Proteomics Identification of Cyclophilin A as a Potential Prognostic Factor and Therapeutic Target in Endometrial Carcinoma*

Zhengyu Li, Xia Zhao‡, Shujun Bai, Zhi Wang, Lijuan Chen, Yuquan Wei, and Canhua Huang§

Endometrial carcinoma is one of the most common malignancies of the female genital tract, and there is an urgent need for discovery of novel factors for prognostic assessment and therapeutic targets to endometrial carcinoma. Herein a two-dimensional gel electrophoresis and MALDI-Q-TOF MS/MS-based proteomics approach was used to identify differentially expressed proteins in endometrial carcinoma. Of the 99 proteins identified, cyclophilin A was one of the most significantly altered proteins, and its overexpression was confirmed using RT-PCR and Western blot analyses. Immunohistochemistry suggested a link between cyclophilin A expression and poor differentiation and decreased survival \( (p < 0.01) \). Knockdown of cyclophilin A expression by RNA interference led to the significant suppression of the cell growth and the induction of apoptosis in endometrial carcinoma HEC-1-B cells \( \text{in vitro} \ (p < 0.01) \) and the inhibition of tumor growth \( \text{in vivo} \ (p < 0.01) \). These data suggest that cyclophilin A may serve as a novel prognostic factor and possibly an attractive therapeutic target for endometrial carcinoma. Molecular & Cellular Proteomics 7: 1810–1823, 2008.

Endometrial carcinoma is one of the most common malignancies of the female genital tract with an approximately 80% 5-year survival for all stages taken together. Annually over 142,000 new cases develop worldwide, and an estimated 42,000 women die from this disease. The incidence of endometrial carcinoma varies among regions; it is 10 times higher in North America and European countries compared with developing countries (1). Recent studies have revealed that endometrial carcinoma is characterized by multiple pathogenetic steps and various genetic and epigenetic interactions (2, 3). A wide variety of genetic molecules characterize the initiation, development, and outcome of this disease, including DNA methylation (4, 5) and altered expression or mutation of p53 (6), K-ras (7), and BRAF (8, 9). Global gene expression analyses have been used to investigate the changes associated with this process (10), including DNA microarray-based approaches for screening genes relevant to carcinogenesis (11, 12). Many novel markers, such as CD171 (13), PTEN (14), and urokinase plasminogen activator receptor (15), have been documented and have been subsequently utilized in diagnosis, prognosis prediction, and subtype classification. However, although the case fatality rate is lower than that of many kinds of malignancies, prognosis for endometrial carcinoma patients remains poor, which suggests an urgent and persistent need for further elucidation of the molecular mechanisms underlying the development of endometrial carcinoma.

Recently it has become apparent that, in addition to the better known genetic and epigenetic alterations, there are also factors relating to molecular changes in translation, post-translational modification, and intracellular mislocalization involved in tumor initiation and growth; and these factors cannot be detected either by measuring the amount of RNA or by studying nucleotide sequence variation. It is thought that the association between protein alterations and malignancy by analysis of the cancer proteome can be more informative than genomics or transcriptomics alone (16). A proteomics approach has been applied with success in the studies of many types of cancer, including brain, lung, liver, stomach, and colon cancers and was considered to be suitable to overcome some limitations of current approaches for the elucidation of the molecular mechanisms of endometrial carcinoma. To date, only a few proteomics studies on endometrial carcinoma have been reported; however, some of the findings have been noteworthy (17–19). These studies reported slightly different findings and conclusions; the discrepancies might be due to ethnic differences among patients or technical problems such as varying ability of mass spectrometry to identify a particular protein.

The aim of the present study was to identify novel tumor-associated molecules for potential biomarkers or molecular therapeutic targets using a 2-DE¹-based proteomic approach. More than 100 differentially expressed proteins were revealed
between carcinoma and normal tissues, and subsequently 99 proteins were identified by MS analysis. Of them, cyclophilin A was further characterized based on the fact that it was already found to have an oncogenic property in several cancers, for example, lung cancer (20, 21) and pancreatic cancer (22, 23). The data presented in this study demonstrate that cyclophilin A may be a novel prognostic factor as well as a potential therapeutic target for the clinical treatment of endometrial carcinoma.

**EXPERIMENTAL PROCEDURES**

**Clinical Specimens**—Fresh endometrial carcinoma and paired adjacent normal tissues were obtained from eight patients suffering from endometrial cancer who underwent surgical resections at the Gynecological Department of West China Second Hospital of Sichuan University from 2005 to 2006. Paraffin-embedded endometrial carcinoma specimens were obtained from 84 patients who underwent surgical resections from 1998 to 2002. Histodifferentiation grading of endometrial carcinoma was assigned according to the criteria established by the World Health Organization in 1975, and surgical pathologic staging was assigned according to the 1988 recommendations of the International Federation of Gynecology and Obstetrics. Paraffin-embedded endometrial intraepithelial neoplasia (EIN) specimens were obtained from 29 patients bearing EIN who underwent hysterectomy. Paraffin-embedded normal endometrium specimens were obtained from 39 patients with normal menstruation who underwent hysterectomy for uterus myoma. 21 cases were in the proliferative phase, and 18 cases were in the secretory phase. All these samples were taken by experienced gynecologists and gynecological surgeons and examined by experienced pathologists for histological confirmation. A summary of clinicopathologic information for these patients is shown in Table I.

