Original contribution

Laser captured microdissection-microarray analysis of the genes involved in endometrial carcinogenesis: stepwise up-regulation of lipocalin2 expression in normal and neoplastic endometria, and its functional relevance

Running title: Endometrial carcinoma and lipocalin2

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Abstract

Background: Endometrial carcinoma often arises from normal endometrial glandular cells via a precursor, atypical endometrial hyperplasia. However, the genetic changes involved in this carcinogenetic process are not fully understood.

Methods: Differentially expressed genes were selected from glandular cells of normal proliferative phase endometria, atypical endometrial hyperplasia and endometrial carcinoma using laser-captured microdissection (LCM) and microarray.

Results: The microarray analysis revealed a total of 51 genes to be up-regulated, and 23 genes to be down-regulated in neoplastic endometrial epithelia. We focused on *lipocalin2* (*LCN2*), which showed the largest magnitude of up-regulation. Immunostaining for lipocalin2 confirmed a stepwise increase in its expression in endometrial hyperplasia and carcinoma. In addition, elevated expression of lipocalin2 was correlated with the poor outcome of endometrial carcinoma patients. The subcellular distribution of lipocalin2 was both cytoplasmic and nuclear, despite reports that lipocalin2 is a secretory protein. Treatment of endometrial carcinoma cells with 5-azacytidine increased the expression of lipocalin2, suggesting the expression to be controlled by methylation of the promoter. The forced expression of lipocalin2 resulted in the enhanced cell proliferation and invasion in vitro.

Conclusions: The expression of lipocalin2 increased with the endometrial carcinogenesis, and accumulation of the protein conferred biological aggressiveness to endometrial carcinoma cells. These results suggest lipocalin2 to be a novel target in the treatment of endometrial carcinoma.

Introduction

Uterine endometrial carcinoma is one of the most common malignancies in the female genital tract (1), accounting for approximately 25% of all deaths ascribed to cancer of the female genital tract in developed countries (2). The number of patients with this tumor has been increasing rapidly in Japan (3). Thus, further understanding of this malignancy in terms of the carcinogenetic process and biological characteristics is important for better management of this disease.

Endometrial carcinoma is clinicopathologically classified into two conceptional subgroups; type 1 and type 2 (1). The type 1 tumor is dominant, making up approximately 80% of all cases, and thought to arise from normal endometrial glandular cells via a precursor, atypical endometrial hyperplasia. Previous papers have reported the accumulation of genetic abnormalities, such as mutations of the PTEN, K-Ras, and p53 genes and microsatellite instability, to be involved in this tumorigenetic cascade (4). However, the percentage of endometrial carcinomas in which such genetic abnormalities have been identified is reportedly 50-60%, therefore, the involvement of other unknown genetic abnormalities has been presumed.

Difficulty in the detection of genetic abnormalities in endometrial carcinogenesis, especially those involved in the early stages of the hyperplasia-carcinoma sequence, is partly attributed to the histological structure of the normal endometrium. Normal endometrial glandular cells, from which endometrial carcinomas develop, are surrounded by ample stromal cells. The early step of neoplastic change is believed to occur in limited loci in endometrial glands as observed in endometrial hyperplasia. Therefore, if DNA is extracted from a tissue block, subtle changes may be missed due to the intermingling of the intervening stroma and normal glands. In this regard, the precise collection of tissue samples from neoplastic glands is mandatory to analyze the early and subtle genetic changes. To solve this

problem, we employed the laser captured microdissection (LCM) technique, which enabled us to obtain histologically specific cells under microscopic observation (5). Using this technique, we exclusively collected normal endometrial glandular cells, hyperplastic cells and carcinoma cells from tissue sections of the same patients to abrogate the effect of gene polymorphism. In addition, to detect the genes responsible for the neoplastic process, differences in gene expression among the three cell types were investigated using a microarray. Accordingly, a group of genes whose expression was up-regulated stepwise as the disease progressed was identified. Among them, we focused on *lipocalin2* (LCN2), because it had the largest magnitude of up-regulation in the neoplastic tissues. Lipocalin2, a small protein of 25kD, reportedly functions as a mediator of inflammation and iron ion transport (6~8). In this study, we examined the expression and possible functions of lipocalin2 in endometrial tissues.

Materials & Methods

Fresh tissue samples and laser-captured microdissection (LCM)

Immediately after the uterus was removed, fresh tissue of normal and neoplastic parts of approximately one hundred endometrial carcinomas was extirpated. The normal part was obtained from a macroscopically "flat" area with underlying myometrium and neoplastic part was mainly from the border of the tumor. These tissues were embedded in OCT compound, and flash frozen and stored at -80^{o}C prior to use. The histological diagnosis of these samples was made using hematoxylin and eosin (H &E)-stained frozen sections. Regarding the diagnostic criteria, endometrial hyperplasia was defined by marked glandular crowding (back to back structure) with cellular atypia such as round and enlarged nucleus, granular chromatin pattern and nucleoli. Endometrial carcinoma was defined by apparent stromal invasion as shown by multiple cribriform patterns. Cases lacking these findings were excluded.

Consequently, three cases that simultaneously contained normal proliferative, hyperplastic and cancerous areas were identified. All three carcinomas were endometrioid adenocarcinoma, grade 1. Likewise, there were only 3 cases that included normal proliferative and hyperplastic sites in the same patient. From these 6 cases, ten to thirty serial sections (10μm-thick for LCM, 5μm-thick for H & E) of each frozen tissue sample were cut using a Cryostat (Sakura Seiki, Tokyo, Japan), fixed in 70% ethanol. One of 5 sections was stained with H&E for reference, and the remainders were stained hematoxylin and used for LCM. Normal, hyperplastic and malignant glands were precisely collected using LCM (LM100, Olympus, Tokyo, Japan). At least 20,000 laser shots were performed to obtain the respective target tissue sample. Each fresh tissue sample was used with the approval of the Ethics Committee of Shinshu University, after obtaining written consent from the patients.

