 (70%) of gastric cancer samples, while its expression was very low in normal gastric tissues. Positive relationships between *KLK10* expression and lymph node metastasis (P = 0.048), depth of invasion (P = 0.034) and histology (P = 0.015) were observed. Univariate survival analysis revealed that gastric cancer patients with positive *KLK10* expression had an increased risk for relapse/metastasis and death (P = 0.005 and 0.002, respectively). Cox multivariate analysis indicated that *KLK10* was an independent prognostic indicator of disease-free survival and overall survival in patients with gastric cancer.

CONCLUSION: *KLK10* expression is an independent biomarker of unfavorable prognosis in patients with gastric cancer.

Key words: Kallikrein gene 10; Gastric cancer; Survival analysis; Prognostic biomarkers

Core tip: The study examined the clinicopathologic and prognostic significance of kallikrein gene 10 (*KLK10*) expression in gastric cancer. Based on collective findings, we hypothesize that *KLK10* expression in gastric cancer tissues may have prognostic/predictive value in patients with gastric cancer. *KLK10* expression is an independent biomarker for predicting unfavorable prognosis in patients with gastric cancer.

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INTRODUCTION

Gastric cancer is the fourth most common cancer, and the second leading cause of cancer death worldwide[1]. Mortality due to gastric cancer has risen in China over the past 20 years, especially in rural areas and in aging populations[2,3]. Although the increased use of screening for early disease diagnosis and the widespread administration of systemic adjuvant therapies have led to a decline in mortality rates, the incidence and mortality of gastric cancer are still second only to lung cancer[4,5].

The kallikrein gene family of secreted serine proteases, consisting of 15 genes, is localized in tandem on chromosome 19q13.4 and shows significant homologies at both the nucleotide and the protein levels[6,7]. Kallikrein-related peptidase 10 is a member of the kallikrein family and has been shown in numerous reports to be upregulated in ovarian cancer[8,9]. The human kallikrein (*KLK*) gene 10 encodes human kallikrein gene 10 (KLK10) protein. Recent studies have shown that human KLKs are involved in human carcinogenesis and that several KLKs are promising biomarkers of prostate, ovarian, testicular and breast cancer[10,11]. For instance, prostate-specific antigen (PSA/hK3) which is encoded by the *KLK3* gene is used as a cancer-specific marker for male population screening, early diagnosis and monitoring of prostate cancer[12]. In addition, quantification of KLK5 expression is critical for both the discovery of early cellular and molecular alterations in breast cancer, as well as the identification of novel diagnostic and prognostic biomarkers[13]. Many other KLKs are also expected to act as tumor biomarkers[14-17]. More recent evidence also implicates the KLKs in many cancer-related processes, including cell growth regulation, angiogenesis, invasion and metastasis[7]. Several authors have reported that *KLK10* mRNA was highly expressed in ovarian cancer tissue and that hK10 may be a useful serum biomarker for the diagnosis and management of ovarian cancer[18]. However, few studies have focused on *KLK10* expression in human gastric cancer.

In the present study, we examined the clinicopathologic and prognostic significance of *KLK10* expression in gastric cancer. Based on collective findings, we hypothesize that *KLK10* expression in gastric cancer tissues may have prognostic/predictive value in patients with this malignancy.

MATERIALS AND METHODS

Study population

Tumor specimens from 80 consecutive patients undergoing surgical treatment for primary gastric cancer at the Department of General Surgery, Tianjin First Central Hospital (Tianjin, China) were analyzed in this study. Patient age ranged from 35 to 74 years, with a median of 51 years (Table 1). All tumor specimens and matched control samples taken from normal tissues at the incision edge were snap-frozen in liquid nitrogen and stored at -80 ℃ for subsequent RNA extraction. Investigations were carried out in accordance with the ethical standards of the 1975 Helsinki Declaration, as revised in Tokyo 2004. The patients had not received hormonal therapy or chemotherapy prior to surgery. After surgery, all patients were treated with oxaliplatin-based chemotherapy regimens based on a platinum compound, alone or in combination with other drugs; grade 1 and stage I patients received no further treatment. Follow-up information (median follow-up period of 31 mo) was available for 80 patients (Table 1). Two time-to-event outcomes after surgery were recorded: disease-free survival (DFS) and overall survival (OS). DFS in each case was defined as the time interval between the date of primary cancer removal and the date of the first documented evidence of relapse. OS was defined as the time interval between the date of surgery and the date of death, or the date of last follow-up for those who were alive at the end of the study.