This study was approved by the Institutional Ethics Committee of Sichuan University. Informed consents were obtained from all patients prior to analysis.

**Two-dimensional Electrophoresis and Image Analysis**—Tissue were ground into powder in liquid nitrogen and lysed in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2 M tributylphosphine, 2 M PMSF, 1 mg/ml DNase I, 0.2 mg/ml RNase A). After centrifugation followed by vortexing and incubation, the supernatant was precipitated with cold acetone/trichloroacetic acid. The pellet was dissolved in rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2 M tributylphosphine, 0.2% carrier ampholytes). IEF was performed using ReadyStrip™ IPG strips (17 cm, pH 3–10 non-linear; Bio-Rad) on a PROTEAN IEF system (Bio-Rad) until a total of 80,000 V-h was reached after passive rehydration for 16 h. The focused strips were then equilibrated twice for reduction and alkylation and transferred onto 12% SDS-polyacrylamide gels using a PROTEAN II xi Cell system (Bio-Rad). The protein spots in gels were visualized by Coomassie Brilliant Blue G-250 staining.

Image analysis was performed using PDQuest 7.1 software (Bio-Rad). Spot intensity was quantified automatically by calculation of spot volume after normalization of the image by taking the ratio of spot intensity of one spot to the total spots and expressed as a fractional intensity. Those spots with 2.5-fold (t test, p < 0.05) or more changes in expression intensity and frequencies higher than 25% were selected for identification.

**In-gel Digestion**—Spots of interest were excised and in-gel digested according to the manufacturer’s instructions (Promega, Madison, WI) with some modifications. Briefly the gel spots were destained twice with 0.2 ml of 100 mM NH₄HCO₃, 50% ACN for 45 min at 37 °C and dehydrated in 100% ACN for 5 min. Then the spots were incubated with 10 μl of 10 μg/ml Trypsin Gold (Promega) at room temperature for 1 h and then at 37 °C overnight covered with 20 μl of digestion buffer (40 mM NH₄HCO₃, 10% ACN). The liquid was then removed and saved, and tryptic peptides were extracted twice with 50 μl of 50% ACN, 5% TFA by sonication for 15 min. Then all extracts were pooled and dried in a SpeedVac at room temperature. Trypsin were desalted using C₁₈ ZipTips (Millipore, Bedford, MA) and reconstituted in 5 μl of 70% ACN, 0.1% TFA.

**Mass Spectrometry Analysis**—1.5 μl of the peptide eluate was mixed with an equal volume of α-cyano-4-hydroxycinnamic acid matrix on the stainless MALDI target and allowed to air-dry. MALDI-TOF MS/MS was performed on a Waters Micromass Q-Tof Premier mass spectrometer (Waters, Manchester, UK). The automatic scan rate was 1 s with an interscan delay of 0.02 s. Spectra were accumulated until a satisfactory signal/noise ratio had been obtained from a range of 900–3500 m/z. After MS acquisition, 10 ions of maximum intensity were selected automatically for MS/MS analysis. Trypsin autolysis products and keratin-derived precursor ions were automatically excluded. The collision voltage of CID was varied between 34 and 161 eV depending on the mass of the precursor. Data from the MS/MS, as PKL (peak list) files acquired by the ProteinLynx software (Version 2.2.5; Waters), were processed with the search algorithm Mascot (Version 2.2.2; Matrix Science, London, UK) against the National Center for Biotechnology Information non-redundant (NCBI nr) protein sequence database (released March 18, 2007). The MS/MS data were retrieved against the Homo sapiens subset of the sequences with the parameters set as follows: enzyme, trypsin; allowance of up to one missed cleavage peptide; mass tolerance, ±0.1 Da; and MS/MS tolerance, ±0.05 Da. Fixed modifications of cysteine carbamidomethylation and variable modifications of methionine oxidation were allowed. The justification of the threshold used was p < 0.05, indicating the identification at the 95% confidence interval for

<table>
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<th>Clinicopathologic features</th>
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<tr>
<td>Normal endometrium</td>
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<tr>
<td>Mean age (range)</td>
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<td>Proliferative phase</td>
<td>21 (53.8)</td>
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<td>Secretory phase</td>
<td>18 (46.2)</td>
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<td>Total</td>
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<tr>
<td>EIN</td>
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<tr>
<td>Mean age (range)</td>
<td>45.3 ± 5.5 (39-52)*</td>
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<tr>
<td>EIN I</td>
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<tr>
<td>EIN II</td>
<td>9 (31.0)</td>
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<td>EIN III</td>
<td>11 (37.9)</td>
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<tr>
<td>Total</td>
<td>29 (100.0)</td>
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<table>
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<th>Endometrial carcinoma</th>
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<td>Pathologic classification</td>
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<td>Endometrioid adenocarcinoma</td>
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<tr>
<td>Mean age (range)</td>
<td>47.8 ± 13.3 (34-73)*</td>
</tr>
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<td>&lt;50 years</td>
<td>45 (53.6)</td>
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<tr>
<td>≥50 years</td>
<td>39 (46.4)</td>
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<td>Surgical pathologic staging</td>
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<td>I (endometrial or muscular layer involved)</td>
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<td>II (cervical portion involved)</td>
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<td>III (pelvic lymphatic metastasis involved)</td>
<td>13 (15.5)</td>
</tr>
<tr>
<td>Total</td>
<td>84 (100.0)</td>
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</table>

* Mean age (range) in years.
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those matched peptides, and MASCOT scores (based on combined MS and MS/MS spectra) over 66 were considered statistically significant. Only those individual MS/MS spectrum with statistically significant ion scores exceeding the threshold (based on MS/MS data) were considered acceptable. To eliminate the redundancy of proteins appearing in the database under different names or accession numbers, the one protein member belonging to the species *H. sapiens* with the highest MASCOT score was further selected out from a multiple-member protein family.

