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High KRT8 expression promotes tumor progression and metastasis of gastric cancer

Jian Fang,[1] Hao Wang,[2,3] Yun Liu,[1] Fangfang Ding,[1] Ying Ni[1] and Shihe Shao[1]

[1]School of Medicine, Jiangsu University, Jiangsu; [2]Second People's Hospital of Wuxi, Wuxi; [3]Nanjing Medical University, Nanjing, China

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Correspondence
Shihe Shao, School of Medicine, Jiangsu University, 301 Xuefu Road, Zhenjiang, Jiangsu 212013, China.
Tel: +86-13951404805; Fax: +86-0511-85038449;
E-mail: shaoshihe2006@163.com

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Keratin8 (KRT8) is the major component of the intermediate filament cytoskeleton and predominantly expressed in simple epithelial tissues. Aberrant expression of KRT8 is associated with multiple tumor progression and metastasis. However, the role of KRT8 in gastric cancer (GC) remains unclear. In this study, KRT8 expression was investigated and it was found to be upregulated along with human GC progression and metastasis at both mRNA and protein levels in human gastric cancer tissues. In addition, KRT8 overexpression enhanced the proliferation and migration of human gastric cancer cells, whereas the knock-down of KRT8 by siRNA only inhibited migration of human gastric cancer cells. Integrinβ1-FAK-induced epithelial-mesenchymal-transition (EMT) only existed in the high KRT8 cells. Furthermore, KRT8 overexpression led to increase in p-smad2/3 levels and TGFβ dependent signaling events. KRT8 expression in GC was related to tumor clinical stage and worse survival. Kaplan–Meier analysis proved that KRT8 was associated with overall survival of patients with GC that patients with high KRT8 expression tend to have unfavorable outcome. Moreover, Cox's proportional hazards analysis showed that high KRT8 expression was a prognostic marker of poor outcome. These results provided that KRT8 expression may therefore be a biomarker or potential therapeutic target to identify patients with worse survival.

Gastric cancer (GC) is a common malignancy of the human digestive system worldwide.[1] It is characterized by a high incidence and mortality rate[2] making it the fifth most common malignancy in the world after cancers of the lung, breast, colorectum and prostate.[3] Surgical resection, chemotherapy and radical therapy, show significant improvement over surgery alone in early-stage GC patients.[4,5] However, approximately 60% of patients with gastric cancer have locally advanced and metastatic disease at the time of surgery resulting in a relatively low therapeutic efficacy with surgical resection.[1] The valid therapeutic methods for advanced gastric cancer with invasion and metastasis remain poor and limited.[6] Therefore, the molecular mechanisms of gastric cancer progression and metastasis should be understood.

Keratins polymerize to form an intermediate filament (IF). This is observed mainly in epithelial cells as an essential cytoskeletal component involved in the maintenance of cell morphology.[7] Usually, keratins are subdivided into Type I (K9-22) and Type II (KRT1–KRT8).[8,9] The expression levels of some Keratins, such as KRT1, 5, 6, 7, 19, 20, were reported to have changed in some tumors including gastric cancer.[10–13] Therefore, the expression of Keratin may alter tumor progression. KRT8, an important Keratin, is expressed in various tumors abnormally.[14] However, the expression level of KRT8 is different in various tumors, for instance, KRT8 expression is reduced in the human breast and colorectal carcinomas.[15,16] It is upregulated in the head and neck,[17] oral cavity carcinoma,[18] and transitional cell carcinoma of the urinary tract;[19] and is associated with unfavorable prognosis.[18]

In recent years, aberrant expression of KRT8 has been found to be associated with multiple tumor progressions such as cell migration,[7,20] cell adhesion[21] and drug resistance.[20,22] However, the mechanisms of these processes caused by KRT8 have not been very clear yet. Earlier reports confirmed the acquisition of KRT7 and loss of KRT20 in gastric carcinoma.[10,11] However, little is known about the expression and functions of KRT8 in GC.

In the present study, it was shown that the expression level of KRT8 mRNA and protein was higher in GC tissues than in normal tissues. A high expression of KRT8 was closely related with poor overall survival of GC patients and promoted proliferation, EMT and migration of GC cells. Additionally, the knockdown of KRT8 inhibited migration and EMT, but had no effects on the proliferation of GC cells. We also showed that the high expression of KRT8 regulated the cell-matrix adhesion by integrinβ1-FAK signaling. Moreover, the results implicated that KRT8 may exert its biological functions through TGF-β/Smad2/3 pathway. Overall, our results showed that KRT8 could regulate gastric carcinogenesis and may serve as a potential target for antineoplastic therapies.

Materials and Methods

Patients and samples. In this study, informed consent was obtained from all patients and approved by the Ethics Committee of the School of Medicine, Jiangsu University. We consecutively enrolled 70 adults with histology-confirmed GC from July 2007 to February 2010 to immunohistochemistry. Fresh

tumor tissues and adjacent normal tissues, from 50 patients who underwent surgical treatment for GC from October 2012 to January 2015, were also obtained for RNA and protein extraction. All gastric cancer samples were collected from the Second People's Hospital of Wuxi, Jiangsu, China. All participants were randomly selected from the patients diagnosed with GC, at Second People's Hospital of Wuxi, who had not received chemotherapy or radiation therapy prior to surgery. Tumor stages of participants were determined according to the Cancer Staging Manual (Seventh Edition) of the American Joint Committee on Cancer (AJCC).

Cell culture. The five human gastric cancer cell lines (BGC-823, AGS, HGC-27, MKN-28, SGC-7901) were cultured and maintained in our laboratory. The cancer cell line AGS was cultured in F12 (Gibco, Grand Island, NY, USA) medium supplemented with 10% fetal bovine serum (FBS). All other cancer cell lines were cultured in RPMI-1640 medium (Gibco), supplemented with 10% FBS. All cells were cultured at 37°C in a humidified incubator with 5% CO_2.

