Clinicopathologic and Biological Significance of Kallikrein 6 Overexpression in Human Gastric Cancer

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Abstract

Purpose: Human kallikrein genes (KLK) have been reported to be involved in human malignancies and several KLKs are promising biomarkers of prostate, ovarian, testicular, and breast cancers. Herein, we investigated the clinicopathologic and biological significance of KLK6 gene expression in human gastric cancer.

Patients and Methods: Using real-time reverse transcription-PCR, we analyzed the KLK6 expression status with respect to various clinicopathologic variables in 66 patients with gastric cancer. In addition, we established a KLK6 stably suppressed gastric cancer cell line (MKN28) using small interfering RNA-mediated gene silencing, and investigated its effects on the cell proliferation rate, cell cycle, and invasiveness.

Results: The KLK6 gene expression in cancerous tissue (0.37 ± 0.53) was significantly (P < 0.000001) higher than that in noncancerous tissue (0.026 ± 0.060). Elevated KLK6 expression was significantly associated with lymphatic invasion (P = 0.03). Furthermore, patients with a high KLK6 expression had a significantly poorer survival rate than those with a low KLK6 expression (P = 0.03). Therefore, we showed that KLK6 gene silencing with KLK6 small interfering RNA effectively suppressed the cell proliferation rate (P = 0.002), cell population in the S phase (P < 0.01), and invasiveness (P < 0.01) in comparison to mock-transfected cells.

Conclusions: The KLK6 gene is markedly overexpressed in gastric cancer tissue and its expression status may be a powerful prognostic indicator for patients with gastric cancer. Our findings also suggest that KLK6 may possibly be a novel target for gastric cancer therapy by gene-silencing procedures.

The kallikrein gene family of secreted serine proteases, consisting of 15 genes, localizes tandemly on chromosome 19q13.4 and shows significant homologies at both the nucleotide and the protein levels (1–5). The human kallikrein gene 6 (KLK6), encoding human kallikrein 6 protein (hK6), has been cloned independently by three groups. Using a differential display PCR technique from breast cancer cell lines, Anisowicz et al. cloned the full-length cDNA of KLK6, named protease M (6). They showed that protease M was strongly expressed in breast cancer cell lines and in ovarian cancer tissues and cell lines. Little et al. cloned the identical cDNA, which they named ZYME, from brain tissue of a patient with Alzheimer’s disease (7). Yamashiro et al. cloned the gene, which they called NEUROSIN, from a cDNA library prepared from a human colorectal cancer cell line (8).

Recent studies have suggested that human KLKs are involved in human carcinogenesis and that several KLKs are promising biomarkers of prostate, ovarian, testicular, and breast cancers (3, 5). For example, the KLK3 gene encodes prostate-specific antigen (hK3), which is a currently available cancer-specific marker, and is widely used for the screening, diagnosis, and management of prostate cancer (9, 10). KLK2 protein (hK2) can be another useful diagnostic marker for prostate cancer (11, 12). Many other KLKs have also been expected to act as tumor biomarkers (13–18). In addition, more recent evidence also implicates KLKs in many cancer-related processes, including cell-growth regulation, angiogenesis, invasion, and metastasis (4, 5). Regarding KLK6, several authors have reported that the KLK6 mRNA was highly expressed in ovarian cancer tissue and that hK6 could be a useful serum biomarker for the diagnosis and monitoring of ovarian cancer (19, 20). However, no information is available on KLK6 expression in human gastric cancer, the second most common cancer in Japan.

In the present study, we therefore examined the clinicopathologic and prognostic significance of KLK6 expression...
in gastric cancers. Furthermore, we investigated the association of such biological behaviors, as cell growth and invasiveness, with \(\text{KLK6}\) gene expression when suppressed by gene silencing with small interfering RNA (\(\text{siRNA}\)) in a gastric cancer cell line.

### Materials and Methods

#### Cell lines and tissue samples

The cell lines derived from human gastric cancer, including AZ521, KATOII, MKN1, MKN7, MKN28, MKN45, MKN74, NUIGC3, NUIGC4, and human fibroblast cell line, KmST6, were obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer (Tohoku University, Sendai, Japan), and maintained in RPMI 1640 containing 10% fetal bovine serum (FBS) and antibiotics at 37°C in a 5% humidified CO\(_2\) atmosphere.

