ABSTRACT

**Purpose:** The purpose of this study was to examine expression levels of the human tissue kallikrein 11 gene (KLK11) in epithelial ovarian tumors and to identify the relationship between KLK11 expression and patient survival.

**Experimental Design:** KLK11 mRNA expression was examined by semiquantitative PCR in 64 epithelial ovarian tumors (7 adenomas, 6 low malignant potential tumors, and 51 adenocarcinomas) and in 10 normal ovaries. Semiquantitative PCR results were correlated with clinicopathologic variables and overall survival. cDNA from human normal tissues and tumor tissues was also analyzed.

**Results:** KLK11 mRNA expression was detected in various human cancer tissues including breast, lung, colon, prostate, pancreas, and ovarian carcinoma. The mean value of relative KLK11 expression ratio was significantly higher in ovarian tumor samples than in normal ovary samples (compared with normal samples: adenoma, $P = 0.0006$; low malignant potential tumor, $P = 0.0049$; and carcinoma, $P < 0.0001$). No statistically significant associations between KLK11 mRNA expression level and clinical stage, histological type, or histological grade were observed. The log-rank test showed that high KLK11 mRNA expression and advanced clinical stage significantly correlated with poor patient survival ($P = 0.0185$ and $P = 0.0043$, respectively).

High KLK11 mRNA expression and clinical stage remained significantly associated with overall survival ($P = 0.0225$ and $P = 0.0202$, respectively) after multivariate analysis.

**Conclusions:** KLK11 expression may play an important role in ovarian cancer development and act as an independent prognostic marker in ovarian cancer patients.

**INTRODUCTION**

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 (1-4).

The human kallikrein gene family, a subfamily of serine proteases, is located at the chromosomal locus 19q13.3-q13.4. Until recently, this family was thought to include only three genes, the pancreatic renal kallikrein gene (KLK1), the human glandular kallikrein gene (KLK2), and KLK3, which encodes prostate-specific antigen (hK3). However, in the past few years several groups have reported the cloning of new serine proteases that colocalized with and were homologous to the three known kallikreins, such that 15 members of human kallikrein gene family are now recognized (5, 6). KLKs share many common structural features, and a universal nomenclature for KLK structure has been established (7). There is now growing evidence that KLKs are involved in human malignancies. Prostate-specific antigen (hK3) has been used successfully as a tumor marker for the early detection of prostate cancer due to its abnormal prevalence in the peripheral blood of affected patients (8). Prostate-specific antigen (hK3) is also recognized as a favorable prognostic factor for breast cancer (9, 10). Recent reports have suggested that hK2 could be another useful diagnostic marker for prostate cancer (11, 12). We reported previously that protease M (KLK6) is highly overexpressed in ovarian tumors (13) and that the stratum corneum chymotryptic enzyme (KLK7) is expressed at abnormally high levels in ovarian cancer (14). We also reported the cloning of tumor-associated differentially expressed gene-14 (TADG-14; KLK8), a novel serine protease overexpressed in ovarian cancer (15). In addition, hK6, hK10, and hK11 have been shown to be potential serum biomarkers in ovarian cancer (16-19).

The KLK11 gene was originally cloned from human hippocampus cDNA, using PCR with primers targeted to conserved serine protease regions, and was originally named trypsin-like serine protease (20). With the newly established kallikrein gene nomenclature, this gene was renamed as KLK11 (7). KLK11 mRNA expression has been demonstrated in many tissues, including brain, skin, salivary gland, stomach, prostate, and intestine (21). Diamandis et al. (19) have developed an immunofluorometric procedure for measuring hK11 in biological fluids and tissue extracts. They reported that elevated serum levels of hK11 were found in 70% of women with ovarian cancer and in...
60% of men with prostate cancer, which suggested that hK11 may be of potential value as an ovarian and prostate cancer biomarker (19). In addition, a recently published article by Borgono et al. (22) reported that higher hK11 concentration in ovarian tumor cytosolic extract is a marker of favorable prognosis in patients with ovarian cancer.

The aim of the present study was to determine the potential role of KLK11 mRNA expression in ovarian cancer development and/or progression. KLK11 mRNA expression in various normal and cancer tissues was investigated by the application of semiquantitative PCR to samples of epithelial ovarian tumors and normal ovaries. The results were analyzed in terms of tumor type, clinical stage, histological grade, and histological type. The prognostic significance of KLK11 mRNA expression level in ovarian cancer patients was also analyzed.

