

RESEARCH ARTICLE

Clinical significance of kallikrein 5 as a novel prognostic biomarker in gastric adenocarcinoma

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Abstract

Backgrounds: Gastric cancer is one of the most common cancers with unsatisfied prognosis. It is challenging to predict gastric cancer prognosis due to its highly heterogeneous nature. Kallikrein 5 (KLK5) belongs to the family of kallikreins, which plays a crucial role in serine proteolysis and exerts diverse physiological functions. The role of KLK5 in human gastric adenocarcinoma (GAC) has not been elucidated. In the present study, we aimed to examine the expression level of KLK5 and dissect whether the KLK5 expression was associated with GAC prognosis.

Patients and methods: Clinicopathological analyses were performed in a retrospective GAC patient cohort ($n = 138$). The expression of KLK5 was tested by quantitative RT-PCR and immunohistochemistry staining. The prognostic role of KLK5 in GAC was assessed by univariate and multivariate analyses. The effects of KLK5 on cell proliferation, migration, and invasion were examined through cellular experiments.

Results: The data showed that KLK5 expression was elevated in GAC tissues compared with normal stomach tissues. Protein expression of KLK5 was positively correlated with tumor invasion depth and lymph node metastasis. Patients with higher KLK5 expression had poorer overall survival. KLK5 was identified to be an independent risk factor according to multivariate analysis. Using human GAC cell lines, we found that KLK5 can promote tumor cell migration and invasion.

Conclusions: Our study demonstrated that higher expression of KLK5 was significantly correlated with a poorer prognosis of GAC patients, implying the potential of KLK5 as a novel prognostic biomarker in GAC.

KEYWORDS

biomarker, gastric adenocarcinoma, kallikrein 5, prognosis

1 | INTRODUCTION

Gastric cancer is one of the most common malignant tumors of the digestive system. According to the latest statistics of the World

Health Organization, both the incidence and mortality rate of gastric cancer ranked seventh among all malignancies.¹ In 2020, the United States expects 27,600 new cases of gastric cancer and 11,010 deaths from gastric cancer.¹ The high incidence and mortality rate of gastric cancer make it a public health concern, especially

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in developing countries.² Although recent advances in endoscopic techniques, imaging techniques, surgical techniques, and targeted drugs have improved the overall survival of patients with gastric cancer, most patients have advanced diseases at the time of diagnoses whose 5-year survival rate is only 5%.³ Therefore, it is important to find sensitive and specific biomarkers for the early detection, targeted treatment, and accurate prognosis prediction of gastric cancer patients.

Kallikreins (KLKs) is a family of serine proteases comprised of 15 members, namely KLK1–KLK15.⁴ KLKs function by cleaving peptide bonds within proteins, thus also named as kallikrein-related peptidases.⁵ According to their diverse substrates, KLKs participate in various physiological functions such as skin desquamation,⁶ blood pressure regulation,⁷ semen coagulation, and liquefaction.⁸ Recently, accumulating evidence suggests circumstantial correlations between KLKs and malignancies.^{9,10} For example, KLK3 was reported to be downregulated in breast cancer, and its expression indicated a better response to tamoxifen and a better prognosis.¹¹ Similarly, a lower KLK13 expression was observed in breast cancer tissues, and its expression level was positively correlated with prognosis.¹² In contrast, several KLKs seem to play oncogenic roles instead of tumor-suppressing roles. For example, higher KLK4 and KLK7 were identified in ovarian cancers and correlated with unfavorable clinical outcomes.^{13,14} Interestingly, even the same KLK may exert different expression patterns and play distinct roles in different cancers. On one hand, a downregulated expression of KLK5 was observed in prostate cancers and played tumor-suppressing roles.¹⁵ On the other hand, KLK5 showed higher levels in ovarian cancers and indicated unfavorable prognosis.¹⁶ Similarly, higher KLK5 was correlated with poorer prognosis of colorectal cancer patients.^{17,18} However, the expression and clinical significance of KLK5 in gastric cancer remain unknown.

Here, we initially investigated the mRNA and protein levels of KLK5 in gastric adenocarcinoma tissues and nontumorous stomach tissues. Our work represents the first report regarding the expression and clinical significance of KLK5 in gastric adenocarcinoma. According to our data, KLK5 was highly expressed and was an independent predictor of poor prognosis in gastric adenocarcinomas. We also revealed that silencing KLK5 could significantly suppress gastric cancer cell migration and invasion, while showed no significant effect on cell proliferation. Our data provide the evidence of KLK5 as a potential gastric cancer treatment target.

2 | METHODS

2.1 | Patients and tissue specimens

Tumor tissues and adjacent normal stomach tissues were collected from gastric cancer patients that underwent curative surgery at the Department of Gastrointestinal Surgery in The First Affiliated Hospital of Xinjiang Medical University. All patients were diagnosed based on pathological tests. None of the patients received

any preoperative radiotherapy or chemotherapy. All specimens were collected under the guidance of the HIPAA protocol and supervised by the Ethics Committee of The First Affiliated Hospital of Xinjiang Medical University. Written informed consents were obtained from all participants. Our study contains two retrospective cohorts. The first cohort is comprised of 35 cases whose tissues were flash-frozen in liquid nitrogen after surgical resection and were subjected to quantitative PCR analyses. The second cohort is comprised of 138 cases whose tissues were formalin-fixed paraffin-embedded (FFPE) and were used for immunohistochemistry staining and survival analyses.

