

## Expression of the Serine Protease Hepsin and Clinical Outcome of Human Endometrial Cancer

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**Abstract.** *Background:* Hepsin is a type II transmembrane serine protease originally identified in the human liver as a cDNA clone. Hepsin was found to be significantly overexpressed in cancer samples compared to matched various tissues (e.g. prostate, renal, ovarian carcinoma). The purpose of the present study was to examine hepsin expression and to evaluate its clinicopathological significance in endometrial cancer. *Patients and Methods:* Hepsin expression was examined by immunohistochemistry in 34 cases with normal endometrium as a control, 11 cases with endometrial hyperplasia, and 128 cases with endometrioid adenocarcinoma. *Results:* Hepsin expression was found to be significantly higher in endometrial cancer compared to normal endometrium and endometrial hyperplasia. High levels of hepsin expression were associated with advanced stage ( $p<0.001$ ), high grade ( $p=0.002$ ), depth of myometrial invasion ( $p<0.001$ ), cervical involvement ( $p=0.007$ ), lymph node metastasis ( $p=0.001$ ), lymph vascular space (LVS) involvement ( $p=0.006$ ), ovarian metastasis ( $p=0.002$ ), and peritoneal cytology ( $p=0.03$ ) of endometrial cancer. *Conclusion:* These findings indicate that hepsin protein expression could be an important indication for high risk of endometrial cancer.

Endometrial cancer is currently the fourth most common gynecological malignancy in Japan, with an estimated incidence of 4,046 new cases in 2003 (1). Clinical parameters such as stage of the disease, nuclear grade, histological subtype, and tumor size correlate with the outcome of the disease. Although it is a relatively common cancer, the molecular genetic factors related to the