RT-PCR—The primer sequences and the expected sizes of PCR products were as follows: cyclophilin A, sense 5′-CAT GGT CAA CCC CAC CGT GTT CTT-3′ and antisense 5′-TAG ATG GAC TTG CCA CCA GTG CCA T3′ (236 bp); CD147, sense 5′-AGC GGT TGG AGG TTG TAG G-3′ and antisense 5′-GGG AGG AAG ACG CAG GA-3′ (311 bp). Total RNA was extracted using TRIzol reagent (Invitrogen), and RT-PCR was performed with conditions as follows: reverse transcription at 48 °C for 30 min and denaturation at 94 °C for 2 min; then amplification for 30 cycles at 94 °C for 0.5 min, annealing at 60 °C (cyclophilin A) or 57 °C (CD147) for 0.5 min, and extension at 72 °C for 0.5 min; and then a terminal elongation step at 72 °C for 10 min and a final holding stage at 4 °C.

Western Blotting—Tissue samples were ground in liquid nitrogen and lysed in RIPA lysis buffer (50 mM Tris-HCl (pH 7.4), 0.25% sodium deoxycholate, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 1 mM phosphatase dehydrogenase were used as positive control for transfection evaluation.

According to the shRNA design principle, oligonucleotide sequences of cyclophilin A-shRNA (sense, 5′-GAT CCA AGA TGA GAA CTT CAT CCT TTC AAG ACG AGG ATG AAG TTc TCT TCA TCT TTT TTT TCT CGA CA-3′; antisense, 3′-GTT CTA CTC TGG TAG AAG AGT CCT GCT CCT ACT TCA AGA GTA GAA AAA AAA CAG CTT TTC GA-5′) were designed to also correspond to nucleotides 265–283. The HK sequence, with no homology to any human gene, was used as negative control. The plasmid Pgenesil-2 containing a kanamycin resistance gene was linearized with BamHI and HindIII, and the annealed oligonucleotide templates were ligated into a plasmid vector using T4 DNA ligase. Chemically competent DH5α *Escherichia coli* were transformed, and positive transformants were isolated by kanamycin selection and amplified using standard methods. Identification of the insert-containing plasmids was confirmed by digestion with SalI, and plasmid DNA from positive clones was extracted and sequenced for additional verification. Once the requirement had been met, a large scale preparation of plasmid DNA was extracted and named Pgenesil-2-cyclophilin A-shRNA or Pgenesil-2-HK-shRNA.

Cell Culture and Transfection—The human endometrial carcinoma HEC-1-B cell line (ATCC, Manassas, VA) was maintained in Roswell Park Memorial Institute (RPMI) 1640 medium. The Lipofectamine 2000 (Invitrogen) and siRNA were diluted in antibiotics-free medium, respectively, and then combined at a ratio of 2.5:1. The combinations were transfected into the cells in the indicated concentrations according to the manufacturer’s recommendation.

Cells Proliferation and Apoptosis Assays—Cells seeded on 96-well plates in triplicate were transfected with siRNA at various concentrations. After incubation at 37 °C for the indicated durations, 20 μl MTT (2 mg/ml; Sigma) was added for another 4-h incubation. The MTT formazan precipitate was then dissolved in 200 μl of DMSO, and the absorbance was measured at a 595-nm wavelength.

For clonogenic formation assay, 100 counted cells transfected with siRNA were seeded in triplicate in a 6-well plate and cultured continuously for 14 days. Clones were stained with Giemsa and counted under a microscope. A cluster with more than 50 cells was considered as a clone.

For flow cytometry analysis, PI-stained cells were analyzed on an EPICS ELITE ESP flow cytometer (Beckman Coulter). DNA-bound PI fluorescence was measured with a 15-milliwatt air-cooled argon ion laser at 488-nm excitation. Analyses of cell cycle distribution and apoptosis profiles were performed with Coulter Elite 4.5 Multicycle software.

TUNEL staining was performed according to the manufacturer’s directions (Promega). The cells were then observed under a fluorescence microscope (Olympus, Tokyo, Japan), and a nucleus with bright green fluorescence staining was recorded as a TUNEL-positive event.

Tumor Xenograft Model and shRNA Treatment—The Institutional Animal Care and Treatment Committee of Sichuan University approved all studies herein. Healthy female nude mice (BALB/c, 6–8 weeks of age, nonfertile, and 18–20 g each) were injected subcutaneously with HEC-1-B cells (5 × 10^6/100 μl of PBS/mouse) via the right flank. After 7 days, when the tumor diameters were about 0.6 cm, the mice were randomly divided into four groups (five per group) for intratumor injection. The groups were as follows: 1) PBS, 100 μl of PBS; 2) Lipo, Lipofectamine 2000 at 62.5 μg/100 μl of PBS; 3) negative control, Pgenesil-2-HK-shRNA at 25 μg/100 μl of PBS; 4) shRNA, Pgenesil-2-cyclophilin A-shRNA at 25 μg/100 μl of PBS. Intratumor injections were performed every 3 days, and tumor volumes were
Data Analysis and Statistics—All quantitative data were recorded as mean ± S.D. Comparisons between two groups were performed by Student’s t test; comparisons among multiple groups were performed by one-way ANOVA, least significant difference t test, or Dunnett’s t test; comparisons of ordinal data between two groups were performed by rank sum test; and relevance analysis of ordinal data was performed by cross-tabs χ² test. Survival curves were generated according to the Kaplan-Meier method, and the statistical analyses were performed using log-rank test. Multivariate analyses were evaluated with Cox proportional hazard models. Statistical significance was defined as p < 0.05.