The 66 tumor samples and the matched control samples taken from normal tissue located far from the tumor site of gastric cancers were frozen in liquid nitrogen immediately after a surgical resection, and were kept at −90°C until RNA extraction. The surgical samples were obtained at the Department of Surgical Oncology, Medical Institute of Bioregulation, Kyushu University, Beppu, Japan. None of these patients received preoperative treatment, such as radiation or chemotherapy. Written informed consent was obtained from all patients according to the guidelines approved by the Institutional Research Board.

#### Clinicopathologic data

All data, including sex, histology, serosal invasion, lymph node metastasis, lymphatic invasion, vascular invasion, and clinical stage were obtained from the clinical and pathologic records.

#### RNA preparation and reverse transcription

The total RNA was isolated by the modified acid guanidinium-phenol-chloroform procedure with DNase (21). CDNA was synthesized from 2.5 μg of total RNA as described previously (22).

#### Oligonucleotide primers for KLK6 gene amplification by PCR

The primer sequences for \(\text{KLK6}\) were:

- 5′-CATGCGGCGACTCCGCGCAGAAGC-3′
- 5′-TGGATCACAGCCCGGACAACAGAA-3′

The forward primer was located in exon 2, whereas the reverse primer was located in exon 3. The length of the amplicon was 215 bp. The amplification was done for 28 cycles of 1 minute at 95°C, 1 minute at 57°C, and 1 minute at 72°C. An 8 μl aliquot of each reaction mixture was size-fractionated in a 2% agarose gel and visualized by ethidium bromide staining. To ensure that the RNA was not degraded, a PCR assay with primers specific for the glyceraldehyde-3-phosphate dehydrogenase (\(\text{GAPDH}\)) gene was carried out in each case, except that only 22 cycles were done under the following cycling conditions: 1 minute at 95°C, 1 minute at 56°C, and 1 minute at 72°C. The primer sequences for the \(\text{GAPDH}\) amplification were:

- 5′-GTGTATCAGGGAAGCTCAGCA-3′
- 5′-GTATCATATTTTGGCCAGGT-3′

The length of this amplicon was 249 bp.

#### Real-time quantitative reverse transcription-PCR

The real-time monitoring of the PCR reactions was done using the LightCycler FastStart DNA Master SYBR Green 1 kit (Roche Diagnostics, Tokyo, Japan). The amplification conditions of the 40 cycles consisted of denaturation at 95°C for 10 seconds, annealing at 60°C for 10 seconds, and elongation at 72°C for 10 seconds. The products were then subjected to a temperature gradient from 68°C to 95°C at 0.1°C/s with continuous fluorescence monitoring to produce a melting curve of the products. After proportional background adjustment, the fit point method was used to determine the cycle in which the log-linear signal was distinguished from the background and that cycle number was used as a crossing-point value. The standard curve was produced by measuring the crossing point of each standard value (2-fold serially diluted CDNAs of MKN28) and plotting them against the logarithmic value of the concentration. Concentrations of each sample were then calculated by setting their crossing points to the standard curve. The expression levels were normalized by the \(\text{GAPDH}\) mRNA expression (23). We classified the 66 cases into two groups using the mean expression level of \(\text{KLK6}\) mRNA in tumor tissue (0.37); i.e., a high-expression group (>0.37, \(n = 20\)) and a low-expression group (<0.37, \(n = 46\)).

#### Immunohistochemistry

Immunohistochemical studies of \(\text{hK6}\) were done on surgical specimens from patients with gastric cancer using the avidin-biotin-peroxidase method (LSAB2 kit, Dako, Kyoto, Japan). We scored the expression as negative for \(\text{hK6}\) when <10% of the carcinoma cells were stained in an examined area of a specimen. We examined \(\text{hK6}\) protein expression in tumor and the corresponding normal tissues from 18 representative gastric cancer cases among 66 cases. In 18 selected cases, 6 cases showed a lower expression level of \(\text{KLK6}\) mRNA in gastric cancer tissues than in noncancerous tissues, whereas the remaining 12 cases exhibited a higher \(\text{KLK6}\) mRNA expression in tumor than normal tissues.

