Clinical Significance of Human Kallikrein Gene 6 Messenger RNA Expression in Colorectal Cancer

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Abstract  Purpose: Human kallikrein gene 6 (KLK6) is a member of the human kallikrein gene family, and recent studies have found that many kallikreins have altered expression patterns in various malignancies. The purpose of the current study was to quantify the expression of KLK6 in malignant and benign colorectal tissues and to statistically analyze whether KLK6 expression levels correlate with clinicopathologic variables and prognosis in patients with colorectal cancer. Experimental Designs: Paired colorectal tissue samples from cancerous and corresponding noncancerous tissues were obtained from 63 patients with colorectal cancer who underwent surgical resection. Quantitative analyses of KLK6 mRNA expression were done using real-time quantitative reverse transcription-PCR. Results: KLK6 mRNA overexpression in cancerous tissues compared with normal counterparts was observed in 57 of 63 (90%) patients. The mean expression level of KLK6 mRNA in cancerous tissues was significantly higher than that in noncancerous tissues (P < 0.0001). Elevated KLK6 expression was significantly correlated with serosal invasion (P < 0.05), liver metastasis (P < 0.05), and advanced Duke’s stage (P < 0.01). Furthermore, patients with high KLK6 expression had a significantly poorer actuarial overall survival than patients with low KLK6 expression (5-year overall survival rates: 54% and 73%, respectively, P < 0.05). Conclusions: The results of this study indicated that KLK6 mRNA expression was significantly higher in cancerous than in noncancerous colorectal tissues, and high expression of KLK6 mRNA correlated with serosal invasion, liver metastasis, advanced Duke’s stage, and a poor prognosis for patients with colorectal cancer.

The kallikrein gene family consists of 15 genes localized in tandem on chromosome 19q13.4, and all of the kallikrein genes encode for secreted serine proteases (1–4). Kallikreins have a similar genomic organization and show significant homology at both the nucleotide and the protein level (1–3, 5). The human kallikrein gene 6 (KLK6), which encodes for human kallikrein 6 protein (hK6), has been cloned independently by three groups. Using a differential display technique from primary and metastatic breast cancer cell lines, Anisowicz et al. (6) first isolated the cDNA of KLK6, which they named protease M. They showed that protease M is strongly expressed at the mRNA level in certain primary breast cancer cell lines and in ovarian cancer tissues and cell lines. Yamashiro et al. (7) cloned this same gene, which they named neurosin, from a cDNA library prepared from a human colorectal cancer cell line (COLO 201). Neurosin was found to be highly expressed in the brain. Finally, Little et al. (8) cloned the same cDNA, which they named zyme, by PCR amplification from the brain tissue of a patient with Alzheimer’s disease. The predominant expression of KLK6 in brain cells suggests the possible involvement of kallikrein 6 in the development and progression of Alzheimer’s disease (6–8).

A growing body of evidence suggests that human kallikrein genes (KLK) are involved in human malignancies and that many KLKs are promising biomarkers of prostate, ovarian, breast, and testicular cancers (1–3). Prostate-specific antigen (hK3) has been used as a tumor marker for the early detection of prostate cancer as a result of studies which have identified elevated serum prostate-specific antigen levels in prostate cancer patients (9). An elevated level of prostate-specific antigens (hK3) is also recognized as a favorable prognostic factor for breast cancer (10). Recent reports have suggested that hK2 could be another useful diagnostic marker for prostate cancer (11). Several authors have reported that KLK6 mRNA is highly expressed in ovarian cancer tissues (6, 12), and recent studies have indicated that hK6 could potentially be a biomarker for the diagnosis and monitoring of ovarian cancer (13, 14). In colorectal cancer, KLK6, KLK8, and KLK10 have

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been found to be up-regulated in cancer tissues compared with normal tissues (15, 16).

Because KLK6 has been found to be highly expressed in colorectal cancer cells as well as in ovarian cancer cells (12, 15, 16), we hypothesize that KLK6 may also have potential applications in the clinical diagnosis and monitoring of colorectal cancer. To the authors’ knowledge, no information has been published concerning the clinical significance of KLK6 mRNA expression in colorectal cancer. In the current study, we quantified the expression of KLK6 in malignant and benign colorectal tissues. We then performed statistical analyses to determine whether KLK6 expression levels correlate with clinicopathologic variables and prognosis in patients with colorectal cancer.

**Materials and Methods**

**Patients and sample collection.** Primary colorectal cancer specimens and adjacent normal colorectal mucosa were obtained from 63 patients who underwent surgery at the Medical Institute of Bioregulation Hospital, Kyushu University. Immediately after resection, the necrotic and ulcerated portions of the tumor were removed, and normal colonic mucosa was dissociated from muscle and connective tissue. All specimens were immediately frozen in liquid nitrogen and kept at −80°C until RNA extraction was done. Written informed consent was obtained from all patients. Whenever possible, specimens were also prepared for immunohistochemical studies. All 63 patients were clearly identified as having colorectal cancer based on the clinicopathologic findings. No patients received chemotherapy or radiotherapy prior to surgery. Fifteen of these 63 patients had synchronous liver metastases. All patients were closely followed after surgery at regular 1-month intervals. The follow-up periods ranged from 2 to 67 months with a mean of 30 months.