2.2 | Quantitative Real-Time Reverse Transcriptase-Polymerase Chain Reaction

Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-qPCR) was performed to test the mRNA level of KLK5 in clinical specimen. Briefly, total RNA was extracted using the TRIzol[®] reagent and reverse-transcribed into cDNA with Superscript II[®] retrotranscriptase. The cDNAs were then applied to qPCR using SYBR master mix (Applied Biosystems) and run on the Step One Plus Applied Biosystems Real-time PCR machine according to the manufacturer's instructions. The qPCR primers were synthesized by Generay Biotech as followings: KLK5 forward 5'-GCAGGTAGAGACTCCTGCCA-3', KLK5 reverse 5'-CACAAGGGTAATCTCCAG-3'; GAPDH forward 5'-GGACCTGACCTGCCGTCTAG-3', and GAPDH reverse 5'-GTAGCCCAGGATGCCCTTGA-3'. All experiments were repeated for three independent times. The mRNA levels were evaluated using 2^{- $\Delta\Delta$ Ct} method and presented as fold changes in tumor tissues vs. paired adjacent stomach tissues.

2.3 | Immunohistochemistry

Immunohistochemistry (IHC) was carried out to evaluate KLK5 protein levels in GAC specimens and nontumorous stomach tissues. Briefly, FFPE tissue sections were firstly de-paraffinized with xylene and ethanol. Secondly, sections were incubated in 3% H₂O₂ for 30 min to inactivate endogenous peroxidase and then incubated in EDTA buffer (pH = 9.0) for antigen retrieval. Thirdly, unspecific antigen binding was blocked by 1% bovine serum albumin (BSA). Fourthly, rabbit polyclonal antibodies to KLK5 (1:150, Ab28565, Abcam) were used to incubate slide sections using nonspecific IgG as negative control. Finally, secondary antibody and DAB solution (Beyotime) were used to visualize immunoreactivities.¹⁹

Immunohistochemistry results were semi-quantified by two independent pathologists based on the positive-stained percentage and staining intensity from five randomly selected fields under a light microscope. The percentage of positive-stained cells was scored as follows: 0 for no staining, 1 for 1%–25% staining, 2 for 26%–50% staining, 3 for 51%–75% staining, and 4 for 76%–100% staining. The staining intensity was scored as follows: 0 for negative staining, 1 for weak staining (light yellow), 2 for moderate staining

(dark yellow), and 3 for heavy staining (dark brown).²⁰ The final IHC score was obtained by multiplying the two scores above, ranging 0–12. The patients were then divided into subgroups with the cut-off (IHC score = 3.5) determined by the receiver operating characteristic (ROC) curve. Accordingly, 73 cases were sub-grouped into negative-KLK5 group (IHC score < 3.5), while the other 65 cases into positive-KLK5 group (IHC score ≥ 3.5).

2.4 | Cell culture and shRNA infection

The human GAC cell lines SGC-7901, MKN-45, BGC-823, MGC-803, and nontumorous GES-1 stomach mucosa epithelium cells were purchased from the Cell Center of Shanghai Institutes for Biological Sciences. All cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Gibco). Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂.

To generate GAC cells with stable KLK5 knockdown, specific lentiviral shRNA constructs NM_012427.31248s1c1 (KLK5-shRNA#1) and NM_012427.3650s1c1 (KLK5-shRNA#2) targeting KLK5 were obtained Sigma-Aldrich). A nonspecific scramble shRNA was used as a negative control. The cells were infected as previously described.²¹ After infection, cells were maintained in DMEM supplemented with puromycin.

2.5 | Proliferation

The cells that were stably infected with KLK5-shRNAs, and the control cells were seeded into 96-well plates at a density of 3000 cells/well and incubated for 24, 48, 72, and 96 h. Then, the culturing medium was discarded and 100 µl MTT solution was added to the culture for another 4 h. Then, 200 µl DMSO reagent was added to each well to resolve the crystals. Finally, the absorbance was measured at 570 nm.²²

2.6 | Migration and invasion

For the wound healing assay, the cells were seeded on six-well plates. When 95% confluence was achieved, the cell monolayer was gently scratched using a sterile 200-µm plastic pipette tip. The wound was then photographed. After culturing for another 24 h, the healing wound was photographed.

For the invasion assays, 4×10^4 cells suspended in medium without serum were seeded in the upper chamber membrane, which was pre-coated with Matrigel (BD Biosciences). Then, 600 µl medium with 10% fetal bovine serum was added to the lower chamber. After 24 h, the underside of the membrane was fixed for 30 min and stained with 0.1% crystal violet. The inner side of the membrane was wiped with a cotton swab. The cells were then quantified under a microscope.

