

Aim of the study: This study aims to investigate the expression of human kallikrein 6 (hK6) in gastric cancer, gastric ulcer and normal gastric mucosa tissues and its biological significance.

Material and methods: The expression of hK6 in 15 normal gastric mucosa (NGM) tissues, 15 gastric ulcer (GU) tissues and 55 gastric carcinoma (GC) tissues was respectively detected by immunohistochemistry. The correlations between the expression of hK6 and the clinical pathological parameters of gastric cancer were also analyzed.

Results: Human kallikrein 6 was mainly expressed in cytoplasm. The positive rate of hK6 was significantly higher in gastric cancer tissues than that in gastric ulcer or normal gastric mucosa tissues (70.9%, 40% and 20%, respectively, $p < 0.01$). With the increase of the invasion depth of gastric cancer cells, aggravation of TNM stage and development of lymph node metastasis, the expression of hK6 increased significantly ($p < 0.05$ and $p < 0.01$). There was no obvious correlation between the expression of hK6 and sex, age, tumor diameter, histodifferentiation degree or primary pathological location of gastric cancer ($p > 0.05$).

Conclusions: The overexpression of hK6 is related to the depth of invasion, lymph node metastasis and clinical stage of gastric carcinoma, which suggests that hK6 may act as a new marker of gastric cancer biological behavior.

Key words: expression, gastric cancer, gastric neoplasm, human kallikrein 6, immunohistochemistry.

Expression and biological significance of human kallikrein 6 in gastric cancer tissues

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Introduction

Gastric carcinoma (GC) is one of the most common malignant tumors in the world. Due to its occult onset and difficult early diagnosis, a specific marker of gastric carcinoma has not been found yet. Human tissue kallikreins (KLKs) are a tumor marker family which was only discovered in recent years. The *KLK6* gene is one member of this family. It is composed of 223 amino acids, and possesses trypsin-like activities [1]. Studies have shown that hK6 participates in the genesis of many kinds of malignant tumors, and its expression is closely correlated with tumor biological behaviors such as invasion and metastasis [2, 3]. But so far, few reports on the correlation between hK6 and gastric carcinoma have been released.

In this study, the expression of hK6 in gastric carcinoma tissues (GC), gastric ulcer tissues (GU) and normal gastric mucosa (NGM) tissues was detected by immunohistochemistry, and the relationships between the expression of hK6 and clinical pathological parameters were also explored.

Material and methods

General data

Fifty-five paraffin-embedded samples were taken from gastric carcinoma patients who received tumor excision in Qilu Hospital of Shandong University between December 2007 and October 2008. Among these patients, 37 were male and 18 were female, with a median age of 56 (range: 26–89). All the cases were primary cases according to pathohistological diagnosis. None of the patients received any chemotherapy or radiotherapy prior to the operation. Among all the cases, 26 were diagnosed as moderately/well-differentiated carcinoma and 29 cases as poorly differentiated carcinoma. The diameter of the tumor was less than 5 cm in 30 cases and 5 cm or above in 25 cases. Lymph node metastasis was found in 38 cases. The primary site of tumor was as follows: the gastric antrum in 25 cases, gastric body in 11 and gastric fundus in 19. The invasion depth of the tumor was as follows: 12 cases were at stages T1 + T2 and 43 cases were at stages T3 + T4. Clinical stage: 21 cases were at stages I + II and 34 were at stages III + IV. Tumors were staged according to the 5th edition of AJCC/UICC TNM classification (1997). Meanwhile, 15 samples of gastric ulcer tissues and normal gastric mucosa tissues were respectively collected as the controls. The gastric ulcer tissues were taken from gastric ulcer patients with gastroscopic biopsies. The normal gastric mucosa tissue was 8–10 cm from the margin of the carcinoma tissue. All samples were fixed with 10% formaldehyde and embedded with paraffin. Consecutive sections with the thickness of 3 μ m were made. Written informed consent was obtained from all patients according to the guidelines approved by the Institutional Research Board.

