KAI1 Metastasis Suppressor Protein Is Down-Regulated during the Progression of Human Endometrial Cancer

Fu-Shing Liu, J-T. Dong, Jung-Ta Chen, Yeun-Ting Hsieh, Esther Shih-Chu Ho, Man-Jung Hung, Chien-Hsing Lu, and Li-Ching Chiou

Division of Gynecologic Oncology, Departments of Obstetrics and Gynecology [F-S. L., Y-T. H., E. S-C. H., M-J. H., C-H. L., L-C. C.] and Pathology [J-T. C.], Taichung Veterans General Hospital, Taichung, Taiwan 40705, Republic of China; Departments of Obstetrics and Gynecology [F-S. L., E. S-C. H.] and Pathology [J-T. C.], Chung Shan Medical University, Taichung, Taiwan 40201, Republic of China; and Winship Cancer Institute, Emory University School of Medicine, Atlanta, Georgia 30322 [J-T. D.]

ABSTRACT

Purpose: KAI1 is a metastasis suppressor gene located on human chromosome 11p11.2. It is a member of the structurally distinct family of cell surface glycoproteins, transmembrane 4 protein superfamily. KAI1 was initially isolated as a gene that suppressed metastasis of rat prostate tumor cells. Decreased KAI1 expression has been observed recently in various human cancers, including pancreatic, lung, hepatic, colorectal, breast, ovarian, esophageal, and cervical cancers. Frequent down-regulation of the KAI1 protein was also observed in endometrial cancer cell lines. The aim of this study was to determine whether this gene is altered in human endometrial carcinoma. In addition, its prognostic significance in this tumor was also evaluated.

Experimental Design: Tumor specimens from 18 cases with various degrees of endometrial hyperplasia, 97 primary endometrial carcinomas with various stages, and 28 metastatic lesions of this cancer were examined in this study. Using the method of immunohistochemistry, we characterized the KAI1 protein expression in the 143 endometrial tumors. Expression of KAI1 at RNA level was also examined in 35 of the 143 samples using a real-time quantitative PCR method. The data from immunohistochemical analysis were correlated with various clinicopathological factors.

Results: High levels of KAI1 protein expression were detected in almost all of the specimens with endometrial hyperplasia (17 of 18). In contrast, loss of KAI1 expression occurred in an increasing frequency (27.8–71.4%) from early stages of primary endometrial carcinomas to metastatic tumors ($P < 0.001$). In addition, more poorly differentiated tumors demonstrated significantly lower KAI1 expression as compared with the well-differentiated tumors ($P < 0.001$). It was also found that patients with KAI1-negative tumors had a lower survival rate than those with KAI1-decreased or positive tumors ($P = 0.0042$ and 0.0286, respectively). However, in multivariate analysis, the prognostic significance of KAI1 expression was inferior to tumor stage.

Conclusion: These data suggest that KAI1 expression is down-regulated in advanced endometrial cancer. Clinically it may be a useful indicator of the tumor progression and may provide prognostic information on the outcome of this disease.

INTRODUCTION

Endometrial carcinoma is the most common malignancy of the female genital tract in the United States and Western nations. Two types of this tumor were recognized recently according to the clinicopathologic observations (1). Type I (endometrioid) tumors are low-grade and estrogen-related, usually having a good prognosis. In contrast, type II (nonendometrioid) tumors are mainly aggressive, unrelated to estrogen stimulation, and with grave outcome.

Genetic and molecular events underlying the development of endometrial cancer have also been studied extensively. At present, a number of oncogenes and tumor suppressor genes have been implicated in this cancer. These genes include $k$-RAS (2–5), $p53$ (6–8), $\beta$-catenin (9–11), and PTEN (12–16). It has also been shown that molecular alterations involved in the two types of endometrial cancer are different. For example, type I tumors often exhibit microsatellite instability and mutations of the PTEN, $k$-RAS, and $\beta$-catenin genes, whereas type II tumors demonstrate more frequent alterations of $p53$ and loss of heterozygosity on several chromosomes (17, 18). Once a tumor has formed, additional molecular abnormalities could occur in different neoplastic subclones; these new alterations are responsible for tumor heterogeneity, invasion, and metastasis (18). However, the genetic and molecular factors contributing to metastatic progression of endometrial cancer are still poorly understood.