2.7 | Western blot

The cancer cells were collected, washed twice with cold PBS, and lysed in NP-40 lysis buffer for 30 min at 4°C. Protein concentrations were measured using a bicinchoninic acid assay kit (Thermo). Protein extracts were separated by mini electrophoresis in a premade 8%–12% sodium dodecyl sulfate-polyacrylamide gel with tris (hydroxymethyl) aminomethane hydrochloride and then transferred to a polyvinylidene difluoride membrane. The membrane was incubated with the indicated antibodies and detected by using the chemiluminescence method.²³

2.8 | Statistics

Statistical analyses were performed using the SPSS Software. The associations between KLK5 protein level and clinical characteristics were evaluated through chi-square test. Kaplan-Meier analysis and log-rank test were used to plot and analyze the overall survival curves of enrolled GAC patients. Independent prognostic factors were identified by using a multivariate Cox regression model. $p < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | Upregulated expression of KLK5 in GAC

The mRNA levels of KLK5 in GAC tissues and adjacent nontumorous stomach tissues were firstly evaluated by RT-qPCR. As a result, 77.1% (27/35) paired tissues showed a higher KLK5-mRNA level in GAC than in adjacent tissue (Figure 1A, $p < 0.001$). We next assessed the protein expression pattern of KLK5 in clinical specimens. KLK5 shows predominant localization in cytoplasm and extracellular matrix (Figure 1B). As expected, the immunoreactivity of KLK5 is distinct in tumor tissues from different patients (Figure 1B,C and Figure S1) using IgG as negative control (Figure 1D). By generating the receiver operating characteristic (ROC) curve, we determined an IHC cutoff value as 3.5 to distinguish positive- or negative-KLK5 expression group (Figure 1E). Accordingly, we divided patients into negative-KLK5 expression group ($n = 73$) and positive-KLK5 expression group ($n = 65$).

3.2 | Clinicopathological characteristics of enrolled GAC patients

As for the 35 cases for RT-qPCR experiments, there were 4 cases with TNM stage I, 13 cases with TNM stage II, 12 cases with TNM stage III, and the other 6 cases with TNM stage IV. The clinicopathological characteristics of the 138 cases for IHC assays and survival analyses were intact. In brief, the median age was 54 years old, ranging 30–79 years old. There were 51 female patients and 87 male patients. Among the 138 cases, 23 cases showed cardia or fundus location, 62