Clinical and pathological information documented at the time of surgery included clinical stage, histology, depth of invasion and lymph node metastasis (Table 2). All pathological factors were established as described by the 2010 National Comprehensive Cancer Network Guideline.

Table 1 Data of the study population

Variable	No. of patients	mean ± SE	Range
Age (yr)	80	51 ± 0.81	35-74
Lymph nodes[1]	80	30 ± 3.11	0-63
Follow-up (mo)[2]	80	31 ± 1.98	7-52

[1]Number of lymph nodes removed during surgery; [2]Follow-up time after surgery.

Table 2 Associations between kallikrein gene 10 status and other variables in 80 patients with gastric cancer

Variable	Total	*KLK10*-negative	*KLK10*-positive	*P* value[1]
Sex				
Male	56	14	42	
Female	24	9	15	0.258
Age (yr)				
< 60	31	10	21	
⩾ 60	49	13	36	0.581
Depth of invasion[2]				
T1	27	12	15	
T2-T3	23	7	16	
T4	30	4	26	0.034
Lymph node metastasis[2]				
N1	21	4	17	
N2	35	15	20	
N3	24	4	20	0.048
Differentiation[2]				
Well	18	10	8	
Moderate	37	7	30	
Poor	25	6	19	0.015

[1]χ^2 test; [2]TNM stage system of American Joint Committee on Cancer of 2012. *KLK10*: Kallikrein gene 10.

Ethics

The study protocol was approved by the Ethics Com-

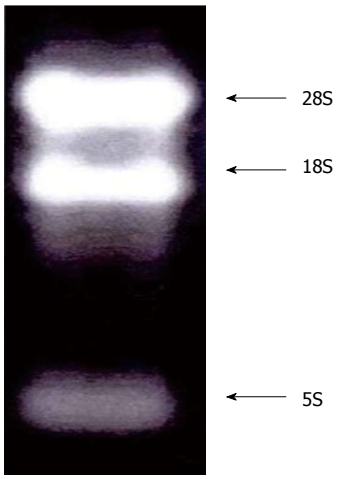

Figure 1 Confirmation of the integrity of total RNA.

mittee of the hospital and written informed consent was obtained from each patient.

Immunohistochemistry

Immunohistochemical studies of hK10 were carried out using the avidin-biotin-peroxidase method (LSAB2 kit, Dako, Kyoto, Japan) on formalin-fixed, paraffin-embedded surgical specimens from patients with gastric cancer. All sections were counterstained with hematoxylin. Primary goat polyclonal antibodies against hK10 (Santa Cruz, United States) were used at dilutions of 1:700.

All sections were independently examined by two researchers (Xin Jiao, Mi-Mi Zhai). The expression of hK10 was scored as positive when the carcinoma cell cytoplasm was stained brown. We examined hK10 protein expression in tumor tissues and corresponding normal tissues from 80 gastric cancer cases.

Total RNA extraction and reverse transcription

Tumor tissues of 100 mg were minced on dry ice using a scalpel and immediately transferred to 2 mL polypropylene tubes. Total RNA was isolated from these samples using TRI-reagent (Ambion Inc., Austin, TX, United States) following the manufacturer's instructions. Total RNA concentration and quality were determined spectrophotometrically at 260 and 280 nm, and RNA integrity was evaluated using agarose gel electrophoresis. Reverse transcription of the mRNA molecules into first-strand cDNA was carried out using 1 μg of total RNA from each tissue specimen, M-MuLV Reverse Transcriptase RNase H (Finnzymes Oy, Espoo, Finland) and an oligo(dT) oligonucleotide as a reverse transcription primer, according to the manufacturer's instructions. Confirmation of the integrity of total RNA is shown in Figure 1.