**Total RNA extraction.** Frozen tissue specimens were homogenized in guanidinium thiocyanate, and total RNA was obtained by ultracentrifugation through a cesium chloride cushion as described previously (17).

**Real-time quantitative reverse transcription-PCR.** The cDNA was synthesized from 8.0 μg of total RNA as described previously (18). Two gene-specific oligonucleotide primers were designed: (KLK6) sense, 5'-CATTGGCCGACCCCGTCGCAAGAC-3'; antisense, 5'-TGATCACA-CAGCCGGCAACAGA-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense, 5'-TTGGTTATCGTGGAAGGACTCA-3'; antisense, 5'-TTGATATGTCGAAAGGACCTCA-3'; antisense, 5'-TTGATATGTCGAAAGGACCTCA-3'; antisense, 5'-TTGATATGTCGAAAGGACCTCA-3'. These primers spanned more than one intron to avoid amplification of any contaminating DNA.

Real-time monitoring of PCR reactions was done using the LightCycler system (Roche Applied Science, Indianapolis, IN). SYBR green I dye (Roche Diagnostics). Monitoring was done according to the manufacturer's instructions, as described previously (19). In brief, a master mixture was prepared on ice, containing 1 μL of cDNA, 2 μL of LC DNA Master SYBR Green I mix, 50 ng of primers, and 2.4 μL of 25 mmol/L MgCl₂. The final volume was adjusted to 20 μL with water. After being loaded into a glass capillary tube, the reaction mixture was exposed to the following cycling conditions: initial denaturation at 95°C for 10 minutes followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at 62°C (60°C for GAPDH) for 10 seconds, and extension at 72°C for 10 seconds. After amplification, the products were subjected to a temperature gradient from 68°C to 95°C at 0.2°C/second under continuous fluorescence monitoring to produce a melting curve of the products. Only one peak for each sample was observed.

We determined the levels of KLK6 and GAPDH mRNA expression by comparisons with cDNA from Human Universal Reference total RNA (Clontech, Palo Alto, CA). After proportional baseline adjustment, the fit point method was employed to determine the cycle in which the log-linear signal was distinguished from the baseline, and that cycle number (threshold cycle) was used as a crossing-point value. The standard curve was produced by measuring the crossing-point of each standard value (4-fold serially diluted cDNAs of Human Universal Reference total RNA) and plotting them against the logarithmic value of concentrations. The standard curve samples were included in each run. The concentrations of all samples were then calculated by plotting their crossing-points against the standard curve. All calculated concentrations were relative to the concentration of the cDNA of Human Universal Reference total RNA, and the amount of the target molecule was then divided by the amount of the endogenous reference (GAPDH) to obtain normalized KLK6 expression values (20, 21). Each assay was done thrice to verify the results, and the mean mRNA expression was used for subsequent analysis.

**Immunohistochemistry.** Immunohistochemical studies of hK6 were done on surgical specimens from colorectal cancer patients using the avidin-biotin-peroxidase method (LSAB2 kit, Dako, Kyoto, Japan) on formalin-fixed, paraffin-embedded tissues. All sections were counterstained with hematoxylin. The primary mouse monoclonal antibodies against hK6 (MCA2158, Serotec, Ltd., United Kingdom) were used at dilutions of 1:500.

**Results**

With regard to KLK6 mRNA expression in clinical samples, 57 of 63 patients (90%) showed a higher expression level of KLK6 mRNA in cancerous tissues than in noncancerous tissues by real-time quantitative reverse transcription-PCR. The mean expression level of KLK6 mRNA in tumor tissues, 0.309 ± 0.500 (mean ± SD), was significantly higher than 0.025 ± 0.092 in the corresponding normal tissues (P < 0.0001, Fig. 1). The median expression levels of KLK6 mRNA in tumor tissues and normal tissues were 0.120 and 0.002, respectively. Immunohistochemical analysis revealed that hK6 was predominantly expressed in cancer cells (Fig. 2).

In the current study, patients with values less than the median expression level of 0.120 in tumor tissues were assigned to the low expression group (n = 31), whereas those with values ≥0.120 were assigned to the high expression group (n = 32). Table 1 shows the clinicopathologic data and KLK6 mRNA expression in tumor specimens from the 63 colorectal cancer patients. The incidence of serosal invasion was significantly higher (P = 0.020) in the high expression group (27 of 32, 84%) than in the low expression group (18 of 31, 58%), and the incidence of liver metastasis was significantly higher (P = 0.011) in the high expression group (12 of 31, 39%) than in the low expression group (3 of 31, 10%). Moreover, the incidence of advanced stage cancer (according to Duke's classification) was significantly higher (P = 0.003) in the high expression group (20 of 32, 63%) than in the low expression group (9 of 31, 29%).