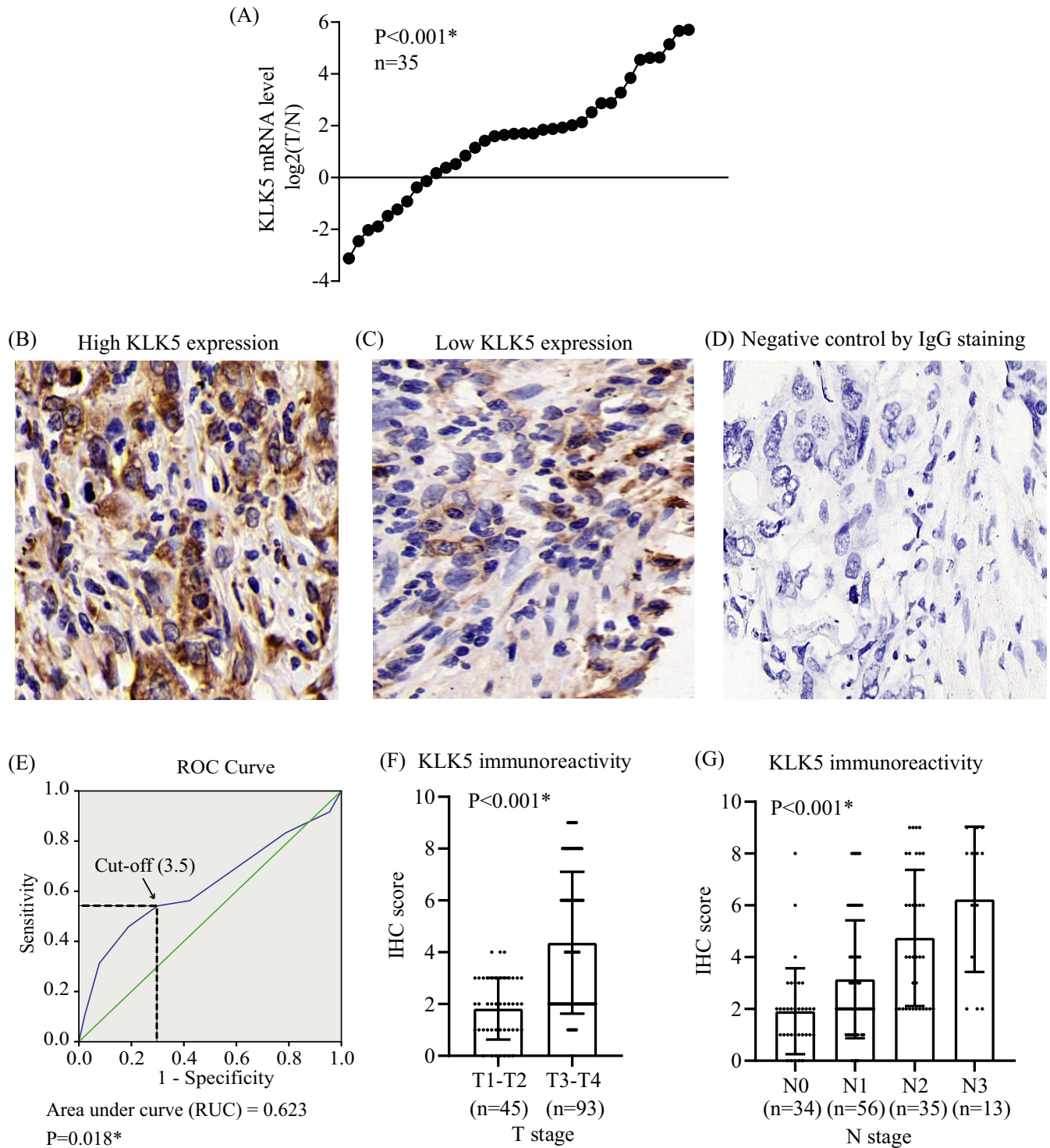


FIGURE 1 KLK5 is highly expressed in GAC tissues. (A) mRNA level of KLK5 was tested by RT-qPCR analysis in 35 paired GAC tissues (T) and adjacent tissues (N), data were exhibited as the relative fold changes in Log2. * $p < 0.05$ by paired Student's t test. Each dot represents the log2 (T/N) ratio of each patient. (B) Representative high protein expression of KLK5 in GAC tissue (T2N1M0). Immunohistochemistry (IHC) score is 9. Magnification: 400 \times . (C) Representative negative expression of KLK5 in GAC tissue (T2N1M0). IHC score is 2. Magnification: 400 \times . (D) Representative negative control of IHC staining in GAC tissue (T2N1M0). IHC score is 0. This IHC staining was conducted by using IgG instead of KLK5-primary antibody to incubate with the tissue slide. Magnification: 400 \times . (E) Based on the IHC scores, a ROC curve was generated to determine a cutoff value to distinguish positive- or negative-KLK5 expression. The IHC score of 3.5 was selected with sensitivity as 0.542 and specificity as 0.700 ($p = 0.018$). (F) Tissues with T stage T3-T4 showed higher KLK5 protein immunoreactivities than those with stage T1-T2. * $p < 0.05$ by unpaired Student's t test. (G) The KLK5 protein level was positively correlated with the N stage of GAC tissues. * $p < 0.05$ by One-way ANOVA test

cases with body location, and the other 53 cases showed antrum or pylorus location. The median tumor size, as presented by the largest diameter, was 2.8 cm, ranging 0.2–9.5 cm. Therefore, 75 cases were grouped as tumor size larger than or equal to 3.0 cm, while the other 63 cases with tumor size less than 3.0 cm. As for the tumor invasion depth, 45 cases were staged as stage T1–T2, and 93 cases with stage T3–T4. There were 34 cases with negative lymph nodes (stage N0), 56 cases with stage N1, 35 cases with stage N2, and the other 13 cases with stage N3. In addition, 17 cases were diagnosed as undifferentiated or poor differentiation, 68 cases with moderate differentiation, and the other 53 cases with well differentiation (Table 1).

3.3 | Correlation between KLK5 expression and clinicopathological parameters

The correlations between KLK5 protein expression levels with tumor stages were next assessed. As shown in Figure 1F, patients with advanced T stages exhibited higher KLK5 protein levels ($p < 0.001$).

Similarly, the protein level of KLK5 was positively correlated with the lymph node metastasis (Figure 1G, $p < 0.001$). The close correlation between KLK5 and tumor stage indicated that KLK5 may participate in GAC progression.

3.4 | Prognostic values of KLK5 in GAC

We further explored whether KLK5 had any correlation with clinical outcomes of GAC patients. The median follow-up time of our enrolled cohort was 51 months (ranging 8–84 months), and 48 cases were dead by the end of the follow-up. Kaplan-Meier analysis revealed that patients with positive-KLK5 expression possessed unfavorable prognosis compared with those with negative-KLK5 expression (Table 2, $p = 0.001$). The average survival time for patients with negative-KLK5 expression was 70.1 ± 2.3 months, while was only 54.3 ± 3.0 months for those with positive-KLK5 expression. Consistently, the 5-year overall survival rate was also significantly higher in negative-KLK5 expression group (75.7%) than that of the

TABLE 1 Correlations between KLK5 expression level and patients' characteristics

Variables	Patients (<i>n</i> = 138)	KLK5 expression		<i>p</i> Value
		Negative (<i>n</i> = 73)	Positive (<i>n</i> = 65)	
Age (years)				
≤50	55	32	23	0.311
>50	83	41	42	
Sex				
Female	51	32	19	0.076
Male	87	41	46	
Location				
Upper 1/3 stomach	23	10	13	0.612
Middle 1/3 stomach	62	34	28	
Lower 1/3 stomach	53	29	24	
Tumor size (diameter, cm)				
≤3.0	75	45	30	0.068
>3.0	63	28	35	
T stage				
T1–T2	45	30	15	0.024*
T3–T4	93	43	50	
Differentiation				
Poor	17	9	8	0.931
Moderate	68	37	31	
Well	53	27	26	
N stage				
N0	34	26	8	0.001*
N1	56	32	24	
N2	35	12	23	
N3	13	3	10	

* indicates $p < 0.05$.