RESULTS

Differentially Expressed Proteins between Endometrial Carcinoma and Normal Endometrial Tissue—Two-dimensional electrophoresis was performed with individual-matched endometrial carcinoma and normal endometrium tissues from eight patients (mean age, 45.63 ± 3.42 years; range, 41–50 years). Image analysis was performed using PDQuest 7.1 software and displayed well resolved and reproducible protein profiles for both human endometrial carcinoma and normal endometrial tissues (Fig. 1A). Coomassie staining for endometrial carcinoma and normal endometrium, respectively, showed a matching rate of 85.6% and averages of 782 ± 28 and 760 ± 33 spots (Fig. 1B). Differentially expressed proteins were defined as statistically significant based on two criteria: 1) intensity alterations >2.5-fold (t test, p < 0.05) and 2) recurrence more than two times in the eight pairs examined. According to these criteria, 112 spots were selected and analyzed using MALDI-Q-TOF tandem mass spectrometry. A total of 99 proteins from the 112 spots were identified. Because different isoforms of a protein might have distinct functions (26), each isoform/spot was considered to be a single protein for analysis in our study. Of these, the majority (79 of 99 proteins) were located in cytoplasm, and most of them had been reported to be involved in cellular functions altered during carcinogenesis, including metabolism (20%), cell transformation (14%), protein folding (12%), proliferation and apoptosis (8%), cell skeleton (6%), and other functions (40%). E, expression profile of 21 significantly altered proteins with recurrence more than four times. The upper portion shows 14 proteins up-regulated in endometrial carcinoma, and the lower portion shows seven proteins that are down-regulated. Each spot volume was quantified from the intensities of spots using PDQuest 2-DE software; values were recorded as mean ± S.D. N, normal endometrium; C, endometrial carcinoma.
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Data of 21 characterized differentially expressed proteins recurring ≥4 times out of the eight pairs of samples

<table>
<thead>
<tr>
<th>Protein description*</th>
<th>Accession no.</th>
<th>Theoretical molecular mass (kDa)/pI</th>
<th>Sequence coverage</th>
<th>Queries matched</th>
<th>MOWSE score</th>
<th>Average ratio (C/N)*</th>
<th>p value</th>
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<tr>
<td>Up-regulated in endometrial carcinoma</td>
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<td>Peroxiredoxin-4 (Prx-IV) (thioredoxin peroxidase)</td>
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<td>36</td>
<td>4</td>
<td>139</td>
<td>27.23</td>
<td>0.014</td>
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<tr>
<td>Stathmin 1 (phosphoprotein p19)</td>
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<td>17.29/5.76</td>
<td>18</td>
<td>3</td>
<td>130</td>
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<td>Glutathione S-transferase P, chain A</td>
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<td>Cyclophilin A</td>
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<td>139</td>
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<td>7</td>
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<td>3</td>
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Down-regulated in endometrial carcinoma

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<th>Protein description*</th>
<th>Accession no.</th>
<th>Theoretical molecular mass (kDa)/pI</th>
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*For several proteins, a few isofoms were identified in the same individual.

**Accession numbers were taken from the NCBInr database.

Table II provides data for 21 characterized differentially expressed proteins recurring ≥4 times out of the eight pairs of samples. All protein spots were identified by MALDI-Q-TOF MS/MS.

The quantified IOD scores for normal endometrium, EIN, and endometrial carcinoma specimens (rank sum test, p < 0.01) (Fig. 3). Together our data demonstrate that this was not attributable to the inflammatory response in the surrounding stroma. In 39 normal specimens, no staining was detected in 23% (nine of 39), weakly positive staining was detected in 77% (20 of 39), and no positive or strongly positive staining was detected. In 29 EIN specimens, no staining was detected in 10% (three of 29), weakly positive staining was detected in 72% (21 of 29), and positive staining was detected in 17% (five of 29). In 52 carcinoma specimens, weakly positive staining was detected in 9.6% (five of 52), positive staining was detected in 44.2% (23 of 52), and strongly positive staining was detected in 46.2% (24 of 52). As shown in Table III, significant differences in staining intensity and positive cells were observed among normal endometrium, EIN, and endometrial carcinoma specimens (rank sum test, p < 0.01). To eliminate potential bias in semiquantitative scoring, we further evaluated the IOD of staining by a computer-assisted system. The quantified IOD scores for normal...
endometrium, EIN, and carcinoma specimens were 5.991 ± 1.515, 11.955 ± 2.299, and 34.028 ± 6.91, respectively, which also suggested remarkable differences of cyclophilin A immunoreactivity among normal endometrium, EIN, and carcinoma tissues (one-way ANOVA, p < 0.05).