Recently a metastasis suppressor gene, KAI1, was identified from the p11.2 region of human chromosome 11 in prostate cancer (19). KAI1 was able to suppress metastasis when introduced into metastatic rat prostate cancer cells, and its expression was reduced in cell lines derived from metastatic human prostate cancer. In addition, KAI1 protein expression was down-regulated during the progression of human prostate cancer (20). A similar role of the KAI1 gene has also been suggested for...
cancers of the lung and pancreas, as down-regulation of KAI1 at RNA level is correlated with poor survival in patients with lung cancer (21, 22), and with lymph node and distant metastases in patients with advanced stages of pancreatic cancer (23, 24).

Additional studies have shown that other common types of human malignancies also reveal decreased expression of KAI1, including bladder, breast, hepatocellular, colorectal, ovarian, esophageal, and cervical cancers (25–32). These observations suggest that the presence of KAI1 might influence the ability of cancer cells to metastasize.

In endometrial cancer, down-regulation of the KAI1 protein was observed in 7 of 8 established cell lines using Western blot analysis, suggesting a possible role for KAI1 in the malignant progression of this tumor (33). However, no comprehensive analysis of KAI1 expression has been reported in human endometrial cancer.

In the present study, we examined protein and RNA expression of KAI1 in various endometrial tumors by using the methods of immunohistochemical staining and molecular analysis. We also correlated KAI1 expression with clinical and histopathological parameters of these tumors.

**MATERIALS AND METHODS**

**Patients and Tumor Specimens.** Ninety-seven patients with endometrial carcinoma were included in this study. Each patient underwent hysterectomy and pelvic lymph node dissection at Taichung Veterans General Hospital between July 1995 and October 2001. These comprised stage I: 65 cases, stage II: 7 cases, stage III: 20 cases, and stage IV: 5 cases. The stage was assigned based on surgical-pathological findings according to the International Federation of Gynecology and Obstetrics surgical staging system for endometrial carcinoma. In addition, 18 samples of endometrial hyperplasia at various degrees and 28 metastatic lesions from 26 patients were also included in this study. The clinical records and histopathological diagnoses were fully reviewed, and representative paraffin blocks were selected for immunohistochemical study.

The median postoperative follow-up for all of the patients was 32 months, with a range of 1–75 months.

**Immunohistochemical Analysis.** The procedure of immunohistochemistry was described previously (30). Briefly, each 4-μm paraffin section was dewaxed, rehydrated, and treated with 3% H2O2. Sections were then microwaved in citrate buffer (pH 6.0) three times for 5 min each time. After being rinsed in PBS (pH 7.6) and preincubated in serum blocking solution (10% goat serum), slides were reacted with the C-16 anti-KAI1 rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at a dilution of 1:50. Slides were then rinsed in three changes of PBS and incubated with the biotinylated secondary antibody (Histostain-Plus kit; Zymed, South San Francisco, CA). After an incubation with peroxidase-labeled streptavidin (Zymed LAB-SA), slides were rinsed again and were developed with the enzyme substrate diaminobenzidine. In each specimen, lymphocytes, macrophages, and endothelial cells in tumor stroma were used as internal positive controls. Normal mouse IgG was substituted for the C-16 KAI1 antibody to serve as a negative control.

Staining intensity on cell membrane was estimated as positive when it appeared to be similar to that of stromal lymphocytes, macrophages, and endothelial cells. The staining pattern of KAI1 expression was classified as positive when >50% of tumor cells within the tumor tissue were positively stained, decreased if 5–50% of tumor cells were positively stained, and negative if <5% of tumor cells were positively stained (30, 32).