Variable	Cases (n = 138)	Survival months (Mean ± SD)	5-year OS (%)	p Value
Age (years)				0.019*
≤50	55	69.7 ± 2.9	78.2%	
>50	83	56.8 ± 2.4	51.5%	
Sex				0.241
Female	51	65.2 ± 3.0	74.1%	
Male	87	63.7 ± 2.9	58.0%	
Location				0.204
Upper 1/3 stomach	23	55.2 ± 4.9	51.0%	
Middle 1/3 stomach	62	67.2 ± 2.8	69.3%	
Lower 1/3 stomach	53	61.6 ± 3.1	63.2%	
Tumor size (diameter, cm)				0.094
≤3.0	75	67.0 ± 2.6	68.2%	
>3.0	63	59.2 ± 3.3	59.3%	
T stage				0.012*
T1-T2	45	72.0 ± 3.2	74.3%	
T3-T4	93	59.5 ± 2.7	58.6%	
Differentiation				0.002*
Poor	17	76.1 ± 4.0	86.5%	
Moderate	68	63.9 ± 2.3	72.4%	
Well	53	52.7 ± 3.1	44.7%	
N stage				<0.001*
N0	34	79.9 ± 2.0	100%	
N1	56	57.4 ± 2.2	56.0%	
N2	35	56.0 ± 2.9	54.0%	
N3	13	31.6 ± 4.6	14.2%	
KLK5 expression				0.001*
Negative	73	70.1 ± 2.3	75.7%	
Positive	65	54.3 ± 3.0	50.4%	

* indicates $p < 0.05$.

positive-KLK5 expression group (50.4%, Figure 2A). Besides KLK5 protein level, the prognostic significances of other clinicopathological parameters were also assessed by survival plots including patients' age (Figure 2B), sex (Figure 2C), tumor location (Figure 2D), tumor size (Figure 2E), T stage (Figure 2F), tumor differentiation grade (Figure 2G), and N stage (Figure 2H).

Multivariate analysis was next conducted by a Cox regression model to figure out independent risk factors. The pathological parameters included age, T stage, differentiation grade, N stage, and KLK5 expression (Table 3). As a result, positive-KLK5 expression showed an independent effect on patients' overall survival (hazard ratio 2.44, 95% confidence interval 1.33–4.48, $p = 0.004$). Similarly, the tumor differentiation grade and lymph node metastasis were also identified as independent risk factors ($p = 0.005$ and $p = 0.007$, respectively).

Furthermore, we analyzed the prognostic value of KLK5 in patients with TNM stage I (Figure 3A), II (Figure 3B), or III (Figure 3C), respectively. Although KLK5 exhibited no prognostic significance in patients with TNM stage I, positive KLK5 was closely correlated

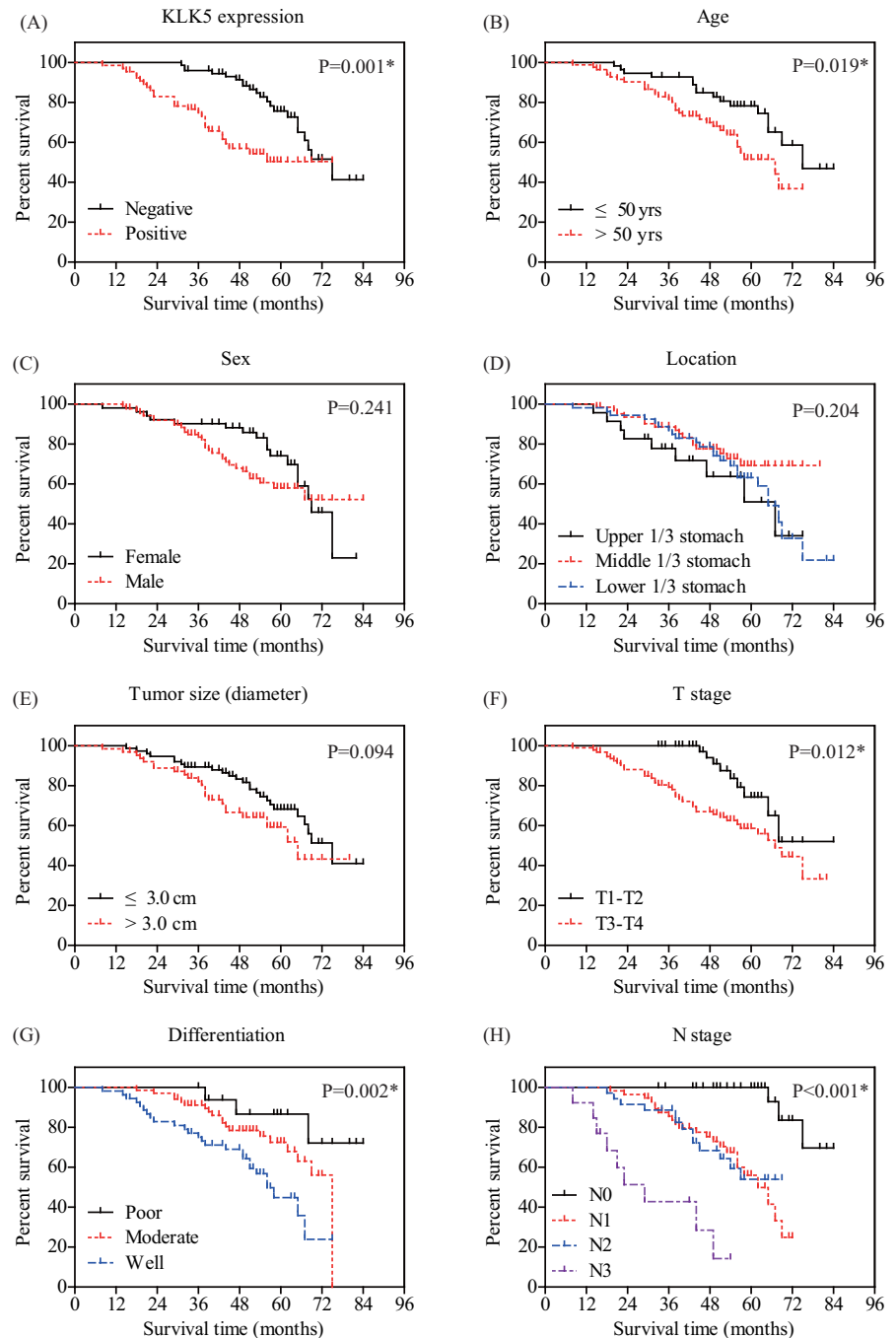
TABLE 2 Kaplan-Meier survival analyses of gastric adenocarcinoma patients