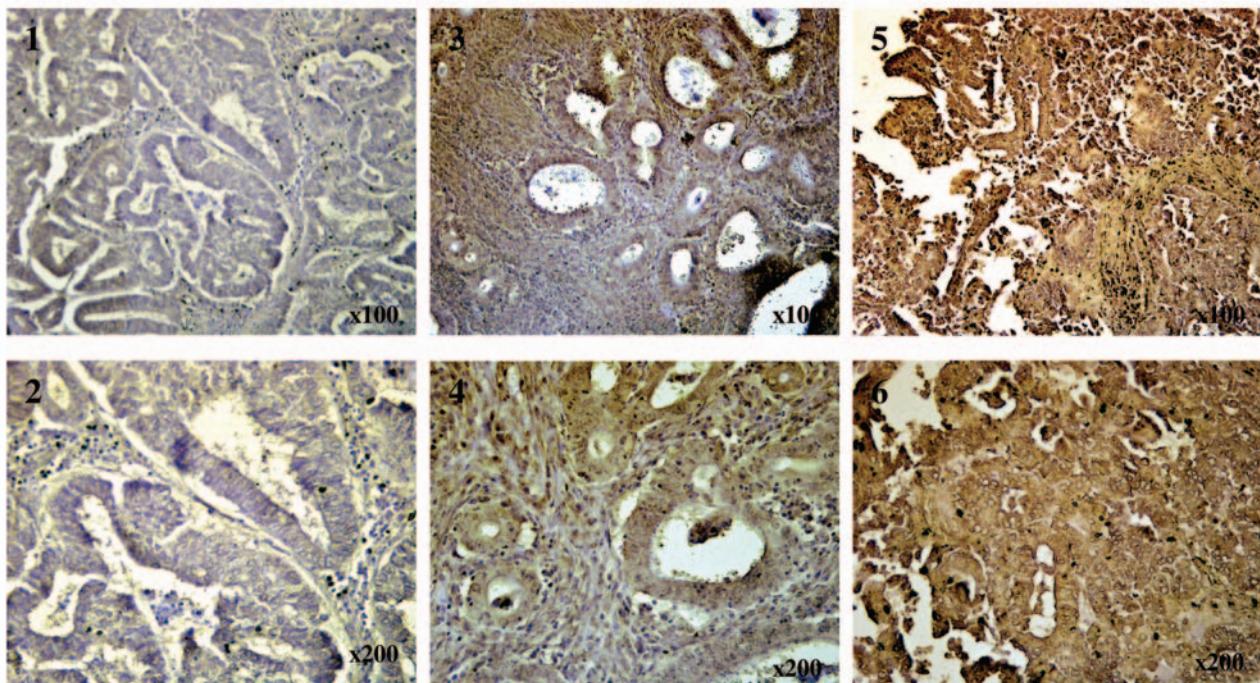
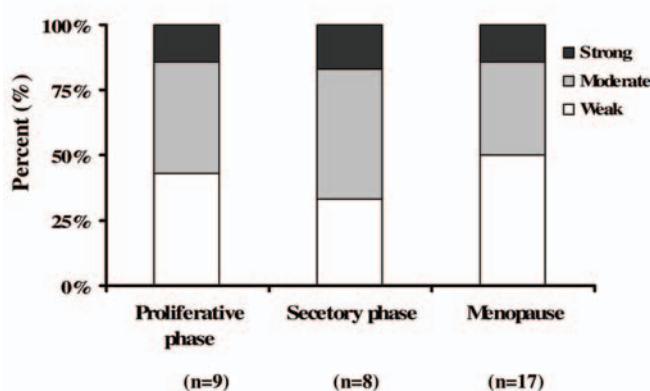
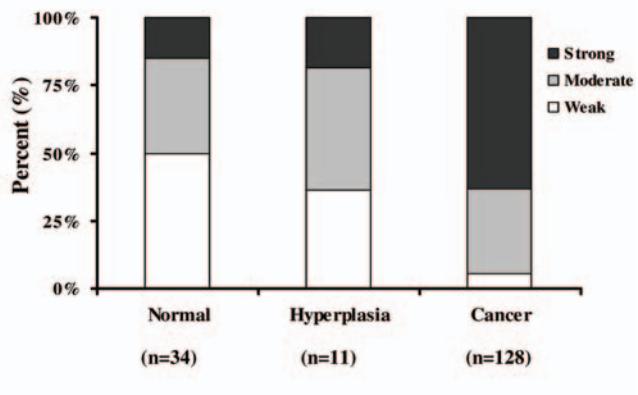
development of endometrial cancer and its prognosis have only recently begun to be investigated. It is hoped that through a better understanding of the molecular genetic alterations implicated in endometrial cancer a more complete profile of risk factors can be developed. The proposed underlying molecular mechanisms implicated in the progression of endometrial cancer include over-expression of oncogenes such as *HER-2/neu* and *myc*, and loss of tumor suppressor genes such as *p53* (2-4).

The serine proteases are one of the largest and most conserved multigene proteolytic families (5). These enzymes are present in a wide range of tissues and biological fluids, and have well-characterized roles in diverse cellular activities, including blood coagulation, wound healing, digestion and immune response. Furthermore, these proteases contribute to a number of diseases and are particularly implicated in tumor growth, invasion and metastasis (6-8). Hepsin is a novel serine protease with a transmembrane domain near the amino terminus (9, 10). This structural feature distinguishes hepsin from most serine proteases of the trypsin superfamily (11, 12). Hepsin is a type II transmembrane serine protease originally identified in the human liver as a cDNA clone (9, 13). A 1.85-kb Hepsin mRNA is highly expressed in normal liver tissues, while it is poorly expressed in other tissues, including normal kidney, pancreas, lung, thyroid, pituitary gland and testis (14). The importance of hepsin in prostate cancer has been demonstrated by a number of independent studies that analyzed the differences in gene expression between cancerous and normal tissue by cDNA microarray analysis. Hepsin was found to be significantly overexpressed in prostate cancer samples compared to matched normal prostate tissues (15-18). Similarly, hepsin overexpression has been reported to occur in ovarian and renal carcinomas (19, 20).