#### Small interfering RNA transfection

The expression vector, \(\text{pSilencer} 3.1-\text{H1 hygro}\) (Ambion Inc., Austin, TX) was used for the expression of \(\text{siRNA}\). A hairpin \(\text{siRNA}\) designed to target the \(\text{KLK6}\) gene (5′-GATCCCGGTATGTTGTCGACAGACTGATCCGCCATGACAAACAGAA-3′) was inserted into the \(\text{pSilencer}\) vector according to the manufacturer's instructions, and then it was transfected into the gastric cancer cell line (MKN28) by the LipofectAMINE method (Life Technologies, Inc., Tokyo, Japan) as described previously (24). Two stably transfected clones were selected after hygromycin treatment (800 μg/mL) and used for the subsequent experiments. A mock vector-transfected clone of the cell line was used as a control.

#### Western blot analysis

Total protein was extracted from the samples with a radioimmunoprecipitation assay buffer. Aliquots of total protein were applied to 12% acrylamide gradient gels. After electrophoresis, the samples were electroblotted onto a polyvinylidene membrane (Immobilon; Millipore, Inc., Bedford, MA) at 0.5 Â for 50 minutes at 4°C. The \(\text{hK6}\) protein was detected using mouse monoclonal antibody (Serotec) at dilutions of 1:1,000. The protein levels of \(\text{hK6}\) were normalized to the level of β-actin protein (Cytoskeleton, Inc., Denver, CO) at dilutions of 1:1,000. The blots were developed with horseradish peroxidase–linked anti-mouse immunoglobulin (Promega, Inc., Madison, WI) at dilutions of 1:5,000. Signals were detected using Supersignal (Pierce, Inc., Rockford, IL).

#### In vitro proliferation assay

Cells were plated at a density of 5 × 10\(^4\) cells per well in three 6-cm plates and were harvested and counted on days 3, 7, and 10. The medium was changed every 72 hours. This experiment was done in triplicate.

#### Cell cycle analysis

Cells (2.0 × 10\(^6\)) were preincubated for 48 hours in serum-free medium at 37°C and then were kept in medium with serum (10% FBS) for 18 hours at 37°C. The cells were harvested and fixed in 70% ethanol at −20°C. Next, the cells were washed and resuspended in propidium iodide staining buffer (5 μg/mL propidium iodide and 0.25 mg/mL RNase) in PBS. The DNA content was evaluated using an EPICS XL flow cytometer (Beckman Coulter, Corp., Tokyo, Japan).

Measurement of bromodeoxyuridine uptake was done as described previously (25). Briefly, after \(\text{siRNA}\)-transfected cells and mock-transfected cells (2.0 × 10\(^3\)/plate) were incubated for 48 hours in serum-free medium at 37°C and 18 hours after addition of 10% FBS at 37°C, bromodeoxyuridine was added to the culture medium (10 μmol/L), and the cultures were incubated for 30 minutes at 37°C. The cells were fixed in 70% ethanol at −20°C. To denature the DNA, the cells were incubated for 30 minutes at room temperature in 2 N HCl with 0.5% Triton X-100. After neutralization with 0.1 mol/L sodium tetraborate (pH 8.5), the cells were incubated with anti-bromodeoxyuridine FITC.
In vitro invasion assay. In vitro invasion assays were done by using 24-well transwell units with polycarbonate filters (pore size, 8 μm) coated on the upper side with Matrigel (Becton Dickinson). Cells (5.0 × 10^4 cells/well) were placed in the upper chamber, and the lower chamber was filled with 750 μL of DMEM with 10% FBS as a chemoattractant. After 48 hours of incubation at 37 °C, the membranes were labeled with Calcein, AM solutions. The invasive cells that had migrated through the membrane to the lower surface were read in a fluorescence plate reader at excitation/emission wavelengths of 485/530 nm.

Statistical analysis. The statistical analysis was done using the χ² method, the Mann-Whitney U test, Student’s t test, and repeated measures ANOVA analysis. Survival curves were drawn according to the Kaplan-Meier method (26), and the log-rank test (27) was applied to compare the survival curves. A probability level of 0.05 was chosen for statistical significance.
significantly higher \((P = 0.03)\) in the high-expression group (17 of 20, 85%) than in the low-expression group (27 of 46, 59%). The incidence of serosal invasion was higher \((P = 0.06)\) in the high-expression group (16 of 20, 80%) than in the low-expression group (26 of 46, 57%). The clinical stage also correlated with the groups \((P = 0.03)\). On the other hand, no significant difference was observed regarding sex, histology, lymph node metastasis, and vascular invasion. The 5-year actuarial overall survival rates in patients with high KLK6 mRNA levels and patients with low KLK6 mRNA levels were 26% and 55%, respectively (Fig. 3). The survival difference between these two groups was statistically significant \((P = 0.03; \text{log-rank test})\).