Immunohistochemical staining

Paraffin-embedded sections were deparaffinized, hydrated in graded alcohols, retrieved by PH with EDTA (pH 8.0) and then washed three times with PBS (PH 7.0) (3 min each time). Cells were incubated with 3% H₂O₂ deionized water at room temperature for 10 min to block the activity of endogenous peroxidase, then washed three times with PBS (3 min each time). A drop of goat serum fluid (reagent A) for blockage use was added. The cells were incubated at room temperature for 10 min, and then the superfluous blood serum on the section was removed. 50 μ l of primary mouse monoclonal antibodies against hK6 (Abnova, Taiwan) at a dilution of 1 : 700 with PBS was added and kept at 4°C overnight, and then rinsed with PBS three times (3 min each time). Biologically labeled goat anti-mouse secondary antibody (Reactant B) was added. The cells were incubated at 37°C for 15 min, rinsed with PBS three times (3 min each time), incubated at 37°C for 15 min after the addition of a drop of horseradish peroxidase-labeled streptavidin fluid (reagent C) onto each section, and then rinsed with PBS three times (3 min each time). The cells were stained with DAB color developing reagent for 3–10 min. Staining time was monitored under the microscope and staining was terminated with running water. The cells were redyed with hematoxylin, dehydrated in graded alcohols, cleared with xylene and mounted with neutral gum. Sections from positive gastric cancer were used as the positive control of hK6, and the negative control was obtained by replacing the primary antibody with PBS.

All sections were independently examined by two pathologists. Ten high power fields of view ($\times 200$) were randomly selected and 100 cells were counted in each field. The average positive cell count was obtained from 10 fields. The immunohistochemical results were graded according to the percentage of the positive cell count, among which negative (–) was < 5%, weakly positive (+) was between 5% and 25%, positive (++) was between 25% and 50%, and strongly positive (+++) was > 50% [4].

Statistical analysis

Data were analyzed by SPSS 12.0 software. For comparisons of the positive rate of hK6 among groups, the χ^2 test was carried out when the total sample was more than 40

while Fisher's exact test was carried out when the total sample was less than 40. $P < 0.05$ was considered significant.

Results

Immunohistochemistry

The immunohistochemical staining of hK6 was generally localized in the cytoplasm and always took a yellowish brown granular appearance, as the arrows show in the figures (Fig. 1, 2 and 3). The expression of hK6 in normal gastric mucosa tissues, gastric ulcer tissues and gastric carcinoma tissues was 20%, 40% and 70.9%, respectively. The expression of hK6 in GC tissues was significantly higher than that in normal gastric mucosa tissues or gastric ulcer tissues ($\chi^2 = 14.436$, $P < 0.01$). No significant difference in the expression of hK6 was found between gastric ulcer tissues and normal gastric mucosa tissues ($P > 0.05$) (Table 1).

Correlations between the expression of hK6 and clinical pathological parameters of gastric carcinoma

The expression of hK6 in GC tissues showed no correlations with sex, age, diameter of the tumor, histodifferentiation degree or the primary site of the tumor ($P > 0.05$). But it is significantly correlated with the invasion depth and clin-

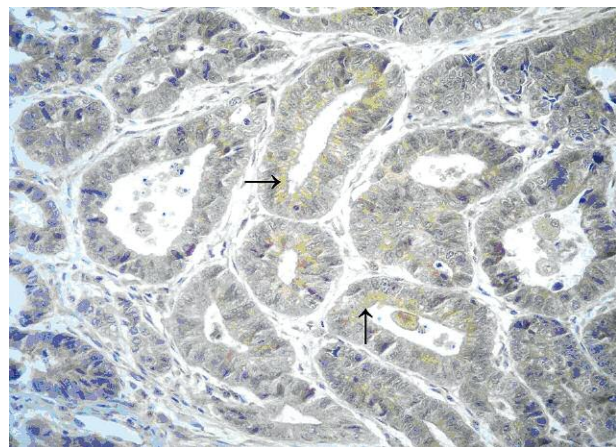


Fig. 1. Positive expression of hK6 in well-differentiated gastric carcinoma tissues by SP method. Magnification 200 \times