In the current study, we examined whether levels of hepsin protein expression are associated with clinicopathological characteristics in patients suffering from endometrial cancer.

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*Key Words:* Hepsin, endometrial cancer, prognostic significance.

**A****B****C**

**Figure 1.** Evaluation of hepsin expression in endometrial tissues. Immunohistochemical staining of hepsin in normal endometrium, hyperplasia, and endometrial cancer using anti-human hepsin protein. (A) 1, Weak epithelial cell staining (original magnification x100). 2, Weak epithelial cell staining (original magnification x200). 3, Moderate epithelial cell staining (original magnification x100). 4, Moderate epithelial cell staining (original magnification x200). 5, Strong epithelial cell staining (original magnification x100). 6, Strong epithelial cell staining (original magnification x200). (B) Histogram of hepsin expression by normal tissue type (proliferative phase, secretory phase, menopause). (C) Histogram of hepsin expression by tissue type (normal endometrium, endometrial hyperplasia, endometrial cancer). The relative strength of hepsin immunohistochemical staining was assessed qualitatively.

## Patients and Methods

**Patients and tissue specimens.** Patients with normal endometrium ( $n=34$ ; proliferative phase ( $n=9$ ), secretory phase ( $n=8$ ), menopause ( $n=17$ )), endometrial hyperplasia ( $n=11$ ; simple endometrial hyperplasia ( $n=2$ ), complex endometrial hyperplasia

( $n=2$ ), atypical endometrial hyperplasia ( $n=7$ )), and adenocarcinoma ( $n=128$ ) were treated at Okayama University Hospital between January 1996 and November 2004. Patients with distant metastasis were excluded from this study.

Tumor specimens were obtained at the time of surgery and immediately fixed in 10% neutral-buffered formalin and embedded

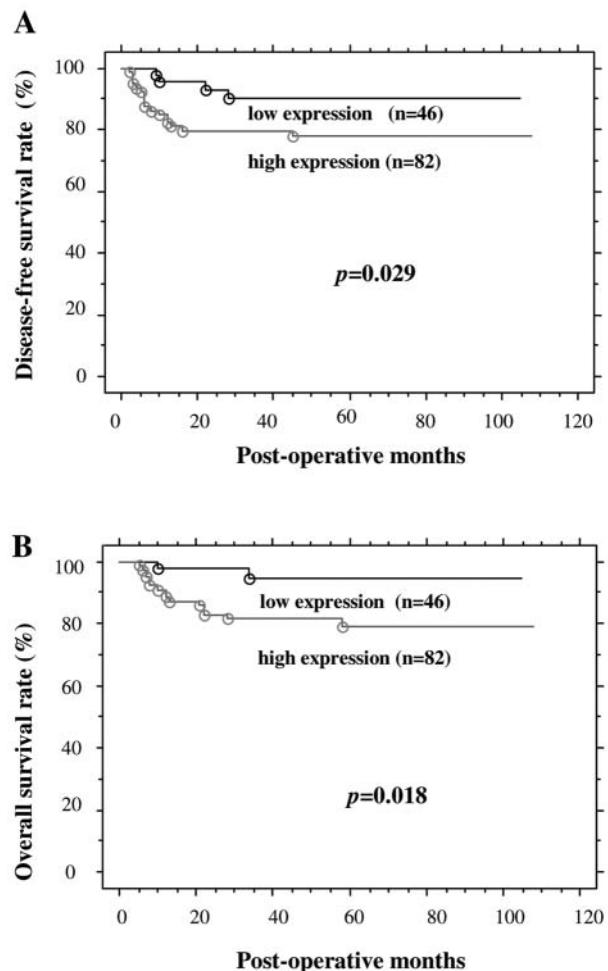
**Table I.** Association between hepsin and clinicopathological factors in endometrial cancer.