**RNA Extraction and cDNA Synthesis.** Thirty-five fresh-frozen endometrial specimens stored in RNAlater (Ambion, Austin, TX) at −20°C were obtained from our tumor bank and were used for KAI1 mRNA expression study. These specimens included 4 cases of hyperplasia, 29 primary invasive carcinomas, and 2 metastatic lesions. Each of these tissue specimens was microscopically confirmed to contain at least 80% of tumor cells. Total cellular RNA was isolated from the specimens using the SV Total RNA Isolation System (Promega, Madison, WI) according to the manufacturer’s protocol. First-strand cDNA was synthesized from 1 μg of total RNA according to the procedure described previously (32). The SW480 colon cancer cell line, which expresses high levels of KAI1, was used as the positive control, whereas the PC-3 prostate cancer cell line was used as the negative control.

**Real-Time Quantitative PCR.** The details of real-time PCR analysis and the determination of relative amounts of indicated transcripts were described previously (34). Primers and probes used in the PCR were chosen with the assistance of the computer program Primer Expression 1.0 (Perkin-Elmer Applied Biosystems, Foster City, CA). Nucleotide sequences of the primers and probe, and the procedure of the PCR amplification were the same as in our previous study (32). A housekeeping gene, 18S rRNA (Perkin-Elmer Applied Biosystems), was used as the internal control. All of the reactions were performed in the ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems), which detects the signal from the fluorogenic probe during PCR. A comparative Ct3 method was used to quantitate KAI1 expression levels (35).

**Statistical Analysis.** Correlations of KAI1 expression with disease progression and differentiation status were examined by χ2 analysis. Data on survival were censored if the patient was still alive at the time of the last follow-up. Survival curves and the median follow-up time among survivors were estimated according to the Kaplan-Meier method. Breslow statistic test was performed to determine whether patients with negative KAI1 expression demonstrated a different survival profile than patients with decreased or positive KAI1 expression. The difference was considered significant when the P was <0.05. To compare the prognostic value among KAI1 expression, cancer cell differentiation, and tumor stage, multivariate analysis was performed with the stepwise Cox regression model using SPSS win 9.0 statistical software.

**RESULTS**

**Immunohistochemical Labeling.** In total, 143 endometrial tumor specimens were examined for KAI1 expression immunohistochemically. Each of them was scored as KAI1-

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3 The abbreviations used are: Ct, threshold cycle.
positive, -decreased, or -negative according to the aforementioned criteria. There were 22 tumors that had two or more types of cells within one specimen. For analysis, the dominant malignant cell type in these tumors was used to represent a tumor for grading and KAI1 expression scoring. Examples of the staining are shown in Fig. 1.

**Correlation between KAI1 Expression and Tumor Progression.** To determine whether KAI1 protein expression was decreased during the progression of endometrial cancer, the tumor samples were classified into four groups: group 1, endometrial hyperplasias ($n = 18$); group 2, stages I and II primary endometrial carcinomas ($n = 72$); group 3, stages III and IV primary endometrial carcinomas ($n = 25$); and group 4, metastatic lesions ($n = 28$). The KAI1 expression for each group is shown in Table 1. Decreased KAI1 protein expression was found to be associated strongly with the progression of endometrial cancers from hyperplasia to metastasis ($P < 0.001$).

**Correlation between KAI1 Expression and Cancer Cell Differentiation.** To evaluate the association of KAI1 expression with cancer cell differentiation, the 97 primary invasive tumors were classified into three groups: well differentiated, moderately differentiated, and poorly differentiated. Loss of KAI1 expression was significantly more frequent in moderately or poorly differentiated tumors as compared with well-differentiated tumors ($P < 0.001$). Positive KAI1 staining was frequent in well-differentiated tumors (78.6%) but infrequent in moderately differentiated tumors (12.1%). None of the 25 poorly differentiated tumors showed positive KAI1 staining. In con-