Plasmid construction, small interference RNA and cells transfection. A full-length human cDNA of KRT8 was synthesized and integrated by Bioworld (Nanjing, China). The product was then sub-cloned into the HindIII and EcoRI sites of the pcDNA3.0(+) vector (Invitrogen, Life Technologies Corporation, Carlsbad, CA, USA) to construct the plasmid pcDNA-KRT8. Later, 2ug pcDNA-KRT8 plasmid was transfected into HGC-27 cells cultured in 6-well plates at 2×10^5 cells/ml using Lipofectamine 2000 (Invitrogen, Shanghai, China). After 24 or 48 h, RNA and protein was extracted, and were then tested by qRT-PCR and Western blotting to analyze the over expression efficiency. 125 nM of either si-KRT8 (Sense: 5′-CUGAGAUGAACCGGAACAUTT-3′; Antisense: 5′-AUGUUCCGGUUCAUCUCAGTT-3′) and control siRNA synthesized by GenePharma Corporation (Shanghai, China) used for targeted downregulation of human KRT8 was transfected into AGS cells cultured in 6-well plates at 3×10^5 cells/mL. After 48 h post-transfection, cells were harvested for qRT-PCR and Western blotting to analyze the knock-down efficiency.

RNA extraction and real-time PCR analysis. Total RNA from cells and tissue samples was islated using TRIzol Reagent (Invitrogen, Life Technologies Corporation), according to the manufacturer's instructions. The cDNA was then synthesized using HiScript QRT SuperMix from qPCR Kit (Vazyme, Nanjing, China). qRT-PCR was performed using TransStart Top Green qPCR Super Mix (TRAN, China) on ABI Step One Plus Real Time PCR System (Applied Biosystems, Foster City, CA, USA). The sequences of the qRT-PCR primers that were synthesized from Invitrogen were as follows: KRT8 forward primer: 5′-CGAGGATATTGCCAACCGCAG-3′, reverse primer: 5′-CCTCAATCTCAGCCTGGAGCC-3′. Data were quantified using a relative quantitative method of the $2^{-\Delta\Delta ct}$ and were normalized by GAPDH expression in each sample.

Western blotting. Cell proteins were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology, Shanghai, China) containing phenylmethanesulfonyl fluoride (PMSF) and phosphatase inhibitors. Then the equal amounts of protein (100 μg) were resolved by 10% SDS-PAGE gels and subsequently electrophoretically transferred to PVDF membranes. Membranes were blocked with 5% non-fat dry milk powder for 2 h at room temperature and then incubated with primary antibodies at 4°C overnight. The membranes were visualized using the appropriate secondary antibody at room temperature for 1 h followed by the enhanced chemiluminescence (ECL) system (Image Quant LAS 4000 mini, Pittsburgh, PA, USA) according to the instructions of the manufacturer. The relative densities of bands were quantified using ImageJ (https://imagej.nih.gov/ij/) software. All experiments were repeated at least three times. Sources of primary antibodies were as follows: anti-KRT8 (Ruiying, Ruiyingbio, SuZhou, China); GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA); His-Tag (Santa Cruz Biotechnology); anti-E-cadherin, anti-N-cadherin, anti-Vimentin, anti-Snail, anti-MMP2, anti-MMP9, anti-TIMP-1, anti-PCNA, anti-Fibronectin, anti-Integrinβ1, anti-FAK, anti-phosphorylated-FAK, anti-Smad2/3, anti-phosphorylated Smad2/3, anti-TGF-β1 (all from Cell Signaling, Danvers, MA, USA). The relative density of bands was quantified using ImageJ (http://rsb.info.nih.gov/ij/) software.

Immunohistochemistry. The gastric tumor tissue was fixed in 10% buffered formalin, embedded in paraffin, and serially sectioned at 4 μm thickness. Endogenous peroxidase activity was suppressed by exposure to 3% hydrogen peroxide for 10 min. Slides were then blocked with 5% BSA (bovine serum albumin; Boster Bioengineering, Wuhan, China). The tissue section was treated with primary antibodies against KRT8 (1:300 dilution). The sections were then incubated overnight in a humidified chamber at 4°C. Sections were visualized with DAB (3,3diaminobenzidine) and counterstained with hematoxylin for microscopic examination.

The expression levels of KRT8 protein in the cytoplasm of tumor cells were scored as the intensity of staining and the percentage of positive-stained cells. The scoring system was as follows: score 0 for no positive cells; and score 1–3 for positive cells presented yellowish, light-brown and dark-brown staining.

Transwell migration assay. Transwell migration assay was performed using CoStar Transwell chambers (8 μm pore size; Corning, Costar, NY, USA). Cells (1×10^5/well) were seeded in the upper chambers of the wells in 300 μl serum-free medium, while the lower chambers were filled with 700 μl medium containing 10% fetal bovine serum to induced cell migration. After incubation at 37°C in 5% CO_2 for 24 h, the cells in the upper surface of the membrane were removed with a cotton swab. Cells migrated to the lower surface of the membrane were fixed with 4% paraformaldehyde and stained with crystal violet. The images were obtained and the cells were counted under a microscope.

The cell proliferation assay. The proliferation of HGC-27 and AGS cells were examined using CCK-8 kit (Tongren, Shanghai, China) according to the manufacturer's instructions. Approximately 3×10^3 transfected cells were seeded in 96-well plates and cultured for 24, 48 and 72 h, respectively. 10 μL CCK-8 solution was then added to each well and incubated for 1 h. The absorbance at 450 nm was measured using a microplate reader at different time intervals (24, 48 and 72 h).

The colony-formation assay. Cells were harvested and seeded into six-well plates (1000 cells/well) and incubated at 37°C in a 5% CO_2 humidified incubator for 14 days. The medium was changed at 3-day intervals. At the end of the incubation period, the cultures were fixed with 4% paraformaldehyde and stained with crystal violet.

Survival analysis. The 70 patients were enrolled in the survival analysis. Follow-up surveys were made by telephone, visits, or letters to update the information and survival data of the patients. The survey period was completed by April 2015, with 5 years of follow-up records obtained for each patient. Overall survival time was defined as the time from the date of surgery to death (for non-censored events) or to the end of the survey period (for censored events).