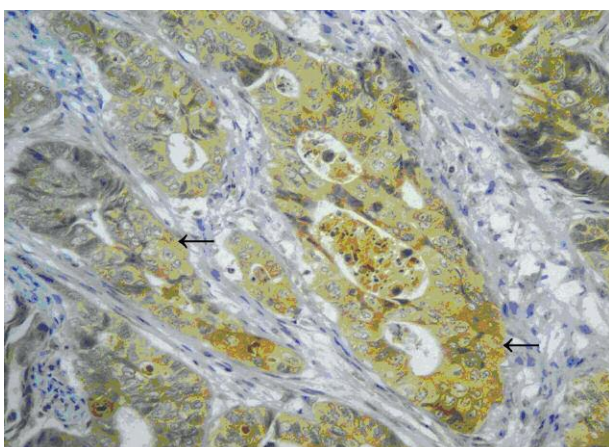


Fig. 2. Strongly positive expression of hK6 in moderately differentiated gastric carcinoma tissues by SP method. Magnification 200 \times

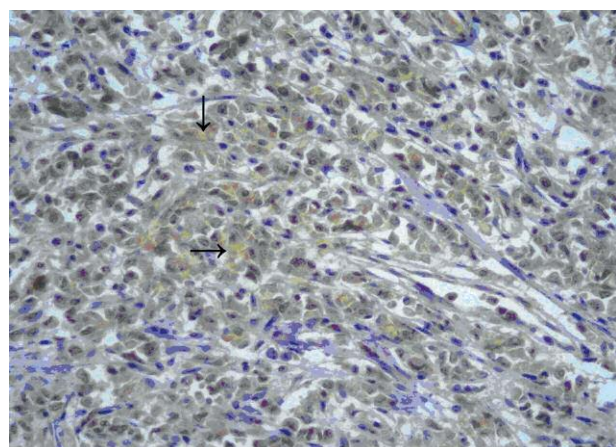


Fig. 3. Strongly positive expression of hK6 in poorly differentiated gastric carcinoma tissues by SP method. Magnification 200 \times

Table 1. Expression of hK6 in normal gastric mucosa tissues, gastric ulcer tissues and gastric carcinoma tissues

Group	Cases	Expression of hK6			Test value	P value
		negative	positive	positive rate (%)		
NGM tissues	15	12	3	20		
GU tissues	15	9	6	40		0.427 > 0.05
GC tissues	55	16	39	70.9	14.436	0.001 < 0.01

Fisher's exact test showed that no significant difference in the expression of hK6 was found between NGM tissues and GU tissues ($P > 0.05$). χ^2 test showed that the expression of hK6 in GC tissues was significantly higher than that in NGM tissues or GC tissues ($P < 0.01$).

Table 2. Correlations between the expression of hK6 and the clinical pathological parameters of 55 gastric carcinoma patients

Clinical pathological parameters	cases	The expression of hK6					χ^2	P value
		-	+	++	+++	positive rate (%)		
Sex								
male	37	9	7	11	10	75.7	1.245	0.264 ^a > 0.05
female	18	7	3	6	2	61.1		
Age (years)								
< 55	31	8	5	11	7	74.2	0.372	0.542 ^a > 0.05
≤ 55	24	8	5	6	5	66.7		
Diameter of tumor (cm)								
≥ 5	25	5	6	8	6	80.0	1.836	0.175 ^a > 0.05
< 5	30	11	4	9	6	63.3		
Histo differentiation								
Moderately well	26	6	5	10	5	76.9	0.865	0.352 ^a > 0.05
poorly	29	10	5	7	7	65.5		
Primary site								
gastric antrum	25	10	4	4	7	60.0	3.090	0.213 ^a > 0.05
gastric body	11	3	2	4	2	72.7		
gastric fundus	19	3	4	9	3	84.2		
Invasion depth								
T1+T2	12	7	1	2	2	41.7	4.679	0.031 ^b < 0.05
T3+T4	43	9	9	15	10	79.1		
TNM staging								
I + II	21	11	2	4	4	47.6	8.933	0.003 ^a < 0.01
III + IV	34	5	8	13	8	85.3		
Lymph node metastasis								
negative	17	9	3	3	2	47.1	5.215	0.022 ^b < 0.05
positive	38	7	7	14	10	81.6		

^auncorrected χ^2 test, and ^bsuccessively corrected χ^2 test.