<table>
<thead>
<tr>
<th>Disease</th>
<th>n</th>
<th>Negative</th>
<th>Decreased</th>
<th>Positive</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperplasia</td>
<td>18</td>
<td>0 (0.0%)</td>
<td>1 (5.6%)</td>
<td>17 (94.4%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Stage I/II</td>
<td>72</td>
<td>20 (27.8%)</td>
<td>35 (48.6%)</td>
<td>17 (23.6%)</td>
<td></td>
</tr>
<tr>
<td>Stage III/IV</td>
<td>25</td>
<td>13 (52.0%)</td>
<td>11 (44.0%)</td>
<td>1 (4.0%)</td>
<td></td>
</tr>
<tr>
<td>Metastatic lesion</td>
<td>28</td>
<td>20 (71.4%)</td>
<td>7 (25.0%)</td>
<td>1 (3.6%)</td>
<td></td>
</tr>
</tbody>
</table>

* $P$ was computed using $\chi^2$ test.
KAI1 Expression in Endometrial Cancer

The KAI1 gene, located on human chromosome 11p11.2, was initially identified as a metastasis suppressor gene for prostatic cancer (19). Expression of KAI1 was reduced in cell lines derived from metastatic human prostate tumors as compared with normal prostate tissue. It was also shown that KAI1 protein expression was down-regulated during the progression of human prostate cancers (20).

KAI1 specifies a protein of 267 amino acids. It belongs to a structurally distinct family of cell surface glycoproteins, transmembrane 4 protein superfamily, which all have four hydrophobic transmembrane domains and one large extracellular N-glycosylated domain. Although the precise biological functions of transmembrane 4 protein superfamily proteins are still to be determined, their membrane localization and extensive glycosylation implicate that these proteins may function via cell-cell and cell-extracellular matrix interactions, thereby potentially influencing the ability of cancer cells to invade and to metastasize (19). Transcript of KAI1 was detected in each of the human tissues tested, including various surface epithelia and activated lymphocytes (19, 39, 40). Loss of expression of this gene has been observed recently in a variety of human cancers (21–32).

Because down-regulation of the KAI1 protein was observed frequently in endometrial cancer cell lines (33), it is possible that this gene is also involved in the malignant progression of this tumor. In the present study, we used immunohistochemical analysis to confirm that the expression of KAI1 protein was down-regulated during the progression of human endometrial cancer. Most endometrial hyperplasias (17 of 18) were well differentiated, whereas 14 of 26 endometrial carcinomas had either decreased or negative expression of KAI1 protein. It was also shown that KAI1 expression was down-regulated during the progression of human endometrial cancers.

The prognostic significance of KAI1 expression in endometrial cancer was analyzed. As was seen in most other human cancers examined, expression of KAI1 appeared to be a favorable prognostic factor in endometrial cancer. The overall survival rates in patients with KAI1-positive and -decreased tumors were better than that in patients with KAI1-negative tumors. However, its prognostic value in multivariate analysis was still inferior to stage. A possible explanation for this phenomenon is that down-regulation of KAI1 occurs with tumor progression and, therefore, its prognostic significance is masked by the traditional factor.

In our previous study of cervical cancer, we demonstrated the correlation between RNA level and protein level of KAI1 expression, as determined by real-time PCR analysis and immunohistochemical study, respectively (32). This correlation was additionally confirmed in the present study. Decreased KAI1 expression at RNA level paralleled with decreased KAI1 protein. Therefore, loss of KAI1 expression in endometrial cancer has been demonstrated to contribute to metastatic progression of tumor cells (38).

The KAI1 expression pattern in endometrial cancer was examined in 97 patients with primary invasive endometrial cancer, which included 14 deaths. The overall survival status information was available for the 97 patients with primary invasive endometrial cancer. Most endometrial hyperplasias (17 of 18) were well differentiated, whereas 14 of 26 endometrial carcinomas had either decreased or negative expression of KAI1 protein. It was also shown that KAI1 expression was down-regulated during the progression of human endometrial cancers.

**Prognostic Implication of KAI1 Expression.** Survival status information was available for the 97 patients with primary invasive endometrial cancer, which included 14 deaths. The relationship between KAI1 expression and overall survival status was examined in these patients. The survival curves for patients with KAI1-positive, -decreased, and -negative tumors were compared, and are shown in Fig. 2. Patients with KAI1-negative tumors had significantly poorer survival than those with KAI1-positive and KAI1-decreased tumors ($P = 0.0286$ and 0.0042, respectively). However, there was no survival difference between patients with KAI1-positive and KAI1-decreased tumors ($P = 0.3657$). When KAI1 expression, cell differentiation, and tumor stage were compared in prognostic significance, stage was the most significant factor for survival ($P < 0.001$; Table 3).