Statistical analysis. All statistical analyses were performed using SPSS 19.0 (SPSS, Chicago, IL, USA). Significant

differences among groups were measured by Student's *t*-test or one-way ANOVA. Correlation between KRT8 expression and clinicopathological factors was estimated by the Fisher's exact test. Overall survival curves were plotted according to the Kaplan–Meier method, with the log-rank test applied for comparison estimated by the Fisher's exact test. A Cox proportional hazards regression analysis was used for univariate and multivariate analyses of prognostic values. $P < 0.05$ was defined as statistically significant.

Results

KRT8 expression is increased in gastric cancer tissues. To determine the expression of KRT8 in gastric cancer, the mRNA levels of KRT8 were first examined in 50 paired GC tissues and the adjacent normal tissues by quantitative real-time PCR (qRT-PCR). The results showed that KRT8 mRNA level increased in 68% (34/50) of gastric cancer tissues (Fig. 1b), which is consistent with the analysis of ONCOMINE dataset (https://www.oncomine.org) (Fig. 1a). The expression of KRT8 protein was next analyzed by immunohistochemical staining in 70 paired tumor tissues and corresponding normal (non-tumorous) tissues. Similar results were obtained from the assessment of KRT8 protein expression (Fig. 1c). As shown in Table 1, 62.8% (44/70) of patients KRT8 staining was dramatically increased in cancerous tissues compared to adjacent normal tissues. Collectively, these data suggested that the expression of KRT8 was aberrantly increased in GC at both the RNA and protein levels.

The expression of KRT8 in human gastric cancer cell lines. To examine the expression of KRT8 in human gastric cancer cell lines, five cell lines (BGC-823, AGS, HGC-27, MKN-28,

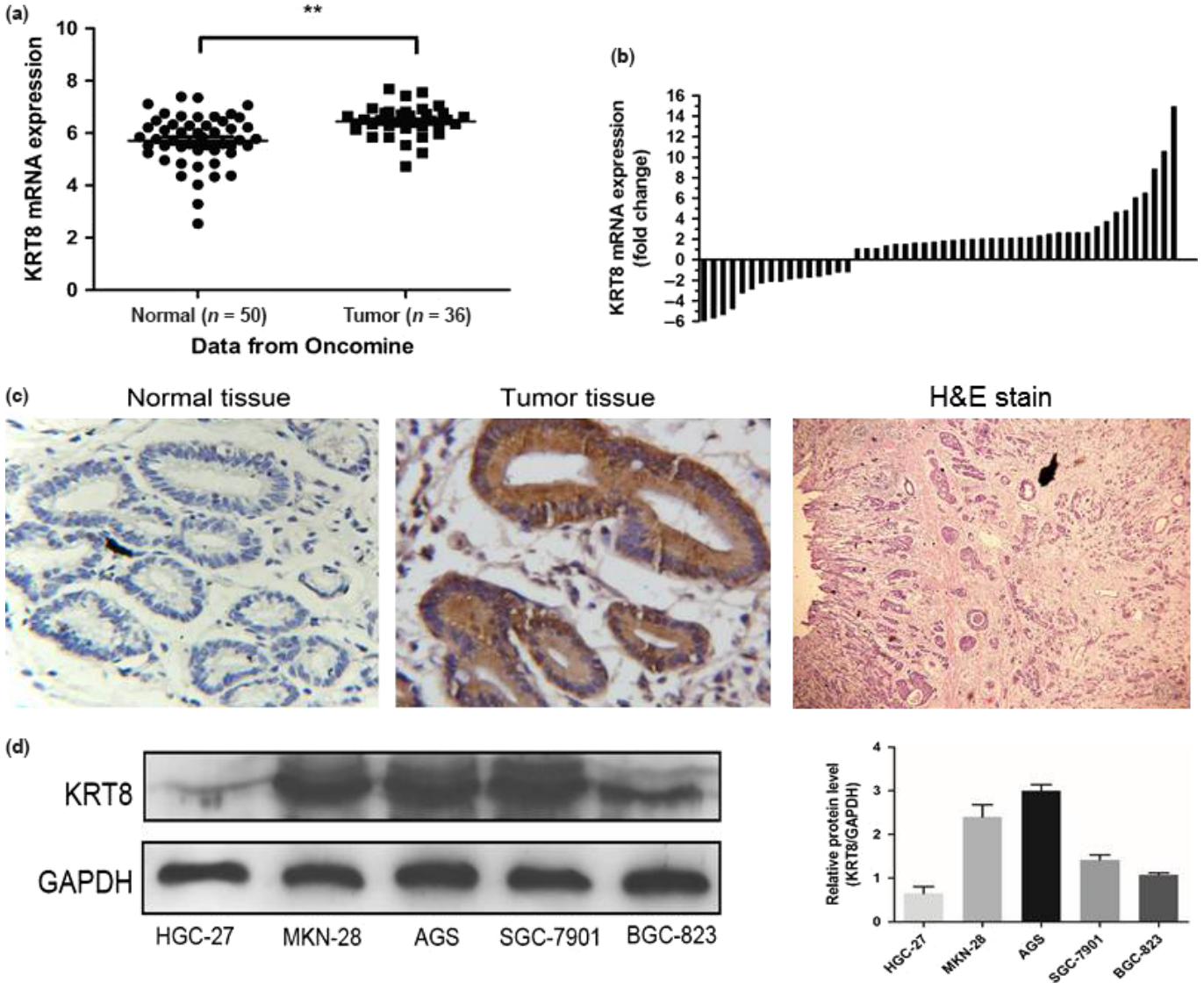

Fig. 1. The expression of KRT8 in gastric cancer tissues and paired adjacent normal tissues and cell lines. (a) KRT8 mRNA expression analysis in GC tissues ($n = 50$) and normal tissues ($n = 36$) by using ONCOMINE. (b) The mRNA level of KRT8 in 50 pairs of GC and normal tissue was detected by qRT-PCR. Upregulation of KRT8 was found in 68% (34/50) of the selected patients. (c) Representative immunohistochemical staining of KRT8 in gastric cancer tissues (middle) and adjacent normal tissues (left) (Original magnification ×200) and H&E staining (Original magnification ×100). (d) Expression levels of KRT8were checked in a panel of five human GC cell lines using immunoblotting. (Scale bar: 100 μM.) $^{**}P < 0.01$.