MATERIALS AND METHODS

Tissue Samples and Clinical and Pathological Data. For semiquantitative PCR analysis, fresh surgical epithelial ovarian tumor tissue samples were collected from 64 patients treated surgically between 1993 and 2001. Patients were diagnosed histologically and received follow-up care in the Department of Obstetrics and Gynecology at Hiroshima University Hospital. In addition, 10 normal ovaries were obtained from patients before undergoing surgery for benign gynecological disease. Informed consent was obtained from each subject according to institutional guidelines. Specimens were obtained immediately after surgery and were cut in half, with one half processed for histological examination to determine the percent-age of tumor cells, which was never <80%, whereas the other half was used for mRNA preparation. Tissues were frozen in liquid nitrogen and stored at −80°C before mRNA isolation.

The histological subtype of each ovarian tumor sample was diagnosed according to accepted criteria (23). The primary pathological diagnoses were adenocarcinoma for 51 patients (26 serous, 10 mucinous, 8 endometroid, and 7 clear cell), low malignant potential tumor for 6 patients (3 serous and 3 mucinous), and adenoma for 7 patients (2 serous and 5 mucinous). All of the adenocarcinoma patients underwent complete surgical staging to exclude the presence of occult metastatic disease. Clinical staging was determined in accordance with the criteria of the International Federation of Gynecology and Obstetrics staging system (24). Initial disease diagnoses were stage I for 21 patients, stage II for 4 patients, stage III for 23 patients, and stage IV for 3 patients. Although most patients with stage Ic, stage II, stage III, or stage IV ovarian cancer had received cisplatin-containing chemotherapy after surgery, none of the patients had received chemotherapy before surgery. The mean follow-up time was 45.0 months (range, 1.7–112.7 months), with 80% of the patients followed for >2 years.

Normal human tissue cDNA from heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, and peripheral blood leukocytes, and human tumor tissue cDNA from breast carcinoma, lung carcinoma, colon carcinoma, prostate carcinoma, pancreas carcinoma, and ovarian carcinoma (Multiple Tissue cDNA Panels; K1420–1, K1421–1, and K-1422–1; Clontech, Palo Alto, CA) were included in the semiquantitative PCR analysis.

Semiquantitative PCR. Extraction of mRNA from ovarian tissue specimens and cDNA synthesis were carried out according to methods described previously (25–28). mRNA was isolated using a RiboSep mRNA isolation kit (Becton Dickinson Labware, Bedford, MA) and the amount of mRNA recovered was measured using a UV spectrophotometer. cDNA was synthesized from 2.0 μg mRNA by random hexamer priming using the Advantage RT-for-PCR kit (Clontech).

KLK11 mRNA expression levels were determined by semiquantitative PCR, which was performed according to a method described previously with some modifications (25–28). The oligonucleotide primer sequences used for PCR were KLK11 sense primer, 5'-CCG CTA CAT AGT TCA CCT GG-3'; and KLK11 antisense primer, 5'-AGG TGT GAG GCA GGC GTA ACT-3'. β-Tubulin cDNA was coamplified with target sequence and used as an internal PCR control. The β-tubulin sense primer was 5’-TGC ATT GAC AAC GAG GC-3' and the antisense primer was 5’-CTG TCT TGA CAT TGT TG-3'. The predicted sizes of the amplified genes were 284 bp for KLK11 and 454 bp for β-tubulin. Thirty cycles of PCR were carried out in a Thermal Cycler (Perkin-Elmer, Foster City, CA). Each PCR cycle involved 30-s denaturation at 94°C, 60-s primer annealing at 60°C, and 60-s extension at 72°C. We confirmed that amplification under these conditions resulted in the linear production of each product. Tubes containing all of the ingredients except templates were included in all of the runs as a negative control. PCR products were separated on a 1% agarose gel, and the density of each PCR product determined using a Printgraph-Densitograph system (ATTO Corp., Tokyo, Japan). The expression ratio of KLK11:β-tubulin was measured by densitometry to evaluate gene expression and the results expressed as the mean ± SD. Differences in the mean value of the KLK11 expression ratio between groups were assessed with respect to tumor type, clinical stage, histological grade, and histological type. In addition, in ovarian cancer cases, cases were classified into the following two groups according to the KLK11 expression ratio, low-expression cases, <median value; and high-expression cases, >median value. A Kaplan-Meier survival curve of ovarian cancer patients was categorized according to the high or low KLK11 expression.

Statistical Analysis. For statistical analysis, an unpaired t test was used to assess the differences in mean values of KLK11: β-tubulin mRNA expression ratios between groups. Statistically significant differences in overall survival rates were determined using the log-rank test. Overall survival time was defined as the period between the time of initial surgery and time of death. Survival times of patients who were still alive were noted along with the date of the last follow-up appointment. For multivariate analyses, we used the Cox proportional hazards model. All of the P values quoted are two-sided. Significance was defined as P < 0.05. The Statview 5 program (Abacus Concepts, Inc., Berkeley, CA) was used to perform all of the statistical analyses.