Microarray analysis

Total RNA was extracted from the tissues obtained by LCM, using an RNeasy Micro Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions, and then a quality of it was checked. The total RNA was then subjected to two rounds of T7-based aRNA amplification and aminoallyle labeling using an Amino Allyl MessageAmp aRNA Kit (Ambion, Austin, TX) according to the instructions. The amplified RNA (aRNA) was coupled with Cye-Dye. Cy5-coupled aRNA of neoplastic cells and cy3-coupled aRNA of normal glandular cells of the same patients were mixed and hybridized with AceGene Human oligo chip array 30k, subset A (Hitachi software, Tokyo, Japan) , containing 10,368 probe sets for 9881 genes, according to the instructions. The hybridized arrays were scanned using a Packard GSI Lumonics Scan Array 4000 (PerkinElmer, Boston, MA) and the results were quantitatively analyzed using QuantArray software (GSI Lumonics, Unterschleissheim, Germany). Data from each microarray were normalized using LOWESS normalization. We

selected genes which were expressed greater than 1.5-fold or less than 0.67-fold in neoplastic tissues compared with corresponding normal tissues.

Validation of gene expression using real time-quantitative PCR

Fresh frozen tissues from four samples each of normal proliferative endometrial glands, atypical endometrial hyperplasia and grade 1 endometrial carcinoma were subjected to analysis. Total RNA was extracted from the tissues using LCM and an RNeasy Micro Kit and treated with DNase I. cDNA was synthesized from each total RNA sample using PrimeScript RT reagent (Takara Bio Inc. Otsu, Japan). Reactions were carried out with the SYBR Green method. A SYBR Green PCR master mix (Takara Bio Inc.) was used in 20-μl reaction mixtures set up in triplicate with each primer. Reactions were run at 95°C for 10 seconds followed by 45 cycles of 95 °C for 3 seconds and 62 °C for 25 seconds. A dissociation curve was then used to ensure that the fluorescence signal was not derived from the formation of primer-dimer. The threshold number of cycles was determined. Gene expression was quantified by the comparative Ct method using β-actin (ACTB) as the internal control. Primer sets used for real time RT-PCR are summarized in Table 1 (Table 1).

Immunohistochemistry

Formalin-fixed and paraffin-embedded tissue specimens of the endometrium obtained by hysterectomy or biopsy were selected from the pathology files of Shinshu University Hospital, and used for immunohistochemistry. One hundred thirty-one cases were endometrioid adenocarcinoma treated between 1987 and 2004 with known age (from 29 to 90 years of age, median 57.3), stage (80: stage I, 12: stage II, 25: stage III, 14: stage IV), histological grade (80: grade 1, 25: grade 2, 26: grade 3), and follow-up survival data. Ten cases were atypical endometrial hyperplasia. Forty cases were normal endometrium (30: proliferative phase, 10:

secretory phase), obtained by hysterectomy for uterine myoma. Each tissue sample was used with the approval of the Ethics Committee of Shinshu University, after obtaining written consent from the patients.

Indirect immunohistochemical staining was performed using a goat-polyclonal anti-LCN2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Histofine SAB-PO detector kit (Nichirei, Tokyo, Japan) with microwave pretreatment as described previously (9). Granulocytes in the same tissue section were used as a positive control. Addition of the blocking peptide (Santa Cruz Biotechnology) prepared by the manufacturer diminished the staining. The immunoreactivity of normal or neoplastic endometrial glandular cells was semi-quantitatively evaluated according to the percentage of positive cells among 500 cells in 5 high power fields by two independent reviewers (T.M. and T.S.), and these results were described as a positivity index (PI), with a maximal score of 100. The significance of differences in PI was examined using the Kruskal-Wallis rank test and Sheffe's test. A P value of less than 0.05 was considered significant. Cumulative survival was also analyzed using the Kaplan-Meier method. The log-rank test was used to evaluate the significance of lipocalin2 for survival. These analyses were made using the SPSS Statistics system (SPSS Inc., Chicago, IL). To confirm the subcellular localization, immunohistochemical expression of lipocalin2 was re-examined using rat-monoclonal anti-human lipocalin2 antibody (R & D systems, Minneapolis, MN) in additional 40 cases of endometrial carcinoma.

Immunofluorescent staining

Immunofluorescent staining for cultured cells was performed as described previously (10), using a goat polyclonal anti-lipocalin2 antibody (x 50) and a Fluorescein Isothiocyanate (FITC)-conjugated anti-goat IgG antibody (x 100, green, Sigma-Aldrich, Saint Louis, MO).

Cell culture and transfection

The endometrial carcinoma cell lines Ishikawa, and HEC1A and HEC1B were gifts from Dr. H. Nishida (Kasumigaura Medical Center, Tsuchiura, Japan) and Dr. H. Kuramoto at Kitazato University (Sagamihara, Japan), respectively. HHUA was purchased from the Riken Cell Bank (Saitama, Japan) with the permission of Dr. Ishiwata at the Ishiwata Laboratory (Mito, Japan). KLE and RL95-2 were purchased from American Type Culture Collection (Rockville, MD). Ishikawa and HHUA were derived from well differentiated endometrial adenocarcinoma. HEC1A and HEC1B were from moderately differentiated endometrial adenocarcinoma. RL95-2 and KLE were from moderately differentiated adenosquamous carcinoma and poorly differentiated endometrial carcinoma, respectively. Primary cultures of normal proliferative phase endometrial glandular cells were prepared from the surgically resected endometrial tissues according to a previous study (9) with written consent from the patients. The human LCN2 expression vector, pCEP4-LCN2, was kindly provided by Dr. Kornelia Polyak (11).

To establish endometrial carcinoma cells that stably express lipocalin2, Ishikawa and HEC1B cells were transfected with pCEP4-LCN2 or pCEP4 alone by lipofection according to the supplier's instructions (Lipofectamine 2000, Invitrogen, Carlsbad, CA). At 48 hours after transfection, the medium was changed to selection medium and a stable expression clone was selected.