Table 1. The correlation of KRT8 expression level to the clinical features of gastric cancer (GC)

Characteristics	*n*	High KRT8 (*n* = 44)	Low KRT8 (*n* = 26)	*P*-value
Gender				
Male	46	29 (63%)	17 (37%)	0.964
Female	24	15 (62.5%)	9 (37.5%)	
Age (years)				
>60	43	26 (60.4%)	17 (39.6%)	0.765
≤60	27	18 (66.7%)	9 (33.3%)	
Tumor differentiation				
Poor or moderate	58	37 (63.7%)	21 (36.3%)	0.722
Well	12	7 (58.3%)	5 (41.7%)	
Primary tumor size (cm)				
≥5	21	16 (76.1%)	5 (23.9%)	0.131
<5	49	28 (57.1%)	21 (42.9%)	
Clinical stage				
I or II	38	19 (50%)	19 (50%)	**0.025**
III or IV	32	25 (78.1%)	7 (21.9%)	
Invasion depth				
T1-2	27	9 (33.3%)	16 (66.7%)	**0.002**
T3-4	43	33 (76.7%)	10 (23.3%)	
Lymph node				
Negative	37	24 (64.8%)	13 (35.2%)	0.713
Positive	33	20 (60.6%)	13 (39.4%)	

P-values < 0.05 are indicated in bold.

SGC-7901) were examined by western blotting. KRT8 protein was expressed in all these five types of the human gastric-cancer cell lines. The expression of KRT8 protein was highest in the HGC-27 cell line and lowest in the AGS cell line (Fig. 1d).

KRT8 overexpression influences proliferation of gastric cancer cells. To explore whether KRT8 could influence cell proliferation in gastric cancer cells, the expression of KRT8 was enhanced in gastric cancer cell lines: HGC-27 cells by transfection with pcDNA3.0-KRT8, HGC-27 cells transfected with pcDNA3.0 (blank vector) were used as controls. The expression of KRT8 in HGC-27 cells was confirmed by western blotting (Fig. 2a,b). Thereafter, the Cell Counting Kit-8 (CCK-8) assay was used in the transfection with pcDNA3.0-KRT8 and pcDNA3.0 (blank vector) HGC-27 cells. It was found that the overexpression of KRT8 significantly increased the proliferation of HGC-27 cells (Fig. 3d) and formed larger clones than the control cells (Fig. 3c).

KRT8 expression in AGS cells was then knocked down by using siRNA (Fig. 2c,d). However, in comparison, the control cells had no difference in the proliferation rate of AGS cells transfected with si-KRT8 in AGS cells (Fig. 3e), as in AGS cells transfected with control siRNA.

KRT8 overexpression induces EMT in HGC-27 cell line. Prior to the migration and invasion of epithelial cells is often a developmental and morphological alternation called epithelial-mesenchymal-transition (EMT).[23,24] The expression of several indicators of EMT was next examined. It was found that the mesenchymal markers N-cadherin Snail and Vimentin were upregulated in KRT8-overexpressed cells, while the epithelial marker E-cadherin was significantly downregulated (Fig. 4a,b). In contrast, the knockdown of KRT8 appeared to cause a modest increase of E-cadherin in AGS cells, while the Snail and Vimentin were reduced significantly as expected (Fig. 4c,d). In summary, these data suggest that KRT8 regulates the process of EMT resulting in the enhanced migratory ability of gastric cancer cells *in vitro*.

Aberrant KRT8 expression influence cell migration *in vitro*. As shown in previous studies KRT8 could alter the ability of migration in various cancers.[7,20,25,26] To better understand the effects of KRT8 on gastric cancer cell motility, the transwell migration assay was used in HGC-27 and AGS cells. Compared with the control cells, KRT8 overexpression markedly increased the number of migrated HGC-27 cells (Fig. 3a). Meanwhile, KRT8 knockdown significantly reduced the number of migrated AGS cells (Fig. 3b).

The expression of a series of molecular markers was then detected by Western blotting in HGC-27 and AGS cells. Just as in Figure 5(a), the expression of MMP-9 was distinctly increased in the HGC-27 overexpression group, while the TIMP-1 was dramatically decreased, when compared to vector groups. However, there were no significant expression discrepancies in MMP-2 (data not shown). In contrast, the knockdown

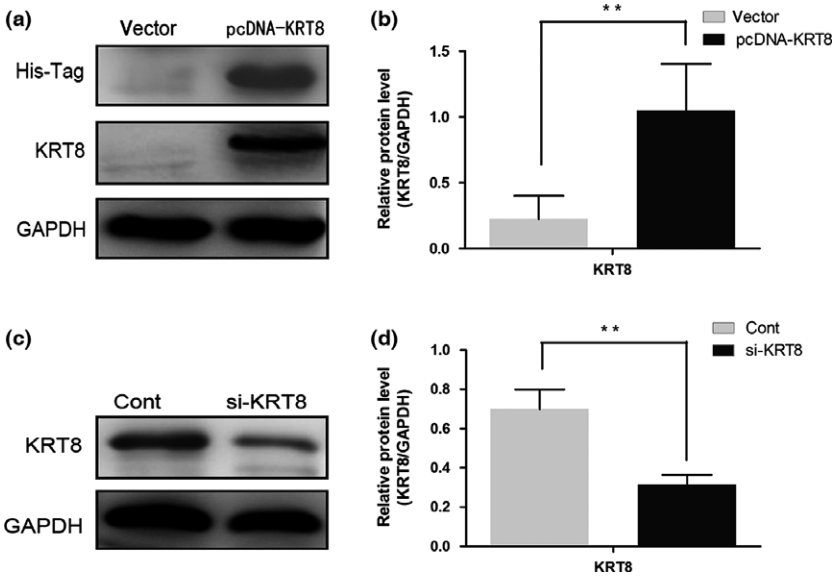

Fig. 2. The expression of KRT8 was enhanced in HGC-27 cells and knockdown in AGS cells. (a) and (b) The expression of KRT8 in HGC-27 cells transfected with pcDNA3- KRT8 increased significantly. $^{**}P < 0.01$ versus HGC-27 cells transfected with pcDNA3.0 HGC-27 cells. (c) and (d) KRT8 siRNA could efficiently inhibit the expression of KRT8 in AGS cells. $^{**}P < 0.01$ versus blank AGS cells.