RESULTS

KLK11 mRNA Expression in Normal Adult Human Tissues and Human Tumor Tissues. Fig. 1A shows the KLK11 mRNA expression pattern in normal adult tissues. KLK11 mRNA expression was detected in normal adult heart, brain, lung, liver, skeletal muscle, kidney, pancreas, spleen,
KLK11 mRNA expression was detected in normal adult heart, brain, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, and colon. Other normal tissues, including normal placenta, ovary, small intestine, and peripheral blood leukocytes did not express KLK11 mRNA expression in all types of human cancer examined, including breast, lung, colon, prostate, pancreas, and ovarian carcinoma.

**KLK11 mRNA Expression in Normal Ovaries and Ovarian Tumors.** To evaluate KLK11 expression in ovarian tumors and normal ovaries, we performed semiquantitative PCR. Typical semiquantitative PCR results are shown in Fig. 2A, and ratios of KLK11 mRNA expression relative to β-tubulin in ovarian tumors and normal ovaries are shown in Fig. 2B. Means and SD of KLK11 mRNA expression ratios determined for various tumor subtypes are shown in Table 1. The KLK11:β-tubulin expression ratio (mean ± SD) was 0.25 ± 0.24 for normal ovaries, 1.25 ± 0.67 for ovarian adenomas, 1.92 ± 1.58 for ovarian low malignant potential tumors, and 1.96 ± 1.07 for ovarian adenocarcinomas. Thus, mean relative KLK11 expression ratios in adenomas, in low malignant potential tumors, and in adenocarcinomas were significantly higher than that in normal ovaries (compared with normal ovaries: adenoma, P = 0.0006; low malignant potential tumor, P = 0.0049; and adenocarcinoma, P < 0.0001). Mean and SD of KLK11 expression ratios determined for the various ovarian adenocarcinoma subtypes are also shown in Table 1. There were no statistical significant differences in KLK11 mRNA expression level with respect to clinical stage, histological type, and histological grade.

**Survival Analysis of KLK11 Expression in Ovarian Carcinoma Patients.** Univariate analysis with respect to clinical stage (stage I versus stage II/III/IV), histological grade (grade 1 versus grade 2/3), and KLK11 mRNA expression status for overall survival of ovarian cancer patients is summarized in Table 2. The log-rank test revealed that advanced clinical stage was significantly correlated with poor patient survival (P = 0.0043), whereas histological grade was not correlated with patient survival (P = 0.2791). When ovarian cancer patients were categorized along a Kaplan-Meier survival curve according to low versus high expression of KLK11, a statistically significant association between high KLK11 mRNA expression levels and poor patient survival was observed (P = 0.0185; Table 2; Fig. 3). Multivariate analysis was performed using representative parameters as listed in Table 2, with clinical stage (stage I versus stage II/III/IV) and KLK11 expression status (low versus high) selected as covariables (Table 3). Both clinical stage and KLK11 expression were identified as significant and independent variables (clinical stage, P = 0.0202; KLK11 expression, P = 0.0225). High KLK11 mRNA expression yielded a hazard ratio of 3.907, with a 95% confidence interval ranging from 1.212 to 12.594.

**DISCUSSION**

A previous study has already documented the expression of hK11 in the serum of patients with prostate or ovarian primary cancers (19). The present study showed that KLK11 mRNA was expressed by a wide variety of human cancer tissues, including breast, lung, colon, prostate, pancreas, and ovarian carcinoma. It should be stressed that our study found that KLK11 mRNA...
expression levels in normal ovaries were quite low, and that KLK11 mRNA expression was increased significantly in ovarian tumor tissues. This suggested that KLK11 expression may play an important role in epithelial ovarian tumor development. The most striking association we observed in our ovarian cancer patient group was a significant correlation between increased KLK11 mRNA expression level and poor prognosis, according to both univariate and multivariate analysis. The present report shows that KLK11 is associated with an aggressive clinical behavior of ovarian carcinoma and that KLK11 is an independent prognostic marker for ovarian cancer patients. Previous studies have reported an association between kallikrein gene mRNA expression and cancer prognosis. KLK4 and KLK5 have been shown to be associated with poor prognosis in ovarian cancer (29–31), and KLK5 has also been associated with poor prognosis in breast cancer (32). In contrast, KLK8 and KLK9 expression have been reported to be markers for favorable prognosis in ovarian cancer (33, 34). In addition, KLK13 is reported to be an independent and favorable prognostic marker for breast carcinoma (35). This study supports the increasing body of literature demonstrating the expression of KLK family gene involvement in the prognosis of human cancers (6).