Variable	No. of cases	Hepsin score (mean±SE)	p-value
Age (years)			0.448
<60	76	1.55±0.60	
≥60	52	1.63±0.56	
FIGO stage			<0.001
I+II	81	1.44±0.63	
III+IV	47	1.83±0.43	
FIGO grade			0.002
1	50	1.36±0.69	
2+3	78	1.73±0.47	
Depth of myometrial invasion			<0.001
<1/2	85	1.46±0.65	
≥1/2	43	1.84±0.37	
Cervical involvement			0.007
Negative	102	1.53±0.62	
Positive	26	1.81±0.40	
Lymph node metastasis			0.001
Negative	111	1.53±0.62	
Positive	17	1.88±0.33	
LVS involvement			0.006
Negative	86	1.48±0.65	
Positive	42	1.81±0.40	
Ovarian metastasis			0.002
Negative	114	1.54±0.61	
Positive	14	1.93±0.27	
Peritoneal cytology			0.03
Negative	102	1.52±0.61	
Positive	26	1.81±0.48	

\*Mann-Whitney U-test. FIGO, International Federation of Gynecology and Obstetrics; LVS, lymph vascular space.

in paraffin. Histological cell types were diagnosed according to the WHO classification (21): 123 were classified as endometrioid adenocarcinomas, four as adenosquamous, and one as serous carcinoma. Histological grades according to the International Federation of Gynecology and Obstetrics (FIGO) staging classification (22) were as follows: 50 were grade 1, 56 grade 2, and 22 grade 3. Surgical staging was reviewed based on the FIGO staging system: 71 were allocated to stage I, 10 to stage II, 37 to stage III, and 10 to stage IV. The median age at the time of surgery was 58 years (range 28–85 years). Disease-free and overall survival rates were defined as the interval between the initial operation and either clinically or radiologically proven recurrence or death, respectively.

**Immunohistochemistry and staining evaluation.** Formalin-fixed, paraffin-embedded sections, at 4 µm thickness, were deparaffinized with xylene and rehydrated in ethanol. Endogenous peroxidase activity was quenched by methanol containing 0.3% hydrogen peroxidase for 15 min. Sections were then incubated at room temperature with a hepsin (Cayman Chemical, Ann Arbor, MI, USA) followed by staining using a streptavidin-biotin-peroxidase kit (Nichirei, Tokyo, Japan). The sections were counterstained with



**Figure 2.** Kaplan-Meier plots for (A) disease-free and (B) overall survival of the 128 patients with endometrial cancer according to their epithelial hepsin expression status. Low epithelial expression, score 0–1; high epithelial expression, score 2.

hematoxylin. The level of hepsin staining in epithelial cells was classified into three groups by scoring the percentage of positive by stained cells: strong score 2, >50% of cells stained; moderate score 1, 10–50% of cells stained; and weak score 0, <10% of cells stained. Microscopic analyses were independently conducted by two independent examiners with no prior knowledge of the clinical data. Final decisions in controversial cases were made using a conference microscope.

**Statistical analyses.** The Mann-Whitney U-test was used to examine the association between clinicopathological factors and hepsin expression. Survival rates were calculated by the Kaplan-Meier method and differences were examined by the log-rank test. Factors found to be significant were then chosen for stepwise Cox's multivariate proportional hazard model to determine their prognostic values. These analyses were performed utilizing StatView 5.0 software (Abacus Concepts, Berkeley, CA, USA). P-values <0.05 were considered statistically significant.

Table II. Disease-free and overall survival analysis of prognostic factor using the log-rank test in endometrial cancer.