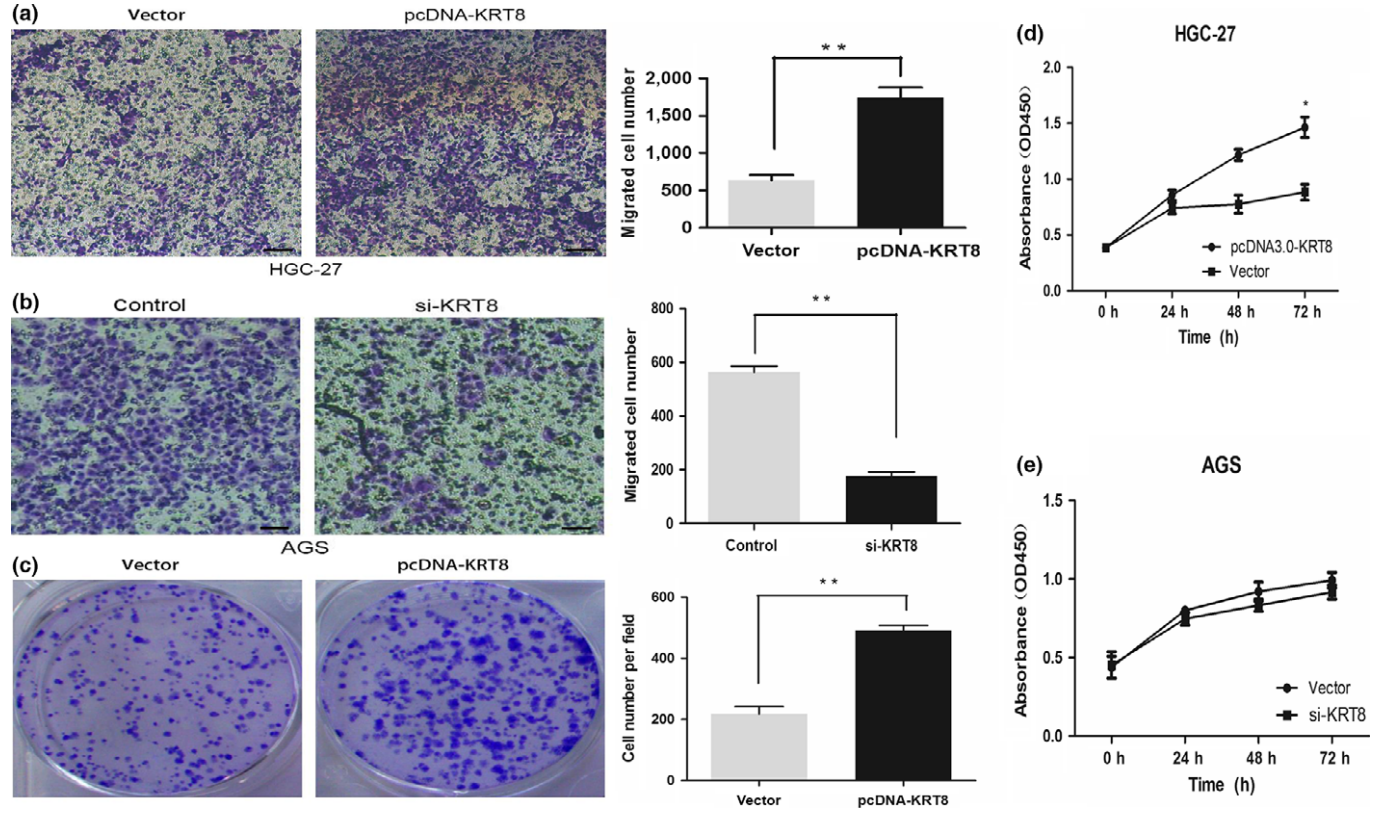

Fig. 3. KRT8 overexpression enhances the proliferation and migration of HGC-27 cells and KRT8 silencing reduces the migration of AGS cells. (a) Overexpression of KRT8 enhanced migratory ability of vector and KRT8-overexpressing HGC-27 cells. (b) The migratory ability of control and KRT8 siRNA-transfected AGS cells was evaluated by using transwell migration assay. Knockdown of KRT8 reduced the migratory ability of AGS cells (200 × magnification). (c) Overexpression of KRT8 increased the colony formation of HGC-27 cells. (d) CCK-8 assay was performed in HGC-27 and AGS cells and indicated that overexpression of KRT8 increases the cell proliferation in HGC-27 cells but no change in AGS cells (e). (Scale bar: 100 μM.) $^{*}P < 0.05$, $^{**}P < 0.01$.