Real-time quantitative reverse transcription-PCR

Based on the mRNA sequences from the NCBI Sequence database, gene specific primers were designed and synthesized for the target *KLK10* gene (NCBI Reference Sequence: NM_002776) and HPRT1 (hypoxanthine phosphoribosyltransferase-1) endogenous reference gene (NCBI Reference Sequence: NM_000194.2) using the Primer Express software (Applied Biosystems, CA, United States). A *KLK10* fragment was amplified using the primers: forward, 5'-CTCTGGCGAAGCTGCTG-3' and reverse, 5'-ATAGGCTTCGGGGTCCAA-3', whereas the primers for HPRT1 were: forward, 5'-TGGAAAGGGTGTTTATTCCTCAT-3' and reverse, 5'-ATGTAATCCAGCAGGTCAGCAA-3'.

Real-time PCR assays of *KLK10* mRNA expression levels were performed using a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, United States), and the reagents TaqMan Fast Universal PCR Master Mix (29) (Applied Biosystems, United States) according to the manufacturer's instructions. With an initial polymerase activation step at 95 ℃ for 10 min, the amplification conditions of the 40 cycles consisted of denaturation at 95 ℃ for 15 s, annealing at 59 ℃ for 30 s, and elongation at 72 ℃ for 30 s. The products were then subjected to a temperature gradient from 55 ℃ to 95 ℃ at 0.1 ℃/s with continuous fluorescence monitoring to produce a melting curve of the products. *KLK10* mRNA expression was calculated from the standard curve, and quantitative normalization of cDNA in each sample was performed using the expression of GAPDH mRNA as an internal control[19]. We classified the 80 cases into two groups using the mean expression level of *KLK10* mRNA in tumor tissues (0.03): *i.e.,* a positive-expression group ($\geq$ 0.03, n = 57) and a negative-expression group ($<$ 0.03, n = 23).

Statistical analysis

Statistical analysis were performed using SPSS for Windows version 19.0 (SPSS, Chicago, IL, United States). Associations between clinicopathological parameters, such as depth of invasion, lymph node metastasis, histology and *KLK10* expression were analyzed by the Chi-square test or Fisher's exact test, where appropriate. Survival analysis were performed by constructing Kaplan-Meier DFS and OS curves and differences between curves were evaluated by the log-rank test (Mantel, 1966), and by estimating the relative risks for relapse and death using the Cox proportional hazards regression model (Cox, 1972). Cox analysis was conducted at both univariate and multivariate levels. Only the patients with known status of all variables were included in the multivariate regression models, which incorporated *KLK10* and all other variables, for which the patients were characterized.

RESULTS

Relationship between KLK10 expression and other parameters

Of the 80 patients included in this study, 57 (70 ℃) were positive for *KLK10* expression in gastric cancer tissues. In normal gastric tissues, the level of *KLK10* was undetectable or low. Table 2 shows the distribution of *KLK10*