The 5-year actuarial overall survival rates in patients with high KLK6 mRNA levels and patients with low KLK6 mRNA levels were 54% and 73%, respectively (Fig. 3). The survival difference between these two groups was statistically significant (P = 0.029).
Discussion

The results of the current study indicated that in colorectal cancer patients, KLK6 mRNA is more frequently overexpressed in cancerous tissues than in noncancerous tissues. Several authors have previously reported that KLK6 mRNA is highly expressed in colorectal cancer tissues, whereas KLK6 mRNA expression levels in normal tissues is quite low (15, 16). Schuster et al. (15) investigated mRNA levels of KLK6 by real-time reverse transcription-PCR of colorectal cancer and normal colon mucosa tissues snap-frozen after surgical resection. They found that the mean KLK6 expression level in normal samples was approximately 2-log lower than that of the cancer samples. Yousef et al. (16) also reported that KLK6 was overexpressed in colorectal cancer tissues compared with normal colon tissues. These findings suggest that KLK6 expression may play an important role in colorectal cancer development.

The current study found that high mRNA expression of KLK6 was significantly associated with serosal invasion, liver metastasis, and advanced Duke’s stage. Furthermore, the actuarial overall survival of patients with high expression of KLK6 was significantly poorer than those with lower expression. To the authors’ knowledge, this is the first report concerning the clinical significance of KLK6 expression in colorectal cancer. With regard to ovarian cancer, several authors

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<th>Low expression</th>
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Fig. 1. KLK6 mRNA expression in cancerous and noncancerous colorectal tissues. Horizontal lines indicate the means. Cancer tissues showed significantly higher KLK6 mRNA expression levels compared to noncancerous tissues (P < 0.0001). The P value was calculated by Student’s t test.

Fig. 2. Representative example of human hK6 expression in colorectal cancer. Immunohistochemical staining with anti-hK6 antibody in colorectal cancer cells (right side) and normal mucosa (left side) indicated that hK6 was predominantly expressed in the cancer cells.

Table 1. Clinicopathologic data and hK6 mRNA expression in the tumor specimens of 63 patients with colorectal carcinoma
have reported that hK6 expression was higher in late stage disease and that tumor or serum hK6 expression was an adverse prognostic marker (13, 14).

KLKs are thought to possess serine protease catalytic activity, and the serine proteases comprise a family of protein-degrading enzymes that serve a variety of biological functions, including induction of blood coagulation, activation of growth and angiogenic factors, and degradation of extracellular matrix components (24–26). Expression levels of serine proteases (such as plasminogen activator) and other classes of proteinases have been shown to correlate positively with invasiveness and metastatic potential of many types of tumor cells (25, 27–31). This behavior is linked to the ability of these tumors to degrade extracellular matrix components, either directly, or indirectly through the proteolytic activation of other enzymes. Magklara et al. (32) 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 part of the basement membrane. Others have shown that hK6 can also digest laminin and fibronectin (33). These findings raise the possibility that KLK6 may be an important factor in pericellular proteolysis and tumor invasion. Lysis of certain components of the extracellular matrix disrupts their dynamic interactions with cells and is linked with altered regulation of cell proliferation that can lead to tumor cell growth and malignant transformation. Such a role for KLK6 is likely in colorectal cancer as well as in ovarian cancer, where elevated KLK6 levels are associated with unfavorable prognosis (14). If serine proteases are involved in cancer progression, they may be suitable candidates for therapeutic targets.

Although KLK6 seems to be a useful biomarker in colorectal cancer, the underlying biological mechanism of possible kallikrein involvement in the progression of colorectal cancer is currently unknown (16). It has been reported that in cancer cell lines, many KLKs, including KLK6, are under steroid hormone regulation (1–3, 34). Certain KLK genes are predominantly up-regulated by androgens and androgenic progestines (e.g., KLK2, KLK3, KLK4, KLK13, and KLK15), whereas others are primarily responsive to estrogens (e.g., KLK5, KLK6, KLK7, KLK9, KLK10, and KLK11; ref. 1). Recent studies also suggest that all of the KLKs may be able to activate each other as well as other molecules (e.g., growth factors and cytokines) in a cascade of events associated with tumorigenesis (1–3). Experimental evidence has shown that hK15 can activate hK3 (prostate-specific antigen), and hK4 has also been shown to activate hK3 (35, 36). In ovarian cancer, at least 10 of the 15 KLKs have prognostic value, and the vast majority of KLKs are coexpressed and presumably coordinately regulated (37). Because KLK8 and KLK10 as well as KLK6 have been found to be overexpressed in colorectal cancer tissues (16), several KLKs may be also be able to activate each other in colorectal cancer. Therefore, it may be worthwhile to clarify the various mechanisms of KLK activation in colorectal cancer.

In conclusion, our results indicated that KLK6 mRNA was overexpressed in colorectal cancer tissues and high KLK6 expression levels were correlated with serum invasion, liver metastasis, advanced Duke’s stage, and poor prognosis. These findings suggest a possible role for KLK6 expression level as a new diagnostic and prognostic biomarker for colorectal cancer. Furthermore, understanding the biological function of KLK6 expression in colorectal tissue may help to delineate its role in colorectal physiology of colorectal cancer.

References

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