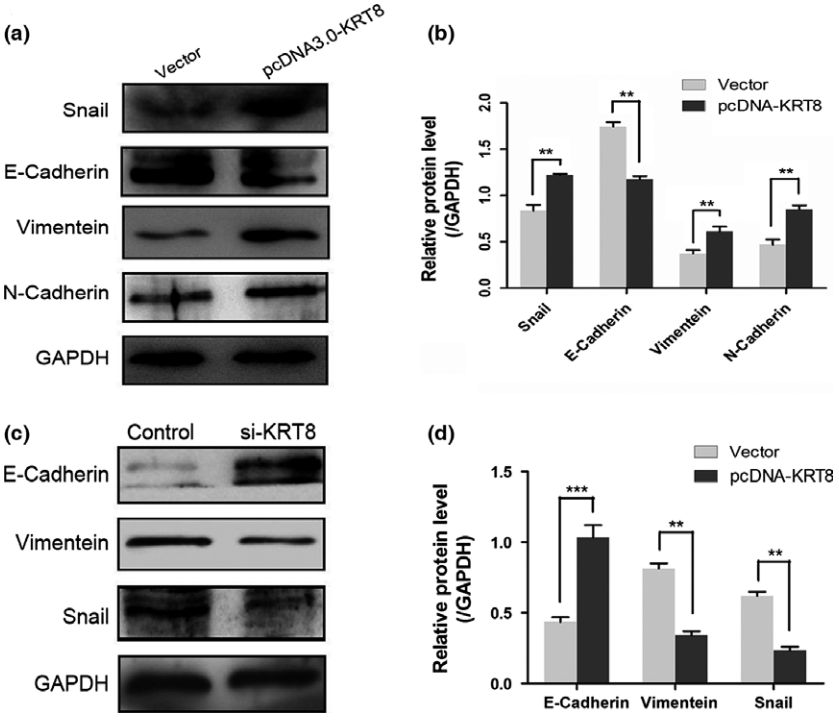

Fig. 4. KRT8 expression affected epithelial-mesenchymal transition (EMT) in HGC-27 and AGS cells. (a) and (b) Western blot showed EMT phenotype appeared in transfected pcDNA3-KRT8 HGC-27 cells. High KRT8 can induce Snail, Vimentein and N-cadherin expression and reduce E-cadherin expression. (c) and (d) EMT phenotype with reduced Snail and Vimentin and increased E-cadherin expression in AGS cells transfected with si-KRT8. $^{**}P < 0.01$, $^{***}P < 0.001$.

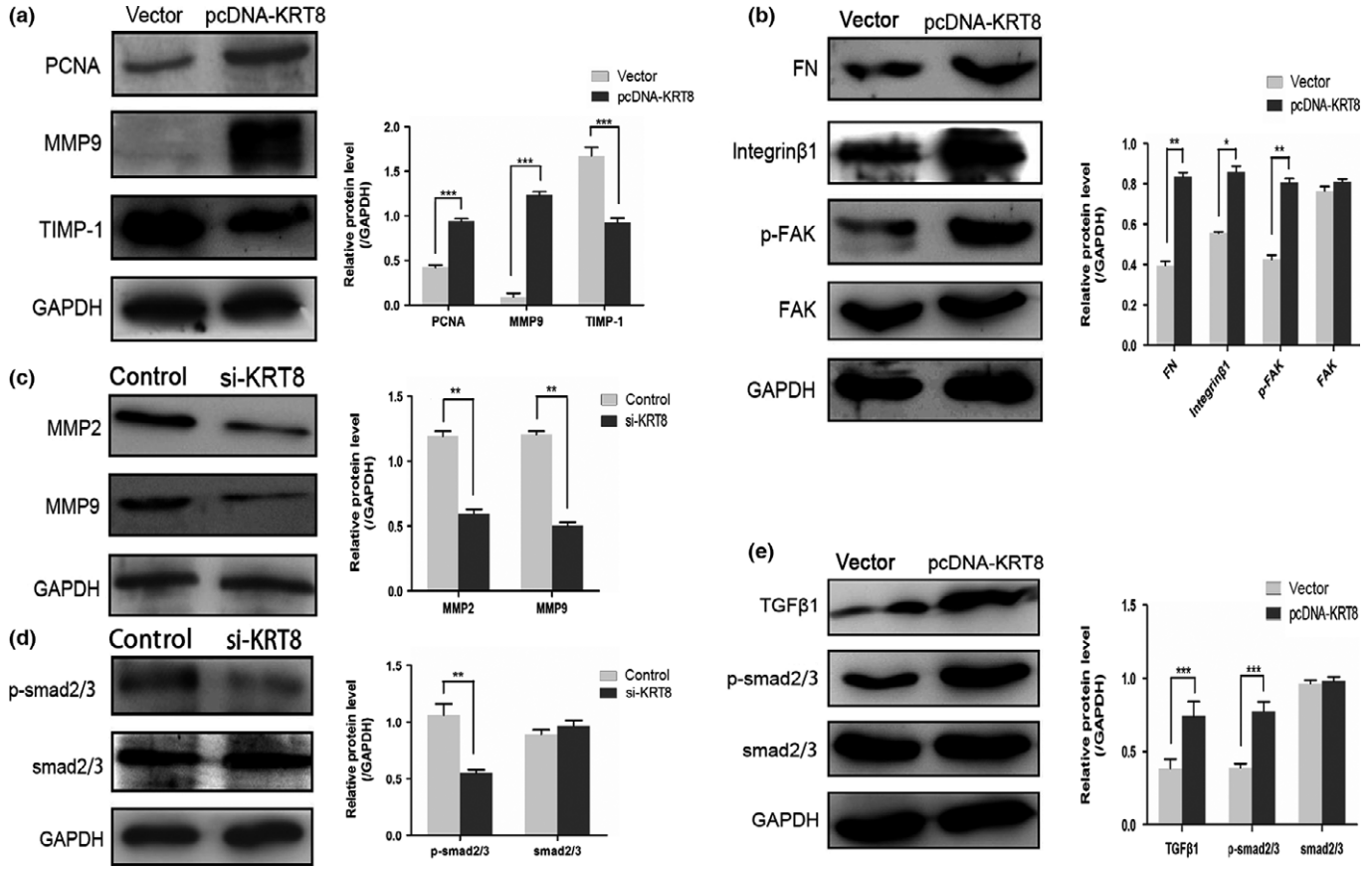

Fig. 5. KRT8 expression affected migration, PCNA, integrinβ1-FAK signaling and TGF-β signaling pathways. (a) and (c) Expression of the proliferation and migration related gene was evaluated by immunoblotting in HGC27 and AGS cells at 48 h after transfected with pcDNA-KRT8 or si-KRT8. (b) Western blotting of FN, integrinβ1, FAK and p-FAK in HGC-27 cancer cells with or without KRT8 overexpression. (d) and (e) Expression of the TGF-β signaling pathway related gene was evaluated by western blotting in HGC27 and AGS cells. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

of KRT8 appeared to cause a modest reduction of MMP-2 and MMP-9 levels in the AGS cells (Fig. 5c). Overall, these data demonstrated that KRT8 expression could influence gastric cancer cell migration *in vitro*.