Following standard histodifferentiation criteria (established by the World Health Organization in 1975), of the 52 carcinoma samples (mean age, 49.4 ± 7.3 years; range, 37–65 years), 20 were well differentiated, 20 were moderately differentiated, and 12 were poorly differentiated; 40 were in stage I, eight were in stage II, and four were in stage III according to the surgical pathologic staging criteria (established by the International Federation of Gynecology and Obstetrics in 1988). Relevance of the clinical characteristics to the overexpression of cyclophilin A by immunostaining is shown in Tables III–V. Overexpression of cyclophilin A was shown to be significantly more likely to present with poor differentiation (cross-tabs χ² test, p < 0.01) (Table IV), but no apparent relationship was observed with surgical pathologic staging in this study (cross-tabs χ² test, p > 0.05) (Table V).

Of the 84 carcinoma cases for survival analysis (31, 37, and 16 for well, moderately, and poorly differentiated, respectively; 55, 16, and 13 for stages I, II, and III, respectively; mean age, 47.8 ± 13.3 years; range, 34–73 years), immunoreactivity

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**Fig. 2.** Cropped 2-DE gel images of cyclophilin A. Up-regulation by 27.23-fold ± 9.52 (t test, p < 0.01) was seen in endometrial carcinoma tissue. MS/MS analysis revealed four matched peptides with 38% sequence coverage and a MOWSE score of 139. N, normal endometrium; C, endometrial carcinoma; N’ and C’, three-dimensional view representation for N or C. expt, experimental; calc, calculated. Data were expressed as mean ± S.D.
of cyclophilin A was weakly positive in 25 patients, moderately positive in 33 patients, and strongly positive in 26 patients. The survival curves were generated according to the Kaplan-Meier method, and survival analysis using log-rank test suggested that immunoreactivity of cyclophilin A was more likely to be associated with poor outcome of patients with endometrial carcinoma (log-rank test, \( p < 0.05 \); Fig. 4B). The 5-year survival rates were 92, 82, and 72% for weakly positive, positive, and strongly positive staining samples, respectively. To determine whether the prognostic value of cyclophilin A immunoreactivity was independent of other risk factors associated with clinical outcome of endometrial carcinoma, multivariate analysis was performed using the Cox proportional hazard model. The risk variables examined included cyclophilin A immunoreactivity (weakly/moderately versus strongly positive), age of patients (<50 versus \( \geq 50 \) years), histodifferentiation (well/moderately versus poorly differentiated), and surgical pathologic staging (stages I/II versus III). These factors are generally known to significantly affect the outcome of endometrial carcinoma. Cyclophilin A immunoreactivity was independently statistically significant as a prognostic factor in survival of patients of endometrial carcinoma (\( p < 0.05 \)).

**Suppression of Cyclophilin A Decreased Cell Proliferation**—Significant suppression of cyclophilin A expression in cyclophilin A-siRNA-transfected cells was observed 72 h after transfection. RT-PCR assay showed that the cyclophilin A expression was reduced by 60.3% at the mRNA level, and Western blot assay showed its expression to be reduced by 84.9%. No apparent inhibition of cyclophilin A expression was observed by simultaneous transfection of scrambled siRNA.

The proliferation activity of transfected cells was examined using the MTT and clonogenic formation assays. MTT results showed that the proliferation activity was suppressed by cyclophilin A-siRNA in both dose- and duration-dependent manners, and the proliferation ratio was decreased to 47.8% of the control value 72 h after transfection (Fig. 5A). In the clonogenic formation assay, upon 14-day continuous culture, the clone numbers were 76 ± 4.6, 67.3 ± 4.5, and 64 ± 4 in untreated control, Lipofectamine 2000, and negative control groups, respectively. Meanwhile the clone number in the cyclophilin A-siRNA group was 23.7 ± 3.1 with an inhibition ratio of 68.8% (Dunnett’s \( t \) test, \( p < 0.01 \); Fig. 5B). Our data confirmed the oncogenic property of cyclophilin A in endometrial carcinoma, and suppression of cyclophilin A by siRNA might lead to a significant decrease in proliferation of HEC-1-B cells.

**Suppression of Cyclophilin A Induced Cell Apoptosis and G1 Arrest**—Tumor growth is often considered to be a balance...
differentiated).
Infection of cyclophilin A-siRNA resulted in an augmentation of apoptosis and a decrease of 15.3% in S phase were observed in S phase in a time-dependent manner. An increase of 16.5% in the sub-G1 values measured by flow cytometry represent cell death or signs of possible toxicity were observed during this period. Although the tumors of all mice were approximately equal in initial volumes, significant differences in tumor growth were observed during treatment as illustrated by the tumor growth curve in Fig. 6A. Over the course of treatment all tumors showed that the sub-G1 values measured by flow cytometry represent cell death or signs of possible toxicity were observed during this period. Although the tumors of all mice were approximately equal in initial volumes, significant differences in tumor growth were observed during treatment as illustrated by the tumor growth curve in Fig. 6A. Over the course of treatment all tumors

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<th>Relevance of endometrial carcinoma clinical characteristics to cyclophilin A immunoreactivity: cyclophilin A immunoreactivity in normal endometrium, EIN, and endometrial carcinoma.</th>
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* Rank sum test, p < 0.01.
* Least significant difference t test, p < 0.01 (well versus moderately and poorly differentiated), p > 0.05 (moderately versus poorly differentiated).