An article reporting the association between hK11 concentration of ovarian cancer cytosolic extracts and clinicopathological characteristics in patients with ovarian cancer has been published recently (22). Interestingly, this report shows a correlation between higher hK11 concentration and favorable outcome in patients with ovarian cancer. It is difficult to explain this discrepancy between the present data showing unfavorable outcome with KLK11 mRNA elevation and the published data showing favorable outcome with elevated hK11 protein levels. Clearly, it is recommended to additionally evaluate the differences between mRNA expression levels and protein expression status in the same series of the ovarian cancer samples.

All of the KLKs are thought to possess serine protease catalytic activity and may be able to activate each other as well as other molecules, such as growth factors and cytokines, in a cascade of events associated with tumorigenesis (5). This cascade seems to be highly complicated, and it will be worthwhile to clarify the various mechanisms that underlie cancer invasion and metastasis. It has been reported that in cancer cell lines many KLKs are under steroid hormone regulation, such that KLK 5–12 are up-regulated mainly by estrogens, whereas KLK 1–4 and KLK 13–15 are mainly up-regulated by androgens (5). Thus, KLK family genes are thought to be associated with endocrine-related malignancies (6). Indeed, prostate-specific antigen (hK3) and human glandular kallikrein (hK2) are widely used tumor markers for prostate cancer diagnosis (8, 11, 12).

| Table 1 KLK11 mRNA expression in epithelial ovarian tumors and normal ovaries |
|---------------------------------|-----------------|-----------------|
|                                 | KLK11 mRNA expression |
|                                 | N    | Mean | SD  |
| Normal ovary                    | 10   | 0.25<sup>a,b,c</sup> | 0.24 |
| Ovarian adenoma                 | 7    | 1.25<sup>b</sup>     | 0.67 |
| Ovarian LMP<sup>+</sup> tumor    | 6    | 1.92<sup>a</sup>     | 1.58 |
| Ovarian adenocarcinoma          | 51   | 1.96<sup>c</sup>     | 1.07 |
| Clinical stage                  |      |      |     |
| Stage I                         | 21   | 1.75 | 1.04 |
| Stage II/III/IV                 | 30   | 2.10 | 1.08 |
| Histological type               |      |      |     |
| Serous                          | 26   | 2.13 | 0.89 |
| Mucinous                        | 10   | 1.88 | 1.15 |
| Endometrioid                    | 8    | 2.01 | 1.46 |
| Clear cell                      | 7    | 1.38 | 1.10 |
| Histological grade              |      |      |     |
| Grade 1                         | 26   | 1.84 | 0.85 |
| Grade 2/3                       | 25   | 2.08 | 1.27 |

<sup>a</sup> Significant; normal ovary versus adenoma: P = 0.0006 (unpaired t test).
<sup>b</sup> Significant; normal ovary versus LMP tumor: P = 0.0049 (unpaired t test).
<sup>c</sup> Significant; normal ovary versus adenocarcinoma: P < 0.0001 (unpaired t test).
<sup>d</sup> LMP, low malignant potential.

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<th>Table 2 Univariate analysis of survival with respect to clinical stage, histological grade, and KLK11 mRNA expression status in ovarian cancer patients</th>
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<td>Factors</td>
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<tr>
<td>Clinical stage (stage I vs. stage II/III/IV)</td>
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<td>Histological grade (grade I vs. grade 2/3)</td>
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<td>KLK11 mRNA expression (low vs. high)</td>
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<sup>α</sup> Log-rank test.

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<th>Table 3 Multivariate analysis of survival with respect to clinical stage and KLK11 mRNA expression status in ovarian cancer patients</th>
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<sup>b</sup> RR, risk ratio; CI, confidence interval.
More recently, hK6 and hK10 have emerged as potential new serum biomarkers for ovarian cancer (16–18). In addition, Diamandis et al. (19) reported that hK11 may be a biomarker for prostate and ovarian carcinoma. Therefore, analysis of hK11 in serum may aid in the diagnosis and monitoring of ovarian carcinoma.

Our present study showed that increased mRNA expression of the KLK11 gene may indicate ovarian adenocarcinomas with aggressive phenotypes. Thus, KLK11 mRNA expression analysis may aid in the prognosis of ovarian cancer. Although the molecular mechanisms underlying increased KLK11 expression levels in many cancer cells have not yet been elucidated, KLK11 may represent a novel molecular target for ovarian cancer prevention or treatment. The results presented here would suggest that the use of KLK11 inhibitors and/or KLK11 regulatory sequences as therapeutic agents for the treatment and prevention of ovarian cancers may be useful.

REFERENCES

Human Kallikrein Gene 11 (KLK11) mRNA Overexpression Is Associated with Poor Prognosis in Patients with Epithelial Ovarian Cancer

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