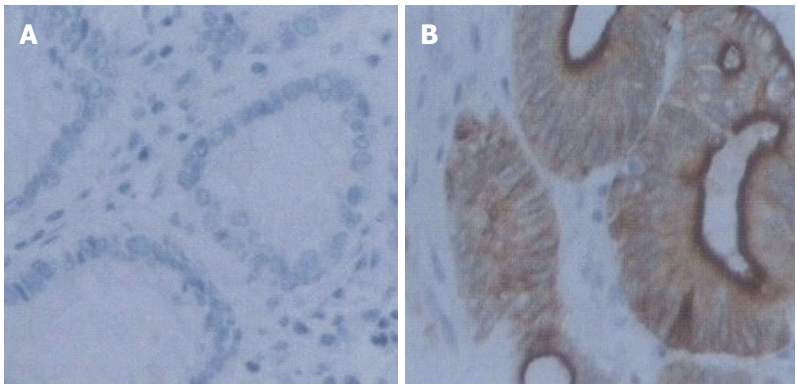

Figure 2 Immunohistochemical analysis of hK10 protein expression in normal gastric tissues and gastric tumor tissues. A: hK10 protein expression in normal gastric tissues; B: hK10 protein expression in gastric tumor tissues.

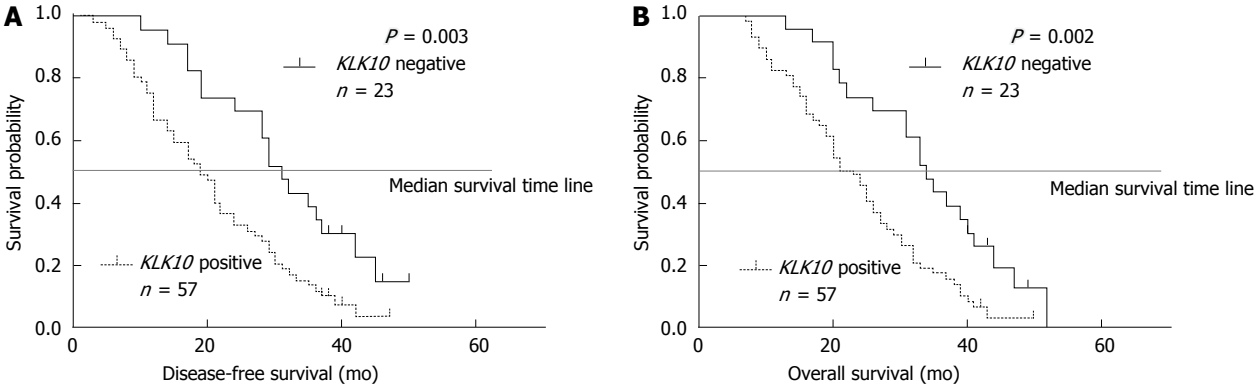

Figure 3 Kaplan-Meier survival analysis of disease-free survival (A) and overall survival (B) in gastric cancer patients who were either kallikrein gene 10 positive or kallikrein gene 10 negative. *KLK10*: Kallikrein gene 10.

expression (positive or negative) in gastric cancer tissues in relation to age, sex, lymph node metastasis, depth of invasion and histology. Patients with *KLK10*-positive gastric cancer more frequently had more lymph node metastasis ($P = 0.048$), greater depth of invasion ($P = 0.034$) and poorer histology ($P = 0.015$). No significant associations between *KLK10* expression and age ($P = 0.581$) and sex ($P = 0.258$) were found.

Immunohistochemistry

In 55 of the 57 patients who were positive for *KLK10* mRNA expression, specific expression of hK10 protein was only found in cancer tissues, but not in the corresponding normal tissues (Figure 2). In 23 cases with negative expression of *KLK10* mRNA, 20 exhibited negative or weak expression of hK10 in cancer tissues. In contrast, 2 cases with high *KLK10* mRNA expression exhibited negative or poor hK10 protein expression in cancer tissues.

Clinicopathologic significance of KLK10 mRNA expression in gastric cancer

The clinicopathologic factors analyzed in relation to *KLK10* mRNA expression in tumor tissues are shown in Table 2. The level of lymphatic invasion was significantly higher ($P = 0.048$) in the positive-expression group than in the negative-expression group. The depth of gastric wall invasion was greater ($P = 0.034$) in the positive-expression group than in the negative-expression group. The histotype also correlated with these groups ($P = 0.015$). In contrast, no significant difference was observed regarding age and sex. The 3-year actuarial OS rates in patients with gastric cancer and positive *KLK10* mRNA expression and in patients with negative *KLK10* mRNA expression were 20% and 42%, respectively (Figure 3). The survival difference between these two groups was statistically significant ($P = 0.002$; log-rank test).