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* Cross-tabs χ² test, p < 0.01.
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* Cross-tabs χ² test, p > 0.05.
* Least significant difference t test, p > 0.05 (well versus moderately and poorly differentiated), p < 0.01 (well versus poorly differentiated).

between proliferation and apoptosis. Flow cytometry analysis showed that the subdiploid peak in transfected cells increased in a time-dependent manner, and the apoptosis/PI-positive percentage reached 25.4, 36.9, and 45.7% at 24, 48, and 72 h after transfection, respectively, whereas the apoptosis/PI-positive percentage reached only 4 and 7.8% in untreated and negative control groups, respectively (Dunnett’s t test, p < 0.01) (Fig. 5C). For the cell cycle distribution, transfection of cyclophilin A-siRNA resulted in an augmentation of cell populations in G0/G1 phase accompanied with a decrease in S phase in a time-dependent manner. An increase of 16.5% in G0/G1 and a decrease of 15.3% in S phase were observed at 72 h after transfection.

As the sub-G1 values measured by flow cytometry represent cells from both apoptosis and necrosis, a more specific TUNEL assay was applied to validate further the apoptosis induced by cyclophilin A-siRNA. Cell nuclei with DNA strand breaks were revealed by labeling free 3'-OH termini and observed to stain dark green as viewed by fluorescence microscopy, indicating apoptosis, and were recorded as TUNEL-positive nuclei. As shown in Fig. 5D, a significant increase of TUNEL-positive nuclei was observed in the cyclophilin A-siRNA-transfected cells in a dose-dependent manner (Dunnett’s t test, p < 0.01) when compared with the control groups: 35.2 ± 4.3% (100 nm), 22.6 ± 4.4% (50 nm), and 17.5 ± 3.0% (12.5 nm) versus 3.2 ± 1.3% (untreated control), 7.2 ± 2.3% (Lipofectamine 2000), and 9.6 ± 2.7% (negative control). These data indicated that inhibition of cyclophilin A expression by siRNA might lead to significant apoptosis induction of HEC-1-B cells and G0/G1 arrest in cell cycle distribution in vitro.

Suppression of Cyclophilin A Inhibited Tumor Xenograft Growth in Vivo—Intratumor injection of Pgenesil-2-cyclophilin A-shRNA resulted in over 70% suppression of cyclophilin A in tumor-bearing mice by immunohistochemical analysis against anti-cyclophilin A antibody. To control for nonspecific effects of the plasmid vector or the liposome, mice treated with Lipofectamine 2000 or Pgenesil-2-HK-shRNA showed no differences in cyclophilin A expression relative to those with PBS injection.
volumes increased, but the tumors in mice treated with Pgenesil-2-cyclophilin A-shRNA presented growth rates significantly smaller in comparison with those for PBS, liposome, or Pgenesil-2-HK-shRNA treatments, respectively. Furthermore the decrease of tumor volumes was observed after 18 days of Pgenesil-2-cyclophilin A-shRNA treatment. At the termination of the experiment, tumor volumes in the three groups reached 603.64 ± 11006.59.68, 613.16 ± 11006.61.87, and 634.17 ± 11006.61.73 mm³ for PBS, LIPO, or Pgenesil-2-HK-shRNA, respectively (analysis of variance, *p* < 0.05). In comparison, tumor volumes in mice with Pgenesil-2-cyclophilin A-shRNA were 159.56 ± 43.33 mm³, which were on average over 73% smaller than those in controls treated with PBS (Student’s *t* test, *p* < 0.01). These data indicated that cyclophilin A suppression by shRNA could significantly decrease tumor growth *in vivo* and with no apparent toxicity at this dose.

**Suppression of Cyclophilin A Decreased Cell Proliferation and Increased Apoptosis in Vivo**—It was apparent that cyclophilin A suppression inhibited cell proliferation and induced apoptosis *in vitro*. To obtain additional insight into the *in vivo* effects, tumor cell proliferation and apoptosis were assessed by PCNA immunoreactivity analysis and TUNEL assay. As shown in Fig. 6B, percentages of PCNA-positive nuclei in the three groups reached 89.4 ± 6.42, 87.6 ± 7.09, 81.6 ± 9.73 for PBS, LIPO, or Pgenesil-2-HK-shRNA, respectively (analysis of variance, *p* > 0.05). In comparison, tumor volumes in mice with Pgenesil-2-cyclophilin A-shRNA were 159.56 ± 43.33 mm³, which were on average over 73% smaller than those in controls treated with PBS (Student’s *t* test, *p* < 0.01). These data indicated that cyclophilin A suppression by shRNA could significantly decrease tumor growth *in vivo* and with no apparent toxicity at this dose.
PBS, LIPO, or Pgenesil-2-HK-shRNA (29.2 ± 7.62 versus 6.6 ± 2.70, 8.2 ± 2.86, or 11.6 ± 4.39; Dunnett’s t test, p < 0.01). In addition, no significant effects on proliferation or apoptosis were observed among control groups PBS (a), LIPO (b), or Pgenesil-2-HK-shRNA (c) (Dunnett’s t test, p < 0.01).

Thus, cyclophilin A suppression by shRNA could significantly inhibit cell proliferation and induce apoptosis in vivo.