Overexpression of KRT8 enhances cell-matrix adhesion by integrinβ1-FAK signaling. Increased cell-matrix adhesion is an important step during tumor cell metastasis.[27,28] To investigate the role of KRT8 in the regulation of cell-matrix adhesion in gastric cancer cells, the expression of fibronectin (FN), Integrinβ1 and FAK within over expressed KRT8 in HGC-27 cell was examined by western blotting. The results showed that the overexpression of KRT8 dramatically promoted the expression of FN, Integrinβ1, as well as the p-FAK (Tyr397) (Fig. 5b). In brief, the adhesion-promoting effects of KRT8 overexpression were most likely mediated by the integrinβ1-FAK signaling.

KRT8-induced EMT and cell migration are mediated by TGF-β signaling. Loss of pathway regulates cancer cell EMT and migration. One of the key mechanisms by which TGF-β promotes cell migration, invasion, and metastasis is through the induction of EMT.[29–31] To investigate the possible potential involvement of TGF-β signaling pathway in KRT8-mediated EMT, cell migration and invasion, the expression of TGF-β1, Smad2/3 and the phosphorylated Smad2/3 (p-Smad2/3) was detected. They are critical downstream regulators of TGF-β signaling pathway. The results showed that the levels of p-Smad2/3 were remarkably increased in KRT8-over expressed HGC-27 cells (Fig. 5e). Consequently, a decreased in KRT8-knockdown AGS cells (Fig. 5d). These data indicated that KRT8 may promote cell EMT and migration partially by activating TGF-β/Smad2/3 pathway.

Clinicopathological features of GC patients with KRT8 expression. In order to investigate the clinical role of KRT8 in GC, we analyzed the relationship between KRT8 expression and clinicopathological factors of the 70 patients. Forty-four of 70 patients with GC defined to high-KRT8 expression carcinomas (Fig. 1c). In Table 1, as compared to the GC with low-KRT8 expression, the GC with high-KRT8 expression can be a more tumor invasion depth ($P = 0.002$) and more advanced clinical stage ($P = 0.025$).

Expression level of KRT8 and the overall survival of patients with GC. To further elucidate the important role of KRT8 in the survival of GC patients, we analyzed the relationship between the KRT8 mRNA expression level and the survival of GC patients from 1928 gastric tumor samples using publicly available datasets.[32] The Kaplan–Meier analyses result demonstrated that higher KRT8 mRNA expression is correlated with the poor overall survival (OS), as well as progression-free (FP) survival of GC patients (Fig. 6a,b). Finally, we used Kaplan–Meier analysis evaluated the correlation of 5-year survival of GC patients with KRT8 expression ($P = 0.0412$) (Fig. 6c). The result indicated that high expression of KRT8 reduced survival rate of GC patients and in accordance with the results of abovementioned dataset. Furthermore, we carried out Kaplan–Meier survival analysis to investigate the clinical