with unfavorable overall survival in TNM stage II or stage III patients. Overall, our results identified KLK5 as a potential prognostic regulator for GAC patients.

3.5 | Silencing KLK5 results in impaired GAC migration and invasion

Since clinical results implied an involvement of KLK5 in GAC progression, we were interested to further validate its cellular effects on the phenotypes of GAC cells. According to the Western blotting data, KLK5 showed a lower protein expression level in nontumorous GES-1 cells than that in GAC cell lines including MKN-45, BGC-823, and MGC-803 (Figure 4A). However, the SGC-7901 cells exhibited comparable KLK5 protein levels with GES-1 cells, further emphasizing the high heterogeneity of gastric adenocarcinoma. We next selected two cell lines, namely the MKN-45 and BGC-823 cells, with the highest KLK5 levels for knockdown assay by shRNA strategy (Figure 4B).

FIGURE 2 Overall survival analyses of enrolled GAC cohort. Kaplan-Meier method was used to generate overall survival plots of GAC patients regarding KLK5 expression status (A), age (B), sex (C), location (D), tumor size (E), T stage (F), tumor differentiation (G), and N stage (H). * $p < 0.05$ by log-rank test



Interestingly, silencing KLK5 did not exhibit significant effect on the proliferation capacity of MKN-45 or BGC-823 cells (Figure 4C). In contrast, the migration and invasion processes were remarkably impaired by KLK5 knockdown in both cell lines (Figure 4D,E).

4 | DISCUSSION

Gastric cancer is a common malignant tumor. In recent years, the significance of immunotherapeutic strategy, such as PD-1 and PD-L1 inhibitors, has been well acknowledged in melanoma, glioblastoma, and hepatocellular carcinoma.^{24,25} However, due to the highly heterogeneous nature of gastric cancer, the efficacy of immunotherapy

drugs and patients' responsiveness to them vary considerably.²⁶ Therefore, it is of great importance to reveal the potential molecular mechanisms of the occurrence and development of gastric cancer, as well as to determine the potential prognostic markers and therapeutic targets to improve the survival outcomes of patients.

Accumulating evidence suggested that many kallikreins (KLKs) play roles in carcinogenesis and cancer progression. Several KLKs have been reported as novel biomarkers for cancers and other diseases. For example, KLK5 was initially suggested to be involved in the desquamation of the epidermis. Recently, the contradictory effects of KLK5 in different malignancies have been discovered.

KLK5 plays pro-oncogenic roles in several cancer types. A higher expression of KLK5 was observed in oral squamous cell cancer

TABLE 3 Multivariate analysis for overall survival of gastric adenocarcinoma patients

Variables	HR	95% CI	p Value
Age			
(> 50 vs. ≤50)	1.75	0.93–3.30	0.081
T stage			
(T3–T4 vs. T1–T2)	1.42	0.66–3.04	0.366
Differentiation			
(Poor vs. Well/moderate)	2.08	1.26–3.46	0.005*
N stage			
(N1–N3 vs. N0)	7.57	1.72–33.23	0.007*
KLK5 expression			
(Positive vs. Negative)	2.44	1.33–4.48	0.004*

* indicates $p < 0.05$.