ical stage of the tumor as well as lymph node metastasis ($P < 0.05$ and $P < 0.01$, respectively). The expression of hK6 in gastric carcinoma tissues at stages T3 + T4 of invasion depth (79.1%) was significantly higher than that at stages T1 + T2 (41.7%) ($P = 0.031$), and the expression of hK6 at clinical stages III + IV (85.3%) was significantly higher than that at stages I + II (47.6%) ($P = 0.003$). In addition, the expression of hK6 in GC tissues with lymph node metastasis (81.6%) was notably higher than that without lymph node metastasis (47.1%) ($P = 0.022$, Table 2).

Discussion

Kallikreins comprise 15 genes located on human chromosome 19q13.4, and encode secreted serine protease. They

show obvious homology in structure [5]. Kallikreins possess the activity of serine proteinase, and they can perform the function of protein degradation, which allows them to play a role in various physiological processes such as inducing hematopoiesis, promoting the formation of growth factors and angiogenesis factors, degrading extracellular matrix, etc. Tumor cells can activate other enzymes via proteins to degrade the extracellular matrix, which is closely correlated with the potentials of tumor cell invasion and metastasis. The degradation of some components in the extracellular matrix damages the intra- and extracellular interaction and changes the cell proliferation cycle, which can lead to the growth and malignant transformation of tumor cells. To date, the roles played by hK2 and hK3 in invasion and metasta-

sis of prostatic carcinoma cells have been elucidated. On one hand, hK2 and hK3 can promote tumor invasion by degrading some components in the extracellular matrix such as collagen IV, laminin, etc. On the other hand, they can activate growth factors (such as TGF- β , etc.) in the microenvironment to regulate tumor cell proliferation and angiogenesis [3].

The results of our study showed that hK6 was overexpressed in gastric carcinoma tissues while its expression in NGM and GU tissues was low, which is consistent with results reported in other studies. The expression of KLK6 mRNA was upregulated in tissues of colon carcinoma, gastric carcinoma, esophageal carcinoma, pancreatic carcinoma, etc. [6], and the expression of KLK6 mRNA in GC tissues was notably higher than that in NGM tissues [7], indicating that KLK6 mRNA might play an important role in the spread of GC.

The correlations between the expression of hK6 and clinical pathological parameters of GC were also explored in this study. The results showed that the overexpression of hK6 was closely correlated with the invasion depth of carcinoma cells, clinical TNM stage of gastric carcinoma and lymph node metastasis, whereas it had nothing to do with sex, age, tumor diameter, the degree of histodifferentiation, or the primary site of the tumor. The protease test *in vitro* showed that hK6 could degrade most components of basement membrane and extracellular matrix (ECM), such as fibrinogen, type-I collagen, type IV collagen, fibronectin, vitronectin, laminin, etc. [8–10]. These results suggest that hK6 may play an important role in the processes of extracellular proteolysis and tumor infiltration. Our results showed that the overexpression of hK6 in GC tissues was closely correlated with the invasion depth, clinical stage and lymph node metastasis, suggesting that hK6 may play a role in the processes of degradation of ECM and tumor metastasis. The increase of the expressions of KLK6 mRNA and hK6 in GC tissues might be closely correlated with cellular cyclical proteolysis and tumor invasion, and the expressions of KLK6 mRNA and hK6 were inhibited and the proliferation and invasiveness of MNK28 cells (a GC cell line with powerful invasiveness) decreased after the cells were transfected with KLK6 siRNA [7], suggesting that KLK6 mRNA and hK6 may play important roles in gastric carcinoma invasion and infiltration.

To sum up, our results showed that hK6 is overexpressed in GC tissues and its overexpression is closely related to the invasion depth of tumor cells, clinical stage of carcinoma and lymph node metastasis. Thus, the expression of hK6 may become a new promising biological marker for GC diagnosis. However, further follow-up study is needed. hK3 has been authorized by the FDA and is widely used for screening, diagnosis and treatment of prostatic carcinoma as a serum tumor marker. In further study, it will be necessary to develop a hK6 ELISA kit to further explore the significance of hK6 in clinical treatment and diagnosis of GC.

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