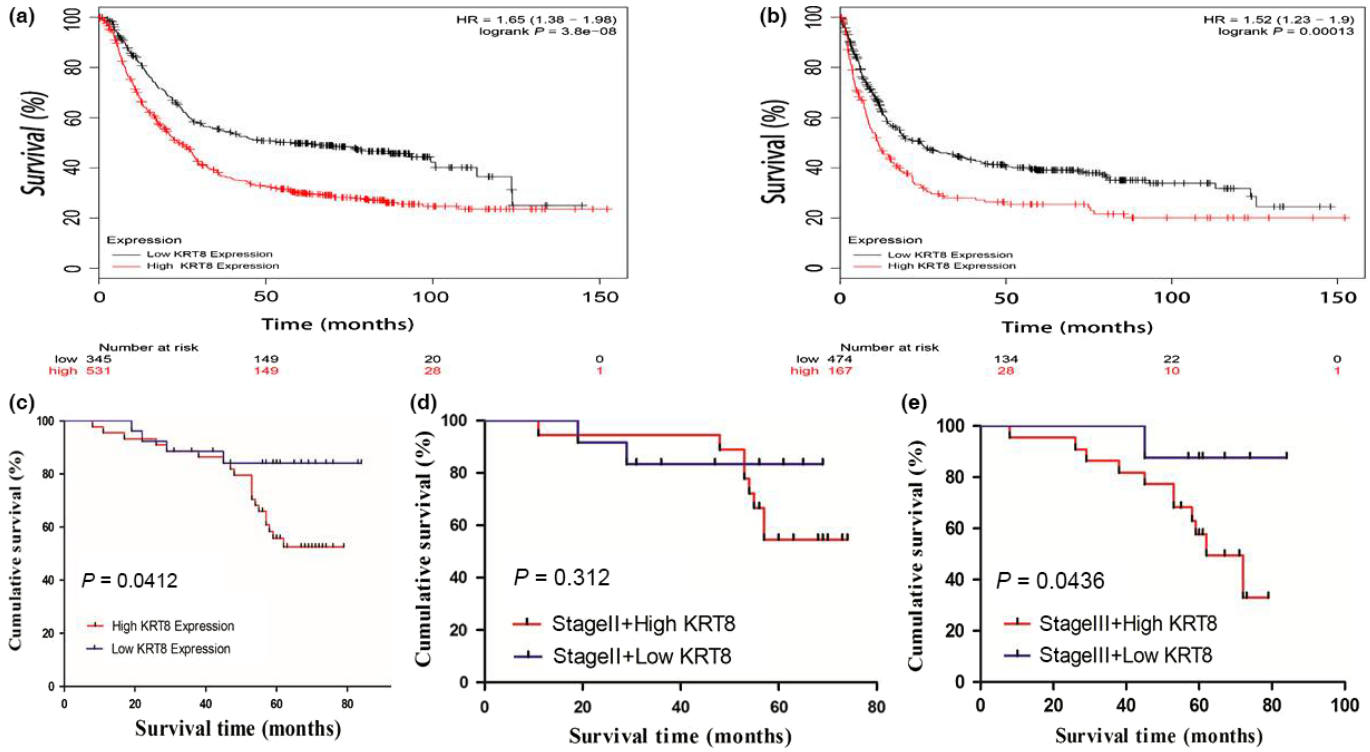

Fig. 6. Prognostic significance of KRT8 in gastric cancer. (a) and (b)The effect of KRT8 mRNA expression level on the overall survival (a) and progression free survival (b) in gastric cancer patients was analyzed and the Kaplan–Meier plots were generated by Kaplan–Meier Plotter (http://www.kmplot.com). (c) Kaplan–Meier survival curve for gastric cancer patients with different KRT8 expression levels. gastric cancer patients with high KRT8 expression showed a significantly poorer prognosis than those with low KRT8 expression ($P < 0.05$). (d) and (e) Kaplan–Meier survival curve for gastric cancer patients with the group of high KRT8 expression and clinical stage II (d) and the group of high KRT8 expression and clinical stage III (e).

outcome of stage II and stage III based on the expression level of KRT8. The data showed the group of stage III and high KRT8 was associated with poor overall survival ($P = 0.0436$), but there was no association between group of stage II and high KRT8 and overall survival ($P = 0.312$) (Fig. 6d,e).

Univariate analysis showed that the relative level of KRT8 expression level, clinical stage and invasion depth were correlated with overall survival rate of patients with GC patients ($P < 0.05$, Table 2). Multivariate Cox regression analysis demonstrated that KRT8 expression level and clinical stage were independent prognostic indicators for the overall survival of patients with GC ($P < 0.05$, Table 2).

Table 2. Univariate and multivariate Cox regression of prognostic factors for overall survival in gastric cancer (GC)

Parameter	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P*	HR	95% CI	*P*
Clinical stage III+IV *vs* I+II	3.15	1.25–7.93	0.015	3.33	1.4–8.1	0.006
Invasion depth T3 + T4 *vs* T1 + T2	2.25	1.57–3.22	<0.001	2.01	0.89–4.53	0.09
KRT8 expression High *vs* Low	4.5	1.35–15.5	0.018	4.75	1.61–14.01	0.0048

CI, confidence interval; HR, hazard ratio.

Discussion

Although KRT8 proteins have been studied in different types of malignancies,[7,20] the knowledge of the aberrant expression and possible role of KRT8 in GC is still unknown. In this study, we confirmed that the expression of KRT8 was astonishingly increased in GC tissues compared to normal controls. We also demonstrated that aberrant KRT8 expression could regulate the production of MMP2, MMP9, TIMP1 and PCNA, resulting in cancer cells migration and proliferation.

Increasing amounts of evidence suggest that the extracellular matrix (ECM) is a key factor for determining whether metastatic tumors spurt or not.[33] The importance of the ECM in primary tumor progression has been reviewed extensively, and it is now widely accepted that the ECM can affect many of the hallmarks of cancer.[34,35] Increased cell adhesion to the ECM is an early step of cell migration.[36] Fibronectin is an important component of the ECM, the changes of fibronectin expression play an important role on cell adhesion.[37] Integrinβ1 is a major adhesion molecule to interact with ECM and regulates the signal transduction between cells.[38] Focal adhesion kinase (FAK) is the downstream target of integrin and is a crucial signaling molecule to modulate cellular responses to integrin-mediated adhesion.[39] Upregulation of integrin and FAK is often observed to correlate with the progression of tumor development implying that the integrin/FAK signaling involves in the regulation of tumor development.[40,41] Given this, the levels of FAK, p-FAK and Integrinβ1 (Fig. 5b) were examined, and all of these were upregulated.

The TGF-β signaling pathway is instrumental in regulating crucial cellular activities such as cell growth, differentiation, motility and invasion. In the occurrence and development of tumors, TGF-β has a dual role. In the early stages of tumor development, TGF-β operates as a tumor suppressor, whereas the opposite is true in late stages, supporting invasion and metastasis.[42] Previous studies in various developmental EMT systems extensively provide convincing evidence that TGF-β signaling is a primary inducer of EMT.[31] These findings present data indicating that the association of KRT8 with EMT and metastasis can be a consequence of KRT8 being involved in the regulation of TGF-β signaling. The expression of the E-cadherin was reduced while MMP9, FN, TGF-β signaling (TGF-β1, p-Smad2/3) pathways-related protein were enhanced in KRT8 overexpressed HGC-27 cells (Fig. 5b). In contrast, E-cadherin was increased while MMP2, MMP9, p-Smad2/3 were decreased in siRNA treated AGS cells (Fig. 5c,d). The data that was generated therefore suggested that the up-regulated expression of KRT8 may enhance EMT to facilitate the development of GC and metastasis by activating TGF-β/Smad2/3 pathway. Thomas *et al.* [43] reported that vimentin expression was inversely associated with keratin expression alone and directly related to worse clinical outcome in breast cancer. We then analyzed the association between KRT8 and vimentin expression, however, we found that vimentin expression was not associated with keratin expression and not related to clinical prognosis (data not show).

Although the current results elucidated an important role of KRT8 in GC progression and is closely correlated with the depth of invasion, lymph node metastasis, and TNM stage, Kaplan–Meier analysis revealed a negative relationship between KRT8 expression and overall survival of GC patients, but the underlying molecular mechanisms remain unclear. The next question that could be addressed is the precise mechanism by which KRT8 regulates GC cell migration and invasion during tumor progression, especially *in vivo*.

In summary, this study reveals that the high expression of KRT8 regulates gastric cancer cells migration, proliferation and EMT and contributes to GC progression such as tumor invasion depth, clinical stage and poor survival. The findings also demonstrate the potential role of KRT8 as a diagnostic and prognostic indicator for GC patients.

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Disclosure Statement

The authors have no conflict of interest to declare.

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