Univariate and multivariate survival analysis

The degree of association between each clinicopathological variable and DFS and OS is shown in Table 3. In univariate analysis, patients with *KLK10*-positive gastric cancer had a significantly increased risk of relapse (decreased DFS) and death (decreased OS) (hazards ratios of 0.46 and 0.43; $P = 0.005$ and 0.002, respectively). Nevertheless, positive *KLK10* expression was weakly associated with an increased risk of death (decreased OS) (hazards ratio of 0.55; $P = 0.03$) in multivariate analysis compared with univariate analysis, while no significant association was found between the positive *KLK10* expression and relapse (decreased DFS) in patients with gastric cancer in multivariate analysis ($P = 0.06$).

Depth of invasion and histotype were the strongest independent indicators of poor prognosis ($P < 0.05$, except for depth of invasion for OS in multivariate analysis). As expected, Kaplan-Meier survival curves (Figure 3) indicated that patients with *KLK10*-positive gastric cancer had shorter DFS ($P = 0.003$) and OS ($P = 0.002$) com-

Table 3 Univariate and multivariate analysis of disease-free survival and overall survival

Survival variable	Disease-free survival			Overall survival		
	95%CI[2]	*P* value	HR[1]	95%CI[2]	*P* value	HR[1]
Univariate analysis						
KLK10						
Negative			1.00			1.00
Positive	0.25-0.74	0.002	0.46	0.26-0.79	0.005	0.43
Age	0.97-1.01	0.46	0.99	0.97-1.01	0.49	0.99
Sex	0.55-1.53	0.75	0.90	0.54-1.51	0.69	0.92
Depth of invasion	1.55-2.81	< 0.001	2.13	1.57-2.89	< 0.001	2.08
Lymph node metastasis	0.97-1.97	0.07	1.42	0.99-2.02	0.051	1.38
Histology	6.42-22.46	< 0.001	10.25	5.73-18.32	< 0.001	12.01
Multivariate analysis						
KLK10						
Negative		1.00			1.00	
Positive	0.31-0.96	0.03	0.58	0.33-1.02	0.06	0.55
Age	0.95-1.00	0.02	0.97	0.95-0.99	0.04	0.97
Sex	0.77-2.22	0.31	1.33	0.77-2.28	0.30	1.31
Depth of invasion	0.99-1.90	0.06	1.42	1.02-1.98	0.03	1.37
Lymph node metastasis	0.84-1.59	0.35	1.21	0.87-1.67	0.24	1.16
Histology	6.55-25.41	0.03	11.11	5.87-21.02	< 0.001	12.90

[1]Hazard ratio (HR) estimated from Cox proportional hazard regression model; [2]Confidence interval of the estimated HR. *KLK10*: Kallikrein gene 10.

pared with *KLK10*-negative patients.

DISCUSSION

Gastric cancer is a common malignant tumor of the gastrointestinal tract. The optimal management of patients with gastric cancer involves a multidisciplinary approach: diagnosis, surgery, and chemotherapy, including the use of biological markers. Several authors have reported that *KLK10* mRNA is highly expressed in human cancer tissues[20-23]. In the current study, we found a significant relationship between *KLK10* mRNA expression and lymph node metastasis, depth of invasion and histology in patients with gastric cancer, as shown in Table 1. These findings indicate that the overexpression of *KLK10* was significantly associated with both an increased incidence of lymphatic invasion and poor histology in patients with gastric cancer. These results suggest that enhanced expression of *KLK10* may play an important role in various pathologic processes of gastric cancer. The results obtained in this study are in agreement with previous studies which examined the association between *KLK10* expression status and the clinicopathological features of patients with gastric cancer[24].