(OSCC), which was correlated with short overall survival. According to their data, knockdown of KLK5 in OSCC cells also led to smaller xenografts.²⁷ Similarly, higher KLK5 has been identified in uterine cervical cancer,²⁸ serous ovarian cancer,²⁹ and colorectal adenocarcinoma.^{17,18} Here, our results firstly identified a significantly higher expression of KLK5 in gastric adenocarcinoma tissues than that in nontumorous stomach tissues on both mRNA and protein levels. Besides, our data confirmed the independent role of KLK5 on help predicting the overall survival of GAC patients. As for the functional mechanisms, long noncoding RNA HEIH depletion can suppress esophageal cancer progression by downregulating KLK5.³⁰ Another upstream regulator of KLK5 is mesotrypsin, which shows similarly prognostic effect for the outcome of lung adenocarcinoma.³¹ It has been reported that silencing KLK5 inhibits skin tumorigenesis by reducing epidermal proteolysis and reinforcing epidermal microstructure.³² However, our knowledge about the downstream signaling of KLK5 in other tumors is limited. Our current data demonstrated that KLK5 enhances GAC cell migration and invasion without affecting its proliferation process. Consistent with our data, KLK5 exhibits parallel expression with vimentin in canine squamous cell carcinoma, suggesting its participation in epithelial-mesenchymal transition process.³³ As a kallikrein-related peptidase, KLK5 has been identified to cleave extracellular matrix (ECM) (collagen type I, II, III, and IV, fibronectin, and laminin) and adhesion molecules (fibrinogen and vitronectin).³⁴ Both ECM and adhesion molecules are critical on modulating tumor invasion and metastasis, and therefore, KLK5 may promote gastric cancer progression via enzymatic cleaving its downstream substrates such as ECM and adhesion molecules. This can also at least partially explain our data that KLK5 enhances gastric cancer invasiveness without significant effect on in vitro proliferation process, which is consistent with a previous finding in bladder carcinoma.³⁵ Of note, KLKs may play synergic roles during cancer progression. For example, the combined elevation of KLK5-8 in endometrial cancer indicated a higher risk of worse survival.³⁶ Indeed, it has been reported that KLK5 can activate other kallikreins such as KLK7 and KLK14, as well as itself.³⁷ The combination effects of

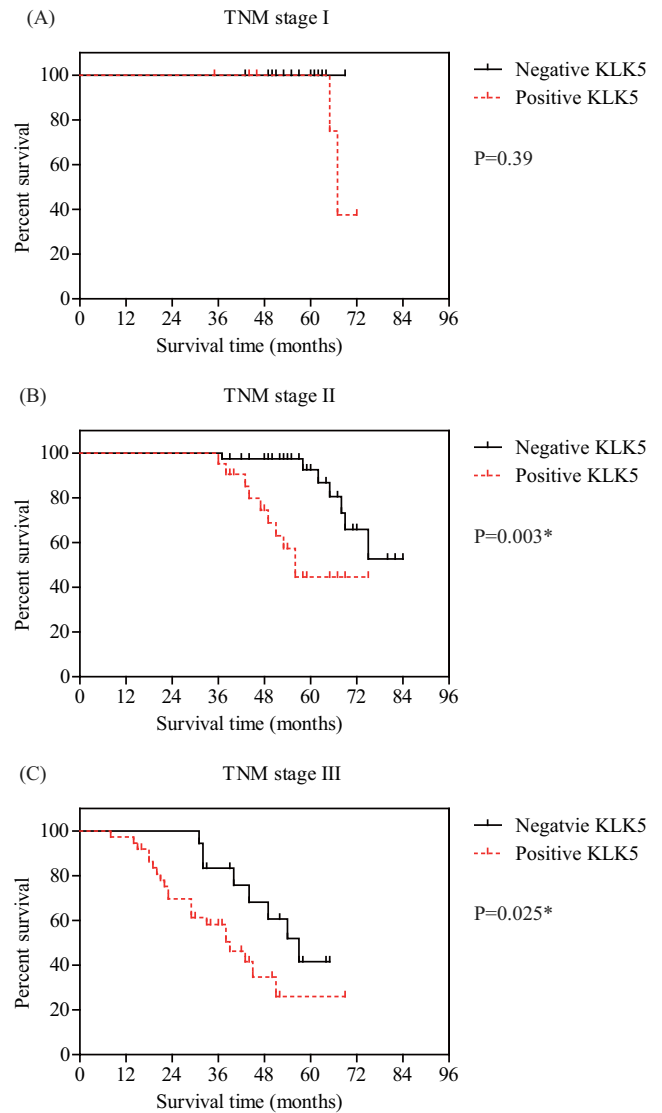
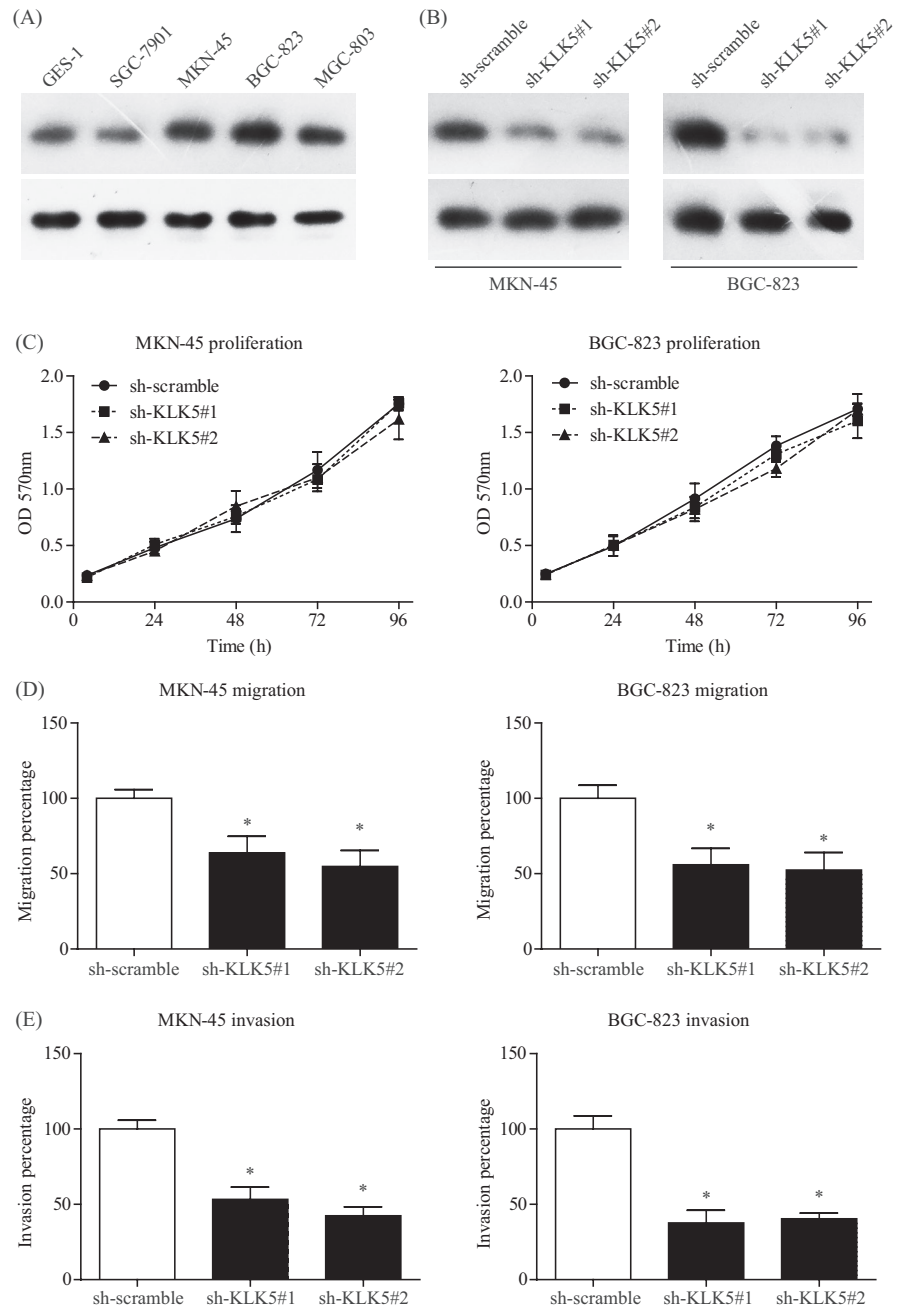