Western blot analysis

Proteins extracted from sub-confluent cultures of endometrial carcinoma cells (Ishikawa, HHUA, HEC1A, HEC1B, KLE) and cultured normal endometrial glandular cells were subjected to a Western blot analysis as described previously (9) using goat-polyclonal

anti-LCN2 antibody for primary antibody. Proteins of cytoplasmic and nuclear fractions were extracted using a NE-PER nuclear and cytoplasmic extraction Kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. Blocking was performed with 5% nonfat milk or 3% bovine serum albmin in PBS-T for 1 hour at room temperature. The membranes were blotted with primary antibody at 4^{o}C overnight and then incubated with a peroxidase-conjugated secondary antibody. Bound antibodies were visualized using the ECL western blot detection reagent (Amersham, Piscataway, NJ). To confirm the results obtained using goat-polyclonal anti-LCN2 antibody, the expression of lipocalin2 protein was re-examined using rat-monoclonal anti-human lipocalin2 antibody in Ishikawa cells.

RT-PCR

Endometrial carcinoma cell lines (Ishikawa, HHUA, HEC1A, HEC1B, KLE) and cultured normal proliferative phase endometrial glands were subjected to RT-PCR. Total RNA extracted by TRIzol reagent (Invitrogen) according to the manufacturer's instructions and primers for lipocalin2 and β-actin (Table 1) were used for RT-PCR. In brief, 1μg of total RNA was treated with 1 U/10 μl DNase I (Life Technologies, Gaithersburg, MD). RT was performed using an RNA PCR Kit (Takara Bio Inc.). The corresponding cDNA fragments were denatured at 94°C for 3 minutes, then subjected to 28 cycles of denaturing at 94 °C for 10 seconds, annealing at 58°C for 10 seconds and extension at 72°C for 20 seconds for lipocalin2, and 24cycles for β-actin.

Effect of the inhibition of methylation on the expression of lipocalin2

Endometrial carcinoma HHUA, Ishikawa, HEC1A, HEC1B, KLE and RL-95-2 cells were treated with a methylation inhibitor, 5-azacytidine **(**5-aza-C**,** Sigma-Aldrich)**,** at 5μM for 72 hours. Cells were harvested and RNA was extracted using TRIzol agent. The expression of

lipocalin2 mRNA was evaluated using a semi-quantitative RT-PCR.

WST-1 assay

Ishikawa and HEC1B cells transfected with pCEP4-LCN2 or pCEP4 alone were plated at a density of 2.0 x 10^3 cells into 96-well plates and cultured under optimal conditions (37 ^oC in a 5% CO_2 incubator) for measurement. The cell viability was measured by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (WST-1 assay) according to the manufacturer's instructions in post-plated day 1 (D-1), 3 (D-3), and 5 (D-5). A_{450} was measured using a microplate reader (Multiskan JX, Thermo Fisher Scientific).

Matrigel invasion assay

Matrigel invasion was performed according to a previous study (12). In brief, polycarbonate membranes (8.0 μm pore size) of the upper compartment of transwell culture chambers were coated with 5% Matrigel (Becton Dickinson Labware, Bedford, MA), and 1×10^5 Ishikawa or HEC1B cells transfected with pCEP4-LCN2 or pCEP4 alone were plated on the upper chamber. Twenty two hours later, the number of migratory cells on the lower surface was counted in arbitrarily selected 10 high power fields. Each experiment was performed in three wells and repeated three times. The statistical analysis was conducted with the Mann-Whitney U test.

Results

According to the laser captured microdissection-microarray analysis, a total of 51 genes were up-regulated, and 23 genes were down-regulated in hyperplastic and cancerous endometrial epithelia compared with normal glandular cells (Fig. 1). Among them, 25 genes were selected according to the possible oncogenetic characteristics on the basis of GenBank data

(http://www.ncbi.nlm.nih.gov/Genbank/), and subjected to real-time RT-PCR to quantitatively ascertain the level of their expression. Accordingly, we selected nine up-regulated genes and one down-regulated gene (Fig. 2) that were expressed greater than 2-fold or less than 0.5-fold in more than three-fourth cases of hyperplasia and/or carcinoma compared with normal endometria. Of the nine up-regulated genes, *lipocalin2* (LCN2) was eventually selected for further analysis, because it showed the largest magnitude of amplification in carcinoma tissues compared to normal and hyperplastic endometria.

Immunohistochemical expression of lipocalin2 protein

Immunoreactivity for lipocalin2 protein using goat polyclonal antibody in formalin-fixed, paraffin-embedded sections was observed both in the cytoplasm and in the nucleus (Figs. 3a-e). Because the nuclear staining of lipocalin2 has not been reported, immunohistochemical expression of lipocalin2 was re-examined using another antibody; rat-monoclonal anti-human lipocalin2 antibody. The result also indicated the similar cytoplasmic and nuclear staining (Fig. 3f).

The positivity index (PI) for cytoplasmic lipocalin2 in normal endometrial glands in the proliferative and secretory phases was 1.6 ± 2.1 and 1.6 ± 2.5 (mean ± standard deviation, SD), respectively (Fig. 3g). The cytoplasmic PI of lipocalin2 increased in a stepwise manner, and the PI of atypical endometrial hyperplasia and endometrial carcinoma was 4.7 ± 4.7 and 10.3 ± 14.1, respectively. The cytoplasmic PI of lipocalin2 in carcinoma was significantly higher than that in the proliferative phase ($p=0.020$, Fig. 3g). There were a few cells with nuclear staining in normal endometrium and hyperplasia, whereas clear nuclear staining was observed in 22 cases of endometrial carcinoma (Fig. 3e). The cytoplasmic PI of grade 2 tumors was significantly greater than that of grade 1($p=0.009$, Fig. 3 h). The nuclear PI of grade 3 tumors and stage III-IV tumors were significantly greater than that of grade 1

(p=0.048) and stage I-II (p=0.002), respectively (Fig. 3h). Regarding the prognostic value of lipocalin2, endometrial carcinoma patients with elevated level of lipocalin2 protein (PI in the cytoplasm≧10, 47/131) had significantly shorter survival periods (p=0.012, Fig. 4a). Likewise, elevated nuclear staining (PI in the nucleus≧10, 22/131) was associated with the poor survival (p<0.0005, Fig. 4b). In stage III-IV patients, the survival of those with elevated cytoplasmic lipocalin2 expression was shorter than that with non-elevated lipocalin2, but the difference was not significant (p=0.108, Fig. 4c). However, the survival of patients with nuclear expression of lipocalin2 was significantly shorter than that with weak or no expression of lipocalin2 (p=0.003, Fig. 4d).