**Biological significance of KLK6 expression in gastric carcinoma:**

The KLK6 small interfering RNA–transfected gastric cancer cell lines stably suppress both KLK6 mRNA and hK6 protein. Although the highest KLK6 expression was found in MKN74 (Fig. 1), this cell line showed an inherently low proliferation rate. Therefore, we used the MKN28 line for subsequent experiments. Among 45 stable KLK6-suppressed clones established using the RNA interference (RNAi) method (with KLK6 mRNA expression <0.3 compared with MKN28 parental cells), we selected two clones (RNAi-1 and RNAi-2) and determined their expression levels of hK6 protein by Western blot analysis. These two stable KLK6-suppressed clones were confirmed to express markedly lower levels (about one fifth) of hK6 protein than the MKN28 parental cells (Fig. 4).

**Lower proliferation activity of KLK6-suppressed cancer cells.** We analyzed whether suppression of KLK6 expression would alter the growth rate of MKN28 gastric cancer cells. As shown in Fig. 5A, there was a significant difference in growth rate between the KLK6-suppressed cells and the mock-transfected cells \((P = 0.002)\). The KLK6-suppressed clones did not reach confluency by 10 days. To investigate whether the KLK6-suppressed cells showed low proliferation activity, we analyzed the cell cycle after serum starvation and after re-feeding with serum (Fig. 5B). The percentage of bromodeoxyuridine-positive cells (cells in S phase) in KLK6-suppressed cells was significantly lower than that in the mock-transfected cells after serum starvation for 48 hours and after addition of serum for 18 hours \((P < 0.01)\). These results suggested that both KLK6 mRNA and hK6 protein expression were closely associated with cell proliferation.

**Low invasive potential of KLK6-suppressed cancer cells.** In a clinicopathologic study, we found that the incidence of lymphatic invasion was significantly higher in the high-expression group than in the low-expression group. To verify these findings in an *in vitro* assay, we examined the invasive potential of the KLK6-suppressed cells using an *in vitro* Matrigel invasion assay (Fig. 6). The KLK6-suppressed cells exhibited significantly less invasive potential than the mock-transfected cells \((P < 0.01)\), suggesting that high expression of KLK6 enhanced tumor invasiveness.

### Table 1. Relationships between KLK6 status and other variables

<table>
<thead>
<tr>
<th>Variables</th>
<th><strong>KLK6</strong></th>
<th><strong>P</strong></th>
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<tbody>
<tr>
<td></td>
<td>Low ((n = 46))</td>
<td>High ((n = 20))</td>
</tr>
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<td>undifferentiated type</td>
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<td>4</td>
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<td>5</td>
</tr>
<tr>
<td>present</td>
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<td>15</td>
</tr>
<tr>
<td>Lymphatic invasion</td>
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<td></td>
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<td>3</td>
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<td>present</td>
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<td>17</td>
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<td>2</td>
</tr>
<tr>
<td>stage IV</td>
<td>5</td>
<td>7</td>
</tr>
</tbody>
</table>

Abbreviation: n.s., not significant.

*Correlation was analyzed by the \(\chi^2\) method.

Fig. 3. Overall survival of patients with gastric cancer according to KLK6 mRNA expression in the cancer tissue. Patients in the high KLK6 mRNA expression group \((n = 20)\) had a significantly poorer prognosis than those in the low KLK6 mRNA expression group \((n = 46)\).