FIGURE 3 Overall survival analyses based on different TNM stages. Kaplan-Meier method was used to generate overall survival plots of GAC patients with different TNM stages, including stage I (A), stage II (B), and stage III (C). * $p < 0.05$ by log-rank test

KLKs further highlighting their clinical significances. However, till now, there is no study screened the expression or prognostic significance of KLK5 in gastric cancer. Therefore, our work would be a good supplementary study that expands our knowledge about KLKs in gastric cancer.

Interestingly, KLK5 seems to suppress tumor progression in several other cancer types. For example, KLK5-knockout mice were prone to develop vaginal tumors compared with wildtype mice, indicating its role as a putative suppressor of vaginal cancer.³⁸ A down-regulated expression of KLK5 was also observed in human breast cancer.³⁹ KLK5 reconstitution in breast cancer cell lines suppressed malignancy by modulating the miRNA network of extracellular matrix and cell-adhesion pathways.⁴⁰ Taken together, the distinct tumor-related roles of KLK5 in different malignancies and underlying signaling pathways deserve further investigation.

FIGURE 4 KLK5 promotes migration and invasion of GAC cells. (A) Western blotting was conducted to test protein expression levels of KLK5 in nontumorous stomach epithelial cell GES-1 and several GAC cell lines. (B) Knockdown of KLK5 in MKN-45 and BGC-823 cell lines were achieved by specific shRNA infection, using nonspecific scramble shRNA as control. (C) Proliferation capacities of MKN-45 and BGC-823 cell lines were tested by MTT assay. (D) Migration capacities of MKN-45 and BGC-823 cell lines were tested by wound healing assay. (E) Invasion capacities of MKN-45 and BGC-823 cell lines were evaluated by Matrigel-transwell assay. All experiments were repeated for three independent times. * indicates $p < 0.05$



5 | CONCLUSION

Our results demonstrated that KLK5 was elevated in GAC tissues and significantly correlated with poor overall survival of GAC patients. KLK5 significantly enhanced migration and invasion processes of GAC cells without affecting the proliferation capacity.

CONFLICT OF INTEREST

None.

DATA AVAILABILITY STATEMENT

Data will be available upon request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Abuduhadeer X, Xu X, Aihesan K, Yilihamu M, Zhao Y, Zhang W. Clinical significance of kallikrein 5 as a novel prognostic biomarker in gastric adenocarcinoma. *J Clin Lab Anal.* 2021;35:e23958. <https://doi.org/10.1002/jcla.23958>