Expression of lipocalin2 in endometrial carcinoma cells

The expression of lipocalin2 protein and mRNA was examined in five endometrial carcinoma cell lines using Western blotting and RT-PCR. In Western blotting, two specific bands (approximately 25kDa and 30kDa), which indicate the difference of glycosylation of lipocalin2 (13), were observed. Similar results were obtained when rat anti-lipocalin2 monoclonal antibody was used (data not shown). The level of lipocalin2 protein correlated well with that of lipocalin2 mRNA. The expression of both lipocalin2 protein and mRNA was relatively strong in endometrial carcinoma Ishikawa and HHUA cells, but weak in HEC1A, HEC1B and KLE cells (Fig. 5a).

Subcellular localization of lipocalin2 protein

Because immunostaining for lipocalin2 in endometrial carcinoma showed both cytoplasmic and nuclear staining, we investigated the subcellular localization of lipocalin2 using endometrial carcinoma cell lines. The expression of lipocalin2 protein recognized by goat-polyclonal anti-LCN2 antibody was ascertained both in the cytoplasmic and in the

nuclear fraction of HHUA and Ishikawa cells (Fig. 5b). Nuclear and cytoplasmic expression of lipocalin2 in Ishikawa cells was also detected by rat-monoclonal anti-human lipocalin2 antibody (data not shown). Immunolabeling of lipocalin2 in HHUA cells also showed both nuclear and cytoplasmic staining (Fig. 5c).

Lipocalin2 expression and lipocalin2 gene methylation

Because the expression of lipocalin2 in pancreatic carcinoma was reportedly enhanced by hypomethylation of the lipocalin2 gene promoter (14), we examined the effect of DNA methylation on the expression of lipocalin2 using a de-methylating agent, 5-aza-C. The results indicated that the expression of lipocalin2 in HEC1A, HEC1B and KLE cells, which showed relatively weak expression of lipocalin2 mRNA before the treatment by 5-aza-C, increased after the treatment (Fig. 5d).

Effect of lipocalin2 on cell proliferation and invasion

We then examined the effect of lipocalin2 on cell proliferation and invasion using endometrial carcinoma Ishikawa and HEC1B cells. Transfection with a lipocalin2-expressing vector, pCEP4-LCN2, was confirmed to increase the expression of lipocalin2 mRNA. The WST-1 assay revealed that the forced expression of lipocalin2 significantly increased cell viability of HEC1B cells by 371 % ($p=0.0001$), and that of Ishikawa cells by 27 % (Fig. 6a, b). The contribution of lipocalin2 to invasive potential was also examined using the Matrigel invasion assay. The results indicated that transfection of the lipocalin2 gene increased the number of invading cells by 89 % ($p=0.0002$) and by 645% ($p<0.0001$) in Ishikawa cells and HEC1B cells, respectively (Fig. 6c).

Discussion

The method of laser captured microdissection (LCM) used in the present study makes it possible to obtain highly specific cells from thin-sliced tissue sections. Therefore, the technique is suitable for collecting small samples of a target tissue, and this advantage is enhanced when the target sites is surrounded by stromal cells such as the endometrium. With the present microarray system, at least 5μg of aminoallyl antisense RNA is required according to the manufacturer. However, the LCM retrieved only 100-500ng of total RNA even when we punched out more than 20,000 laser shots in one tissue sample. Therefore, we were obliged to perform two rounds of preparatory T7-RNA polymerase amplification for the microarray analysis. The first round yielded about 1μg of aRNA, while the second amplification generated more than 50 μg, enough for a microarray analysis. However, the possibility that long genes might have been lost during the two rounds of amplification must be taken into account. Also, there is a possibility that genetic changes such as point mutations in exons cannot be detected. However, when the transcription of a gene is regulated by a point mutation of the promoter sequence, detection is theoretically possible by detecting changes in the transcription of downstream genes. In the present study, we eventually focused on *lipocalin2*, because the increase in the expression of lipocalin2 during endometrial carcinogenesis was the largest observed.

Lipocalin2, also referred to as neutrophil gelatinase-associated lipocalin (NGAL), belongs to the lipocalin group. Lipocalins are a diverse family of over 20 small soluble, and often secreted, proteins (6~8). They have a large degree of diversity at the sequence level (only 20% identity), although most share three conserved motifs. These proteins are defined as lipocalins largely on the basis of their three-dimensional structure, which comprises a single eight-stranded, continuously hydrogen-bonded anti-parallel β-barrel. This antiparallel structure forms an enclosing cavity that is thought to bind a wide variety of low-molecular-mass molecules, including retinoids, arachidonic acid, various steroids and

iron. Thus lipocalins are generally considered as transporters with several functions. These functions include the regulation of immune responses, modulation of cell growth and differentiation, transportation of iron and synthesis of prostaglandin. To our knowledge, this is the first report of lipocalin2 expression in normal and neoplastic endometria.

In mouse uterus, the expression of lipocalin was reportedly induced by estradiol and suppressed by progesterone (15). The expression of lipocalin2 in breast epithelial cells was also stimulated by estrogen (11). These studies suggested the hormone-dependent expression of lipocalin2 and its involvement in estrogen-dependent growth of estrogen receptor (ER)-positive cells. However in the present study, the expression of lipocalin2 protein was limited both in the proliferative and secretory phases in normal endometria, suggesting that lipocalin2 protein does not directly contribute to the estrogen-induced growth of normal endometrial glands. The role of weak expression of lipocalin2 in normal endometrial glands remains undetermined. However, the expression of lipocalin2 may be involved in remodeling of the endometrium after menstrual shedding, because lipocalin2 repaired drug-induced damage in mouse gastrointestinal mucosa (16).