**DISCUSSION**

Endometrial carcinoma remains one of the leading causes of death among women, and therefore the discovery of novel molecular targets for its diagnosis, prognosis, and treatment has the potential to improve the clinical strategy and outcome for this disease. In the present study, we compared the global protein profiles between endometrial carcinoma and matched normal endometrium using a 2-DE- and MS/MS-based approach. This approach makes no assumptions about known or unknown molecules, allowing the process to be independent of any presupposed hypotheses. A total of 99 differentially expressed proteins were identified, most of which were involved in biological processes, including metabolism, cell transformation, protein folding, cell proliferation and apoptosis, and cytoskeleton, that usually play crucial roles in the initiation and development of malignancy. Some identified proteins differed in their theoretical M_r and pI values as a result of post-translational modifications, and some of these migrated on the 2-DE gels as multiple spots with opposed changes. For example, of the five spots identified for stathmin, three were up-regulated and two were down-regulated in carcinoma tissues. This suggested that the different isoforms might have distinct functions during the course of carcinogenesis, and thus each isoform/spot was considered to be a distinct protein for analysis in our study. Only for protein of which changes of isoforms were all consistent, its overall level could be determined.

This comparison of protein profiles between carcinoma and normal tissues yielded interesting and interpretable results. However, biomarkers are known to have the potential to dramatically alter options and strategies of diagnosis and treatment, and consequently a complex process with multiple steps of confirmatory analyses is required for translating our proteomics observations into clinical applications. 21 of the 99 proteins with recurrence over four times were further selected as candidates involved in carcinoma-associated pro-
cesses, including thioredoxin peroxidase, mutant desmin, cyclophilin A, stathmin 1, glutathione S-transferase P, calcyphosine (CAPS), PEBP-1, epidermal fatty acid-binding protein (E-FABP), triose-phosphate isomerase 1, heat shock protein 27, transferrin, etc. E-FABP was shown to be increased 6.56-fold in carcinoma with three matched peptides, 22% sequence coverage, and a MOWSE score of 131. It is regarded to be involved in cellular signaling affecting differentiation, regulation of growth, and gene expression (28). It was identified as a cDNA down-regulated in nodal metastasis relative to the primary breast cancer (29) and up-regulated in esophageal squamous cell cancer (30). Proteomics analysis indicated decreased E-FABP in less differentiated bladder cancer (31). Recently E-FABP was shown to be a major target of c-Myc, which is induced by EpCAM (32). The kinetics of c-Myc and E-FABP induction following EpCAM expression suggested that EpCAM induces E-FABP via the transcription factor c-Myc (33). Thus, E-FABP might be identified as a target of EpCAM, contributing to the cellular changes during carcinogenesis and metastasis.

CAPS was shown to be increased 3.66-fold in carcinoma with seven matched peptides, 37% sequence coverage, and a MOWSE score of 305. It is a calcium-binding protein initially identified as a major phosphorylated substrate for cAMP-dependent protein kinase after stimulation of thyroid cells by thyrotropin (34). As it is phosphorylated in a cAMP-dependent manner, it is considered to be implicated in the cross-signaling between these cascades to coordinate cellular proliferation and differentiation (35). In a recent proteomics analysis, CAPS was found to be overexpressed in ependymomas compared with neuroectodermal tumors (36). Although the exact functions of CAPS remain to be elucidated, its activities in cAMP signaling and cell proliferation and differentiation suggest that it might be involved in an important aspect of carcinogenesis.

In the present study, cyclophilin A was further characterized as a potential prognostic biomarker and therapeutic target for endometrial carcinoma based on several selection criteria. (a) It is reported primarily as being involved in endometrial carcinogenesis. (b) It shows one of the most significant changes between normal and carcinoma tissues. (c) It is reliably identified by mass spectrometry. (d) It shows ubiquitous overexpression in carcinoma tissues (excluding the possibility of the effect being specific for rare subtypes and indicating that this is likely to be a common alteration).

2-DE analysis revealed that expression of cyclophilin A was significantly higher in endometrial carcinoma than in normal endometrium tissues, and its alteration was further confirmed by RT-PCR and Western blot analyses. As a member of the immunophilin family, cyclophilin A is an 18-kDa protein with a wide variety of functions. It is a peptidyl-prolyl cis-trans isomerase and an important component in protein folding. Initially it was thought to be present intracellularly but has been found to be secreted from cells in response to inflammatory stimulation (37, 38). Many studies have focused on its role in protein folding, immune response, and human immunodeficiency virus, type 1 infection (39–45) and demonstrated it to be the intracellular receptor for cyclosporin A. The cyclophilin A-cyclosporin A complex could inhibit the calcineurin activity and nuclear translocation of nuclear factor of activated T cells in T lymphocytes and other cells (46, 47).

As overexpression of cyclophilin A was routinely observed in endometrial carcinoma, studies were performed to examine whether there were any correlations among cyclophilin A expression, histodifferentiation/surgical pathologic staging, and survival rate, which would indicate potential usefulness as a biomarker for the prognosis, progression, or other aggressive behaviors of endometrial carcinoma. Our results showed that overexpression of cyclophilin A was significantly more likely to present with a low degree of differentiation, and survival analysis also showed the correlation of its overexpression with decreased survival. The findings suggested a potential value of assaying cyclophilin A expression for prognosis prediction and as a possible biomarker for clinical treatment of endometrial carcinoma. Consistent with a previous study in lung cancer (48), no significant correlation was observed between expression of cyclophilin A and surgical pathologic staging, and this may have been due in part to insufficient samples for statistical significance. It was striking that nearly all carcinoma specimens in this study with a broad range of stages commonly showed moderate or strongly positive staining against cyclophilin A, strongly suggesting that cyclophilin A overexpression might be involved in the process of carcinogenesis but not in advanced invasion or metastasis. In addition, given the ubiquitous expression of cyclophilin A in endometrial carcinoma, it might have some value in acting as a potential molecular therapeutic target.