Some members of the KLK family have been identified as potential biological markers of prognosis, including KLK5, KLK14 and KLK7[25]. For example, KLK5 expression is an indicator of poor prognosis in ovarian cancer[26]. Furthermore, stratifying patients based on the presence or absence of such markers may result in a different prognosis in individuals. For example, both KLK8 mRNA and KLK6 mRNA are highly expressed in human breast cancer tissues, however, it is unknown whether a breast cancer patient with high expression of KLK8 has a good/poor prognosis compared to a breast cancer patient with high expression of KLK6.

In this study, we identified *KLK10* as a new biomarker of poor prognosis in gastric cancer. Patients with *KLK10*-positive tumors were more likely to have poor histology and advanced stage disease. Our findings demonstrate that *KLK10* expression can reduce DFS in patients with gastric cancer in univariate, but not in multivariate analysis (Table 2). In addition, when assessing *KLK10* expression to predict survival outcomes, we found an increased risk of death in patients with *KLK10*-positive tumors in both univariate and multivariate analysis (Table 2). This indicates that *KLK10*-positivity may be an independent prognostic factor in patients with gastric cancer. That is, *KLK10* may induce gastric cancer cell growth and proliferation. However, the function of the *KLK10* signaling pathway is unclear. Some reports indicate that serine proteinases (the KLK gene family of secreted serine proteases includes 15 genes) participate in tumor growth and invasion by cleaving and activating proteinase-activated receptors (PARs: PAR-1 and PAR-2)[27-29]. A recent study demonstrated that KLK4 is aberrantly expressed in colon cancer and capable of inducing PAR-1 signaling in cancer cells[30]. KLK4 is a tumorigenic factor. Another report showed that KLK14 induced significant extracellular signal-regulated kinases 1 and 2 (ERK1/2) phosphorylation and HT29 cell proliferation, presumably by activating PAR-2. A PAR-2 cleavage and activation-blocking antibody markedly reduced KLK14-induced ERK1/2 signaling[31]. Our lack of knowledge of *KLK10* function and regulation in gastric cancer tissues does not allow us to formulate reasonable hypothesis to explain these observations. More studies with a larger group of patients are necessary to substantiate these findings.

The current study indicates that *KLK10* mRNA was significantly overexpressed in gastric cancer tissues and high *KLK10* expression levels were associated with lymphatic invasion, tumor invasion and poor patient prog-

nosis. hK3 has been well documented to be an excellent tumor marker for prostate cancer. Moreover, hK10 is a promising serum biomarker for ovarian cancer. Therefore, studies are now underway to investigate whether hK10 may also be a useful biomarker for gastric cancer using serum samples from the patients treated at our hospital.

COMMENTS

Background

Gastric carcinoma is one of the most common tumors worldwide. The expression of kallikreins is involved in cancer cell formation. Abnormal expression of kallikrein gene 10 *(KLK10)* is associated with carcinogenesis, and it is a promising serum biomarker for cancers.

Research frontiers

Several authors have reported that *KLK10* mRNA is highly expressed in ovarian cancer tissue and that hK10 could be a useful serum biomarker for the diagnosis and management of ovarian cancer. However, there is little information on *KLK10* expression in human gastric cancer.

Innovations and breakthroughs

This study assessed the clinicopathologic and prognostic significance of *KLK10* expression in gastric cancer. Furthermore, based on the collective findings, this study investigated whether *KLK10* expression in gastric cancer tissues may have prognostic/predictive value in patients with gastric cancer.

Applications

By exploring the relation between the expression of *KLK10* and clinicopathology in gastric cancer, this study may provide a strategy for predicting the prognosis of gastric cancer patients.

Peer review

It is a well written paper. The authors investigated the clinicopathologic and prognostic significance of *KLK10* expression in gastric cancer, which helps understand the pathogenesis and predict the prognosis of gastric cancer. The experimental procedure is quite well performed.

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