The expression of lipocalin2 increased step-wisely in endometrial hyperplasia and carcinoma, suggesting that the protein is oncogenic. Likewise, lipocalin2 was reported to be increased in luminal epithelial cells compared with myoepithelial cells in breast tissues (17), and a majority of breast carcinomas are thought to arise from the luminal epithelium (18). In addition, lipocalin2 is required for BCR-ABL-induced leukemia (19). These studies indicated that lipocalin2 may participate the tumorigenetic process of several cancers. However, transfection of the lipocalin2 gene into mouse NIH3T3 cells did not induce cell transformation (data not shown). Therefore, further studies are needed to clarify the tumorigenetic role of lipocalin2.

Recent studies revealed the overexpression of lipocalin2 in cancers of the lung, colon,

pancreas, breast and ovary (20~22). The present study revealed that the expression of lipocalin2 was increased in endometrial carcinomas. In addition, the expression of lipocalin2 protein was increased in histologically higher grade and advanced stage tumors. These results suggest lipocaln2 to be involved in the acquisition of malignant potential of endometrial carcinoma cells. In addition, the patients with elevated expression of lipocalin2 showed significantly shorter survivals, indicating the possibility of lipocalin2 to be a novel therapeutic target. Lipocalin2 has been reported to be expressed in the cytoplasm and to act as a secretory protein. However interestingly, the present study clearly demonstrated its nuclear localization, especially in endometrial carcinoma cells both in vivo and in vitro. Lipocalin2 is a small molecule and able to pass through the pores of the nuclear membrane, thus, nuclear transportation signaling is unlikely to be needed (23). However, more studies are warranted to elucidate the distinct roles of lipocalin2 based on the differences in its subcellular localization.

The mechanisms up-regulating lipocalin2 expression are not fully understood. However, in thyroid cancer, the expression of lipocalin2 was regulated by NF-κB (24). In addition, Sato et al. reported that the overexpression of lipocalin2 in pancreatic cancer cells was associated with hypomethylation of the lipocalin2 gene promoter. In addition, lipocalin2 was overexpressed in esophageal squamous cell carcinoma, and regulated by hypomethylation of the promoter (25). Actually, lipocalin2 genomic sequence in Entrez Gene (http://www.ncbi.nlm.nih.gov/gene) revealed 25 CpG sites in 1500bp 5’-upstream from transcription initiation site. In the present study, treatment with 5-aza C of endometrial carcinoma cells that showed weak expression of lipocalin2 resulted in the elevated levels of lipocalin2 mRNA. This result suggested the expression of lipocalin2 mRNA to be, at least partly, regulated by the promoter’s hypomethylation.

The in vitro experiments in this study indicated that lipocalin2 stimulated the

proliferation of endometrial carcinoma cells, but the growth-stimulatory effect of lipocalin2 differed between Ishikawa and HEC1B cells. This is in part because the effect of forced expression of lipocalin2 might have been diluted in Ishikawa cells which had abundant intrinsic lipocalin2 protein compared to HEC1B cells. However, the growth- stimulatory mechanisms of lipocalin2 are largely unknown. The present study also revealed that lipocalin2 enhanced the invasive potential of endometrial carcinoma cells. This result may explain the increased expression of lipocalin2 in stage IV tumors, and the association of the lipocalin2 overexpression with poor outcome of the patients. The effect of lipocalin2 on invasive/metastatic potential has been controversial. In colon carcinoma, lipocalin2 reportedly decreased E-cadherin-mediated cell-cell adhesion and increased invasion via Rac1 (26). In addition, Yang J, et al reported the involvement of lipocalin2 in epithelial to mesenchymal transition (EMT) in breast carcinoma and thus metastasis (27). In contrast, Lee et al. reported that lipocalin2 suppressed the invasion and liver metastasis of colon cancer cells (13). Hanai reported that lipocalin2 diminished the invasiveness and metastasis of Ras-transformed cells (28). These reports suggest the function of lipocalin2 in cell invasiveness to be influenced by cell context. It is worth noting that the effect of lipocalin2 can be controlled in an autocrine/paracrine mechanism, because a recent study identified the lipocalin2 receptor (29).

In conclusion, we identified lipocalin2 using a LCM-microarray technique as a protein whose expression was up-regulated during tumorigenetic and developmental processes of endometrial carcinoma. Experiments in vitro revealed that the expression of lipocalin2 affected the growth and invasive potential of endometrial carcinoma cells. Although further studies are warranted to clarify the molecular mechanisms behind the expression and function of lipocalin2, the results of the present study indicate lipocalin2 to be a potential molecular target in the treatment of endometrial carcinoma. .

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References

(1) Ronnett BM, Zaino RJ, Ellenson LH, Kurman RJ. Endometrial carcinoma. In: Kurman RJ editor. Blaustein's pathology of the female genital tract. 5th edition. New York, USA: Springer-Verlag New York, Inc.; 2002. p. 501-59.

(2) Barakat R, Grisby P, Sabbatini P. Corpus. In: Epithelial tumors. Principles and practice of gynecologic oncology. 3rd edition. Philadelphia, USA: Lippincott Williams & Wilkins; 2000. p. 919-59.

(3) Konishi I. Japan society of obstetrics and gynecology, Annual report. Acta Obstetrica et Gynecologica Japonica 2009; **61**: 939-63.

(4) Lax SF. Molecular genetic pathways in various types of endometrial carcinoma: from a phenotypical to a molecular-based classification. Virchows Arch 2004; **444**: 213-23.

(5) Fend F, Raffeld M. Laser capture microdissection in pathology. J Clin Pathol 2000; **53**: 666-72.

(6) Flower DR, North AC, Sansom CE. The lipocalin protein family: structural and sequence overview. Biochim Biophys Acta 2000; **1482**: 9-24.

(7) Grzyb J, Latowski D, Strzalka K. Lipocalins - a family portrait. Journal of Plant Physiology 2006; **163**: 895-915.