Recent studies have indicated cyclophilin A expression in several cancer cells and that it might play a role in apoptosis through caspase activation and involvement of apoptosis-inducing factor. In support of this, cyclophilin A overexpression has been observed in lung cancer (20, 21), pancreatic cancer (22, 23), hepatocellular cancer (49), and buccal squamous cell carcinoma (50) by proteomics approaches. A novel cyclophilin, similar to cyclophilin A, was also found to be involved in metastasis with overexpression in bladder cancer, hepatic cancer, and breast cancer (51). This study was the first identification of cyclophilin A overexpression in endometrial carcinoma, and its precise role in carcinogenesis needs further investigation.

Previous reports have shown that inhibition of cyclophilin A in vivo led to lung cancer growth suppression and cell proliferation inhibition (52), and exogenous cyclophilin A promoted cancer cell growth through interaction with CD147 and activation of extracellular signal-regulated kinases ERK1/2 and p38 mitogen-activated protein kinases (MAPKs) (21, 53). Consistent with these results, our data also showed that inhibition of cyclophilin A expression led to marked suppression of cell proliferation in vitro as measured by MTT and clonogenic
formation assays. In contrast, a recent study reported that no differences in cell proliferation were observed between cyclophilin A transfectant and knockdown samples in various cancer cells (54), and loss of cyclophilin A in mouse embryonic stem cells had no effect on cell growth or viability (55). The discrepancies concerning the involvement of cyclophilin A on cell proliferation may be based on cell type-specific differences or experimental methods and conditions.

Apoptosis inhibition is considered essential for tumor growth, and therefore increasing sensitivity to apoptosis induction is considered a promising strategy for clinical treatment. Flow cytometry analysis and TUNEL assay demonstrated that cyclophilin A inhibition induced significant apoptosis in endometrial carcinoma cells accompanied by G1 arrest in cell cycle. Consistent with our results, another report showed that inhibition of cyclophilin A in vivo led to apoptosis induction in lung cancer cells that might be independent of the Bcl-2 pathway (52). Whereas in the context of atherosclerosis, several studies suggested that cyclophilin A promotes vascular smooth muscle cell growth and endothelial cell apoptosis in cooperation with apoptosis-inducing factor in apoptosis-associated chromatinolysis (56–59). The discrepancy between cancer and normal cells concerning the effect of cyclophilin A on apoptosis remains to be further elucidated.

The present data clearly indicated that suppression of cyclophilin A inhibits cell proliferation and induces apoptosis in vitro. To obtain additional insight into the in vivo effects, we observed the effects of cyclophilin A suppression on a tumor xenograft model. It was noteworthy that, consistent with the outcomes obtained in vitro, suppression of cyclophilin A in vivo was shown to result in decreased tumor growth accompanied with inhibition of cell proliferation and induction of apoptosis. The finding represents the first report of the remarkable activity of cyclophilin A both in vitro and in vivo against human endometrial carcinoma. In addition, no changes in gross measures, such as weight loss, feeding, behavior, or other signs of possible side effects, were observed during the in vivo treatment period, suggesting that cyclophilin A suppression by RNA interference seems to be safe and without detectable toxicity at least at the dose used. Consequently there is a clear indication that cyclophilin A is a promising novel therapeutic target against endometrial carcinoma. In addition, RNA interference treatment might be more effective in the clinic in combination with chemotherapy, which also provides us with an attractive approach for further investigation.

Angiogenesis is also known to be essential for tumor growth and might be enhanced by increased endothelial cell survival and activation. Recent studies indicated that cyclophilin A could induce extracellular signal-regulated kinase, c-Jun NH2-terminal kinase, and p38 kinase activation (57) and increase human umbilical vein endothelial cell proliferation, migration, invasive capacity, and tubulogenesis (60). In contrast, other studies have reported no correlation between angiogenesis and cyclophilin A knockdown using RNA interference as measured by CD31 staining and microvessel density (52). Clearly further studies should be conducted to clarify the precise role of cyclophilin A in angiogenesis.

Recent reports also indicated that up-regulation of cyclophilin A could render resistance to chemotherapeutic-induced apoptosis in cancer cells (54, 62). As an inhibitor of cyclophilin A peptidyl-prolyl isomerase activity, cyclosporin A had been showed to inhibit multidrug resistance in chemotherapy induced by P-glycoprotein and induce apoptosis in cancer cells (61, 63, 64); however, the precise mechanism of action remains to be established. Inhibition of cyclophilin A using cyclosporin A derivatives without immunosuppressant activity or novel inhibitors of cyclophilin A may likely improve the management of and the prognosis of patients with endometrial carcinoma. In the present study, we observed the over-expression of cyclophilin A in endometrial carcinoma. Inhibition of cyclophilin A expression using RNA interference might significantly suppress proliferation and induce apoptosis in endometrial carcinoma cells both in vitro and in vivo. The present findings suggest that cyclophilin A is a promising novel prognostic factor and possibly also a potential therapeutic target in the treatment of endometrial carcinoma.

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