Variable	No. of cases	Estimated 5-year DFS (%)	p-value	Estimated 5-year OS (%)	p-value
Age (years)			0.79		0.63
<60	76	82.9		85.5	
≥60	52	84.6		88.5	
FIGO stage			<0.001		<0.001
I+II	81	96.6		98.8	
III+IV	47	61.7		65.9	
FIGO grade			0.01		0.003
1	50	94		96	
2+3	78	76.9		79.5	
Depth of myometrial invasion			0.004		0.005
<1/2	85	91.6		94.1	
≥1/2	43	67.4		72.1	
Cervical involvement			<0.001		<0.001
Negative	102	91.2		93.1	
Positive	26	53.9		61.5	
Lymph node metastasis			<0.001		<0.001
Negative	111	91.2		94.6	
Positive	17	29.4		35.3	
LVS involvement			<0.001		<0.001
Negative	86	94.2		95.4	
Positive	42	61.9		69	
Ovarian metastasis			<0.001		<0.001
Negative	114	89.5		93	
Positive	14	35.7		35.7	
Peritoneal cytology			0.005		0.003
Negative	102	88.2		91.2	
Positive	26	65.4		69.2	
Hepsin			0.03		0.02
Low (0-1)	46	94		95.6	
High (2)	82	71.4		81.7	

\*Mann-Whitney U-test. FIGO, International Federation of Gynecology and Obstetrics; LVS, lymph vascular space; DFS, disease-free survival; OS, overall survival.

## Results

**Hepsin expression in human endometrial tissues.** Expression of hepsin was examined in human endometrial tissues by immunostaining; Figure 1A illustrates representative immunostaining patterns of hepsin. Weak epithelial staining was observed in 28 cases (16.4%), moderate staining in 55 cases (32.2%) and strong staining in 88 cases (51.4%). The mean score of epithelial staining was 0.64 for normal human endometrium, 0.82 for hyperplasia and 1.57 for cancer samples. Interestingly, endometrial cancer had the strongest expression of hepsin compared to normal endometrium and endometrial hyperplasia, as shown in Figure 1C (Mann-Whitney U-test,  $p<0.05$ ). However, the expression levels of hepsin were not significantly different between proliferative and secretory phase, and between simple and complex endometrial hyperplasia (Figure 1B and C).

**Clinicopathological parameters.** Table I shows the distribution of cases scored as positive for each of the biological parameters examined, according to clinicopathological characteristics in the overall population. As expected, the level of hepsin expression positivity showed a statistically significant association with clinicopathological parameters such as advanced FIGO stage ( $p<0.001$ ), high FIGO grade ( $p=0.002$ ), depth of myometrial invasion ( $p<0.001$ ), cervical involvement ( $p=0.007$ ), lymph node metastasis ( $p=0.001$ ), lymph vascular space (LVS) involvement ( $p=0.006$ ), ovarian metastasis ( $p=0.002$ ), and peritoneal cytology ( $p=0.03$ ), but age was not statistically significant.

**Univariate survival and multivariate analysis.** Figure 2 shows the disease-free and overall survival curves of 128 patients with endometrial cancer, according to hepsin expression status. The disease-free and overall survival rates of patients exhibiting a high hepsin expression (score 2) were

significantly lower than those of patients exhibiting a low hepsin expression (score 0-1) ( $p=0.029$  and 0.018, respectively). The results of the univariate survival analyses of other variables are shown in Table II. Multiple analysis showed that LVS involvement was the strongest independent prognostic factor for both disease-free survival and overall survival; hepsin expression was not an independent prognostic factor for either disease-free or overall survival (Table III).

## Discussion

This is the first study to examine the status of hepsin expression and its possible roles in conjunction with clinical outcome in patients with endometrial cancer. In endometrial carcinoma, conventional clinicopathological factors, such as FIGO grade and stage, histological type, LVS invasion, and depth of myometrial invasion, are well-known prognostic factors. The proposed underlying molecular mechanisms implicated in the progression of endometrial cancer include overexpression of oncogenes such as *HER-2/neu* and *myc*, and loss of tumor suppressor genes such as *p53* (2-4). This study evaluated the prognostic significance of hepsin overexpression in relation to endometrial cancer.