(8) Bratt T. Lipocalins and cancer. Biochim Biophys Acta 2000; **1482**:318-26.

(9) Shiozawa T, Horiuchi A, Kato K, Obinata M, Konishi I, Fujii S, Nikaido T. Up-regulation of p27Kip1 by progestins is involved in the growth suppression of the normal and malignant human endometrial glandular cells. Endocrinology 2001; **142**: 4182-8.

(10) Kikuchi N, Horiuchi A, Osada R, Imai T, Wang C, Chen X, Konishi I. Nuclear expression of S100A4 is associated with aggressive behavior of epithelial ovarian carcinoma: an important autocrine/paracrine factor in tumor progression. Cancer Sci

2006; **97**: 1061-9.

(11) Seth P, Porter D, Lahti-Domenici J, Geng Y, Richardson A, Polyak K. Cellular and molecular targets of estrogen in normal human breast tissue. Cancer Res 2002; **62**: 4540-4.

(12) Horiuchi A, Kikuchi N, Osada R, Wang C, Hayashi A, Nikaido T, Konishi I. Overexpression of RhoA enhances peritoneal dissemination: RhoA suppression with Lovastatin may be useful for ovarian cancer. Cancer Sci 2008; **99**: 2532-9.

(13) Lee HJ, Lee EK, Lee KJ, Hong SW, Yoon Y, Kim JS. Ectopic expression of neutrophil gelatinase-associated lipocalin suppresses the invasion and liver metastasis of colon cancer cells. Int J Cancer. 2006; **118**: 2490-7.

(14) Sato N, Fukushima N, Matsubayashi H, Goggins M. Identification of maspin and S100P as novel hypomethylation targets in pancreatic cancer using global gene expression profiling. Oncogene 2004; **23**: 1531-8.

(15) Huang HL, Chu ST, Chen YH. Ovarian steroids regulate 24p3 expression in mouse uterus during the natural estrous cycle and the preimplantation period. J Endocrinol 1999; **162**: 11-9.

(16) Playford RJ, Belo A, Poulsom R, Fitzgerald AJ, Harris K, Pawluczyk I, Ryon J, Darby T, Nilsen-Hamilton M, Ghosh S, Marchbank T. Effects of mouse and human lipocalin homologues 24p3/lcn2 and neutrophil gelatinase-associated lipocalin on gastrointestinal mucosal integrity and repair. Gastroenterology 2006; **131**: 809-17.

(17) Jones C, Mackay A, Grigoriadis A, Cossu A, Reis-Filho JS, Fulford L, Dexter T, Davies S, Bulmer K, Ford E, Parry S, Budroni M, Palmieri G, Neville AM, O'Hare MJ, Lakhani SR. Expression profiling of purified normal human luminal and myoepithelial breast cells: identification of novel prognostic markers for breast cancer. Cancer Res 2004; **64**: 3037-45.

(18) Taylor-Papadimitriou J, Lane EB. Keratin expression in the mammary gland. In: Neville KC, et al, editors. The Mammary Gland. Development, Regulation and Function. New York, USA: Plenum Press; 1987. p. 185-215.

(19) Leng X, Lin H, Ding T, Wang Y, Wu Y, Klumpp S, Sun T, Zhou Y, Monaco P, Belmont J, Aderem A, Akira S, Strong R, Arlinghaus R. Lipocalin 2 is required for BCR-ABL-induced tumorigenesis. Oncogene 2008; **27**: 6110-9.

(20) Friedl A, Stoesz SP, Buckley P, Gould MN. Neutrophil gelatinase-associated lipocalin in normal and neoplastic human tissues. Cell type-specific pattern of expression. Histochem J 1999; **31**: 433-41.

(21) Stoesz SP, Friedl A, Haag JD, Lindstrom MJ, Clark GM, Gould MN. Heterogeneous expression of the lipocalin NGAL in primary breast cancers. Int J Cancer 1998; **79**: 565-72.

(22) Cho H, Kim JH. Lipocalin2 expressions correlate significantly with tumor differentiation in epithelial ovarian cancer. J Histochem Cytochem 2009; **57**: 513-21.

(23) Paine PL, MooreLC, Horowitz SB. Nuclear envelope permeability. Nature 1975; **254**: 109-14.

(24) Iannetti A, Pacifico F, Acquaviva R, Lavorgna A, Crescenzi E, Vascotto C, Tell G, Salzano AM, Scaloni A, Vuttariello E, Chiappetta G, Formisano S, Leonardi A. The neutrophil gelatinase-associated lipocalin (NGAL), a NF-kappaB-regulated gene, is a survival factor for thyroid neoplastic cells. Proc Natl Acad Sci U S A 2008; **105**: 14058-63.

(25) Cui L, Xu LY, Shen ZY, Tao Q, Gao SY, Lv Z, Du ZP, Fang WK, Li EM. NGALR is overexpressed and regulated by hypomethylation in esophageal squamous cell carcinoma. Clin Cancer Res 2008; **14**: 7674-81.

(26) Hu L, Hittelman W, Lu T, Ji P, Arlinghaus R, Shmulevich I, Hamilton SR, Zhang W.

NGAL decreases E-cadherin-mediated cell-cell adhesion and increases cell motility and invasion through Rac1 in colon carcinoma cells. Lab Invest 2009; **89**: 531-48.

(27) Yang J, Bielenberg DR, Rodig SJ, Doiron R, Clifton MC, Kung AL, Strong RK, Zurakowski D, Moses MA. Lipocalin 2 promotes breast cancer progression. Proc Natl Acad Sci U S A 2009; **106**: 3913-8.

(28) Hanai J, Mammoto T, Seth P, Mori K, Karumanchi SA, Barasch J, Sukhatme VP. Lipocalin 2 diminishes invasiveness and metastasis of Ras-transformed cells. J Biol Chem 2005; **280**: 13641-7.

(29) Devireddy LR, Gazin C, Zhu X, Green MR. A cell-surface receptor for lipocalin 24p3 selectively mediates apoptosis and iron uptake. Cell 2005; **123**: 1293-305.