Fig. 4. Real-time reverse transcription-PCR and Western blot analysis of two KLK6-suppressed clones. The KLK6 mRNA expression is about 80% decreased in both treated clones (RNAi-1 and RNAi-2). The protein levels are measured by the NIH image with \(\beta\)-actin protein normalization. We also observed a reduced expression of hK6 protein in both clones.
Fig. 5. Proliferation activity and cell cycling of KLK6-suppressed cells. The RNAi-1 clone in Fig. 4 was used for the following experiments: A, in vitro growth rate of KLK6-suppressed cells in the presence of 10% FBS. The cell number was counted on days 3, 7, and 10 in triplicate; bar, SD; \( P < 0.01 \); repeated measures ANOVA analysis. B, cell cycle of KLK6-suppressed cells after 48 hours of serum starvation followed by 18 hours of re-feeding with 10% FBS. (a) mock cells after 48 hours of serum starvation followed by 18 hours of re-feeding. (b) KLK6-suppressed cells after 48 hours of serum starvation followed by 18 hours of re-feeding. (c) mock and KLK6-suppressed cells after 48 hours of serum starvation followed by 18 hours of re-feeding. (d) mock and KLK6-suppressed cells after 48 hours of serum starvation. Each experiment was done in triplicate.
Invasion potential of KLK6-suppressed cells. The invasive cells migrating through the membrane to the lower surface were counted by a fluorescence plate reader at excitation/emission wavelength of 485/530 nm. bar: SD; *P < 0.01. Student’s t test.

Discussion

In the current study, we clearly showed a sharp contrast between gastric cancer tissue and the corresponding normal counterpart with respect to KLK6 mRNA expression, as illustrated in Fig. 2. To date, several authors have reported that KLK6 mRNA is highly expressed in human cancer tissues (28–30). Among such gastrointestinal malignancies as colorectal cancer, Schuster et al. investigated the gene expression levels of KLK6 by real-time reverse transcription-PCR of colorectal cancer and normal colon mucosa tissues (31). They found the mean KLK6 expression value in normal samples to be approximately two logs lower than that of the cancer samples. Yousef et al. also reported that KLK6 was overexpressed in colorectal cancer tissues compared with normal colon tissues (30). Moreover, our study showed a drastic change in the KLK6 mRNA expression between the gastric cancer tissue and the normal mucosa. However, none of the previous studies investigated the clinicopathologic significance of the KLK6 expression in cancer tissues. Thus, in this study, we examined the clinicopathologic correlation to the KLK6 expression status in gastric cancer. The findings indicate that the overexpression of KLK6 was significantly associated with both an increased incidence of lymphatic invasion and a poor prognosis for patients with gastric cancer. These findings suggest that an enhanced expression of KLK6 might play an important role in various pathologic processes of gastric cancer.

To ascertain the contribution of KLK6 to cell proliferation and invasiveness, we used MKN28 cells, which inherently expressed high levels of both KLK6 mRNA and hK6 protein, and established the relevant suppressed clones by gene silencing using RNAi techniques. RNAi is mediated by siRNAs that are produced from the long dsRNAs of exogenous or endogenous origin by an endonuclease of the RNase-III type, called Dicer (32, 33), and has emerged as a powerful tool for understanding the gene function. With the aid of RNAi techniques, we showed that the KLK6-suppressed clones had markedly reduced cell growth, proliferation, and invasiveness. Regarding other KLKs, such as KLK2 or KLK3, several reports have indicated that hK2 and hK3 might stimulate the growth and survival of tumor cells by degrading insulin-like growth factor binding proteins (IGFBP2, 3, 4, and 5), thereby liberating the mitogenic growth factor insulin-like growth factor I (IGF-I), which binds to its cell-surface receptor (IGF-IR) and induces cell proliferation and prevents apoptosis (34, 35). Furthermore, Magklara et al. reported that hK6 can degrade in vitro fibrinogen and collagen type I, basic constituents of the extracellular matrix, as well as collagen type IV, a major component of the basement membrane (36). Another group has also shown that hK6 can digest laminin and fibronectin in the tumor parenchyma (37). The lysis of certain components of the extracellular matrix disrupts their dynamic interactions with cells and is linked with an altered regulation of cell proliferation that can lead to tumor cell growth and malignant transformation. The results of our studies suggest that the increased hK6 expression may be associated with pericellular proteolysis and tumor invasion. Because our in vitro experiments showed that KLK6 gene silencing successfully reduced tumor cell proliferation and invasiveness, siRNA-mediated gene silencing of KLK6 might conceivably be a suitable candidate in therapy for patients with gastric cancer.

Finally, the current study indicates that KLK6 mRNA was remarkably overexpressed in gastric cancer tissues and high KLK6 expression levels were associated with lymphatic invasion and poor patient prognosis. hK3 has been well-documented to be an excellent tumor marker for prostate cancer. Moreover, hK6 is a promising serum biomarker for ovarian cancer. Therefore, studies are now under way to investigate whether hK6 may also be a useful biomarker for gastric cancer using serum samples from patients at our institute.

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