The basement membrane is a specialized extracellular matrix structure that separates the epithelial and stromal cell compartments. To accomplish local invasion, tumor cells use extracellular and cell surface proteolytic enzymes to degrade the basement membrane proteins (23, 24). Several studies have demonstrated a critical role of matrix metalloproteinases (MMPs), which can degrade the extracellular matrix and basement membrane proteins and facilitate the initial invasion events (23). MMPs and serine proteases have been implicated in degradation of the extracellular matrix and modulation of cell-substratum adhesion in tumor cells. Type II transmembrane serine proteases are a specialized group of cell surface proteolytic enzymes (24). Hepsin is a novel serine protease with a transmembrane domain near the amino terminus that was originally identified in a cDNA clone from human liver (9, 13). Previous studies have shown that several DNA microarray studies of gene expression have revealed marked overexpression of hepsin in human prostate carcinomas. Hepsin mRNA is up-regulated in 90% of prostate tumors, and exclusively expressed in tumor cells (15). However, it is not clear how hepsin mRNA levels correlate with different stages/grades of prostate cancer. Although initial studies have shown that hepsin levels were highest in prostatic intraepithelial neoplasia and decreased with prostate cancer progression (15), other studies have demonstrated that hepsin mRNA levels increase with prostate cancer progression, reaching maximum levels in those with advanced prostate carcinomas (16, 17). These results indicated that

Table III. Prognostic factors for disease-free and overall survival selected by Cox's multivariate proportional model analysis.

	Hazard ratio	95% CI <i>p</i> -value	Cox's test
Disease-free survival			
FIGO stage	3.4	0.69-16.67	0.13
FIGO grade	3.94	0.95-16.13	0.06
Depth of myometrial invasion	2.44	0.81-7.35	0.43
Cervical involvement	1.52	0.53-4.33	0.09
Lymph node metastasis	5.52	1.73-17.54	0.04
LVS involvement	9.43	1.63-22.22	0.007
Ovarian metastasis	1.47	0.36-5.91	0.58
Peritoneal cytology	1.61	0.57-4.54	0.38
Hepsin expression	1.15	0.18-5.52	0.85
Overall survival			
FIGO stage	4.37	0.45-41.66	0.21
FIGO grade	9.43	0.93-90.91	0.06
Depth of myometrial invasion	2.15	0.67-6.89	0.19
Cervical involvement	3.11	0.95-10.20	0.06
Lymph node metastasis	3.83	1.17-12.5	0.025
LVS involvement	6.25	1.69-21.27	0.005
Ovarian metastasis	1.69	0.44-6.45	0.44
Peritoneal cytology	2.28	0.74-7.24	0.16
Hepsin expression	1.11	0.21-6.41	0.55

CI, confidence interval; FIGO, International Federation of Gynecology and Obstetrics; LVS, lymph vascular space.

hepsin might contribute to endometrial disorders. The increase in the level of hepsin expression in endometrial cancer compared with normal endometrium and endometrial hyperplasia (Figure 1C) might indicate that a rise in hepsin level may act as a switch in the conversion from endometrial hyperplasia to endometrial cancer.

In our study, a significant association between hepsin expression and some of the traditional prognostic factors for endometrial carcinoma, such as advanced stage, poorly differentiated tumor of histology, depth of myometrial invasion, cervical involvement, lymph node metastasis, LVS involvement, ovarian metastasis, and peritoneal cytology, was demonstrated (Table I). Interestingly, a strong hepsin immunostaining pattern was significantly associated with poor prognosis in endometrial cancer (Figure 2 and Table II). Furthermore, multivariate analysis showed that hepsin expression is not an independent prognostic factor for disease-free or overall survival (Table III). These results suggest that expression of hepsin in endometrial cancer may be associated with aggressive biological characteristics and may play an important role in prognosis and/or recurrence.

These findings all indicate that hepsin protein determined by immunohistochemistry could be an important tool for identifying patients with poor prognosis of endometrial cancer.

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