Figure legends

Fig. 1 Results of the microarray analysis. A total of 51 genes were up-regulated greater than 1.5-fold, and a total of 23 genes down-regulated less than 0.67-fold, in hyperplastic and/or malignant endometrial epithelia compared with corresponding normal endometrial glands in more than two-third cases.

Fig. 2 Results of the real-time quantitative RT-PCR. mRNA No.1 to No.4 were obtained from normal endometrial glandular epithelium, No.5 to No.8 were from atypical endometrial hyperplasia, and No.9 to No.12 were from endometrial carcinoma. The mRNA expression levels were indicated by the relative ratio against case No.1. Gray-colored genes indicated those expressed greater than 2-fold or less than 0.5-fold in more than three-fourth cases of hyperplasia and/or carcinoma compared with case No.1.

Fig. 3 Results of immunostaining for lipocalin2. Photomicrographs of immunostaining in normal endometrium in the proliferative phase (**a**), normal endometrium in the early secretory phase (**b**), atypical endometrial hyperplasia (**c**), grade 1 endometrial carcinoma (**d**), grade 2 endometrial carcinoma (**e, f**). **a-e** are stained with goat anti-lipocalin2 polyclonal antibody, and **f** with rat anti-lipocalin2 monoclonal antibody. Immunoreactivities for lipocalin2 are negligible in normal endometrium (**a**, **b**), weakly positive in atypical hyperplasia (**c**) and strongly positive in endometrial carcinoma (**d, e**). Nuclear staining for lipocalin2 is predominantly observed in grade 2 endometrial carcinomas (**e**, **f**). Graphic demonstration of the results of immunostaining for lipocalin2 (**g**, **h**). The cytoplasmic (gray boxes) staining increased with as the disease progressed from normal endometrium, through endometrial hyperplasia to endometrial carcinoma. Nuclear immunoreactivity (white boxes) was observed predominantly in carcinoma (**g**). Graphic demonstration of the results in endometrial

carcinoma according to histological grade and stage (**h**). ＊1, ＊2 and ＊3: significant difference from grade 1 (p=0.009), grade 1 (p=0.048) and stage I-II (p=0.002), respectively. The error bars indicated standard deviation.

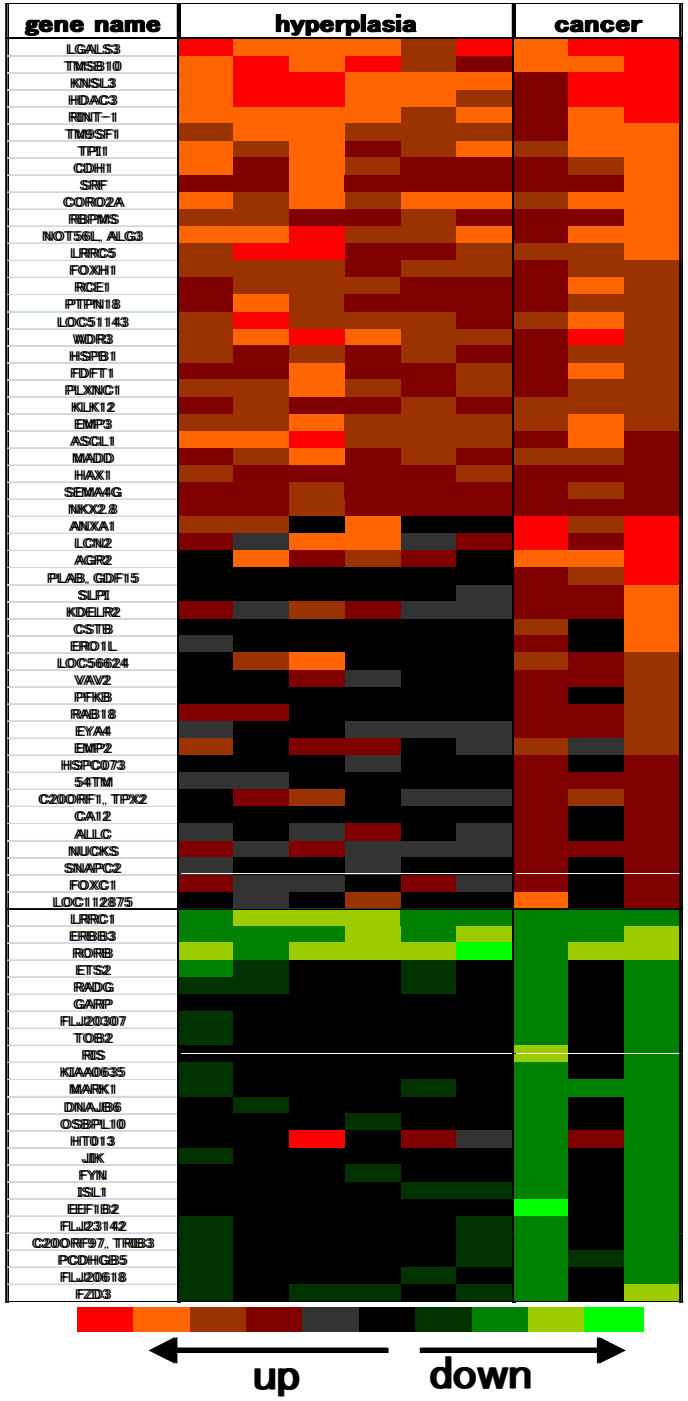
Fig. 4 Cumulative survival (Kaplan-Meier method) of the patients with endometrial carcinoma according to the PI of lipocalin2 immunoreactivity. Cumulative survival of all endometrial carcinoma patients according to the cytoplasmic PI (**a**) and nuclear PI (**b**). Patients with increased PI showed the poor survival with a significant difference. Cumulative survival of stage III-IV patients according to cytoplasmic PI (**b**) and nuclear PI (**c**). Patients with nuclear PI≧10 showed a significantly shorter survival.