**Real-Time PCR Analysis of KAI1 Expression and Correlation with Immunohistochemistry.** cDNA from 35 endometrial tumors were obtained for real-time quantitative PCR analysis. The expression of KAI1 was presented as $\Delta C_t$ (KAI1 C_t - 18S rRNA C_t). Real-time quantitative PCR confirmed a high KAI1 expression level in the positive control (SW 480) with the value of $\Delta C_t = 4.22$. In contrast, very low KAI1 expression level was observed in the negative control (PC-3) with the value of $\Delta C_t = 9.92$. Using $\Delta C_t$ of 6 as the cutoff, the immunohistochemical data of the 35 tumors showed a good correlation with that of real-time quantitative PCR analysis. All of the 9 KAI1-positive tumors had $\Delta C_t < 6$. Among the 26 tumors with $\Delta C_t \geq 6$, 25 (96%) had either decreased or negative expression of KAI1 in immunohistochemical analysis. The only discordant tumor was a moderately differentiated stage IC carcinoma, which had a $\Delta C_t$ value of 6.29 but a positive immunohistochemical score.

**Table 2** Correlation between loss of KAI1 protein expression and cellular differentiation of endometrial cancer

<table>
<thead>
<tr>
<th>Differentiation*</th>
<th>n</th>
<th>Negative</th>
<th>Decreased</th>
<th>Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>WD</td>
<td>14</td>
<td>0 (0.0%)</td>
<td>3 (21.4%)</td>
<td>11 (78.6%) &lt;0.001</td>
</tr>
<tr>
<td>MD</td>
<td>58</td>
<td>12 (20.7%)</td>
<td>39 (67.2%)</td>
<td>7 (12.1%)</td>
</tr>
<tr>
<td>PD</td>
<td>25</td>
<td>21 (84.0%)</td>
<td>4 (16.0%)</td>
<td>0 (0.0%)</td>
</tr>
</tbody>
</table>

* $P$ was computed using $\chi^2$ test.

a WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated.

**DISCUSSION**

It is established that in the oncogenic process some genetic changes result in an imbalance of cellular growth regulation, and this will lead to uncontrolled proliferation. However, unrestrained growth does not, by itself, result in invasion and metastasis. The ability of tumor cells to invade and metastasize often requires additional genetic changes (36). This may include both the activation of metastasis stimulating genes and the inactivation of metastasis suppressor genes (37). Loss of function for metastasis suppressor gene(s), in particular, has been demonstrated to contribute to metastatic progression of tumor cells (38).
carcinoma likely occurred at the transcriptional level rather than translational level.

In this study, loss of KAI1 expression occurred mostly in the advanced and metastatic endometrial cancers. Therefore, its involvement in the progression of this disease could occur as a later event when the endometrial tumor has acquired an invasive character.

Histopathologically, only 4 cases in our patient population belonged to nonendometrioid type (1 serous papillary carcinoma and 3 clear cell carcinomas). Two of these cases exhibited decreased KAI1 expression and the other 2 exhibited negative KAI1 expression. Because the case number was too small, we could not conclude if the frequency of KAI1 loss is different between endometrioid and nonendometrioid carcinomas.

In summary, we found that KAI1 was highly expressed in precancerous endometrial lesions, was down-regulated in advancing primary endometrial tumors, and was additionally lost in metastases of endometrial cancer. In addition, more poorly differentiated tumors demonstrated significantly lower KAI1 expression; and patients with KAI1-negative tumors had a significantly poorer survival rate. These findings suggest that down-regulation of KAI1 is associated with the progression of endometrial cancer. Additional study is needed to determine whether an aggressive treatment strategy and closed follow-up are necessary in patients with KAI1-negative endometrial cancer.

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