Fig. 5 Expression of lipocalin2 mRNA and protein in five endometrial carcinoma cell lines (**a**). Lipocalin2 protein was detected in both the cytoplasmic (α-tubulin) and nuclear (lamin B) fractions of HHUA and Ishikawa cells (**b**). Abbreviations; NE: cultured normal endometrial glandular cells, T: total protein, C: cytoplasmic fraction, N: nuclear fraction. (**c)** Immunofluorescent staining for lipocalin2 in HHUA cells. Both cytoplasmic and nuclear staining was observed. (**d)** Effect of a methylation inhibitor, 5-aza-C, on the expression of lipocalin2 mRNA in six endometrial carcinoma cell lines. The expression of lipocalin2 mRNA recovered after the treatment with 5-aza-C in three cell lines (HEC1A, HEC1B, and KLE) showing weak expression of lipocalin2.

Fig. 6 Effect of the forced expression of lipocalin2 on the proliferation of endometrial carcinoma Ishikawa (**a**) and HEC1B (**b**) cells assessed by a WST-1 assay in post-plated day 1 (D-1), 3 (D-3), and 5 (D-5). The cell viability was indicated by the relative ratio against D-1. (**c**) Effect of the forced expression of lipocalin2 on the invasive potential of Ishikawa and

HEC1B cells assessed by a Matrigel invasion assay. Lipocalin2 stimulated the proliferation and invasion of Ishikawa and HEC1B cells compared to control cells. *: $p<0.05$. The error bars indicate standard deviation. Abbreviations; LCN2: the cells transfected pCEP4-LCN2, pCEP4: the cells transfected pCEP4 empty vector, HPF: high power fields

Figure 1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RBPMS
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JIK
FYN
ISL1
EEF1B2
FLJ23142
C20ORF97,, TRIB3
PCDHGB5
FLJ20618
FZD3
up
down

Fig. 2

Gene \ Case	Normal				Hyperplasia				Carcinoma			
	1	2	3	4	5	6	7	8	9	10	11	12
LCN2/ACTB	1.00	1.45	0.56	5.79	30.06	7.88	128.03	16.90	8.90	10.66	602.87	339.17
SLPI/ACTB	1.00	3.06	0.27	1.17	18.42	8.05	10.39	8.01	0.94	9.27	43.13	14.92
TRIB3/ACTB	1.00	1.81	1.41	1.95	9.83	9.13	1.12	5.37	40.28	5.94	4.05	2.04
ERO1L/ACTB	1.00	0.82	0.84	1.09	2.74	4.19	1.68	1.99	4.51	3.62	4.44	4.14
ANXA1/ACTB	1.00	0.96	0.32	1.04	3.69	2.91	3.69	0.45	0.76	1.31	7.01	2.33
EMP2/ACTB	1.00	0.60	0.86	1.08	3.00	2.45	3.12	0.64	1.98	1.66	5.80	2.47
SNAPC2/ACTB	1.00	2.15	0.78	1.18	1.34	5.23	2.12	3.25	0.48	1.24	3.08	0.80
ERBB3/ACTB	1.00	0.97	0.84	1.07	2.03	2.77	1.22	3.00	2.04	1.29	1.85	0.93
PTPN18/ACTB	1.00	1.30	1.37	1.17	2.49	1.94	2.13	2.42	0.72	1.10	2.90	1.11
HT013/ACTB	1.00	1.99	2.12	1.44	1.32	6.17	0.95	8.69	0.63	1.46	1.48	2.29
CORO2A/ACTB	1.00	1.36	0.71	0.71	1.11	2.73	1.62	1.59	1.06	1.57	3.90	2.57
RINT1/ACTB	1.00	0.97	1.06	0.95	2.36	3.14	1.09	1.84	0.81	1.51	2.08	1.64
HSPB1/ACTB	1.00	1.61	1.14	0.84	2.19	1.88	3.57	1.87	0.52	1.54	2.59	1.49
RAB18/ACTB	1.00	0.64	0.93	0.91	1.06	3.43	0.53	0.62	1.49	1.43	0.55	3.42
HDAC3/ACTB	1.00	0.74	1.00	0.91	0.71	2.60	0.59	0.80	0.92	1.23	0.66	1.70
RCE1/ACTB	1.00	1.17	1.07	1.31	1.46	2.75	1.03	1.69	0.91	1.17	1.41	1.46
LRRC1/ACTB	1.00	0.82	1.11	1.60	2.18	2.26	0.71	1.02	0.78	1.81	1.09	1.25
SRF/ACTB	1.00	1.30	0.81	1.05	0.88	1.47	0.65	1.06	0.76	0.95	0.94	1.16
TM9SF1/ACTB	1.00	1.07	0.91	1.23	0.75	1.92	0.76	1.46	0.55	1.14	0.63	1.02
WDR3/ACTB	1.00	0.91	0.82	1.12	0.88	2.06	1.04	1.36	0.63	0.81	1.93	0.95
FOXH1/ACTB	1.00	1.17	0.37	0.11	2.35	1.48	0.71	0.31	2.29	1.14	4.79	0.39
AGR2/ACTB	1.00	4.14	2.25	1.42	23.67	7.48	0.14	2.48	0.10	5.74	0.30	4.45
KNSL3/ACTB	1.00	0.87	0.56	0.51	0.28	4.02	0.23	1.62	0.09	#VALUE!	0.63	1.21
RORB/ACTB	1.00	0.54	1.18	1.90	0.18	0.66	0.15	0.56	0.06	0.06	0.36	3.44
TPX2/ACTB	1.00	0.47	0.84	0.96	0.51	0.12	0.58	0.02	0.96	1.22	0.22	0.29

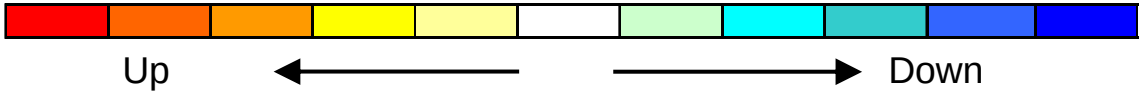
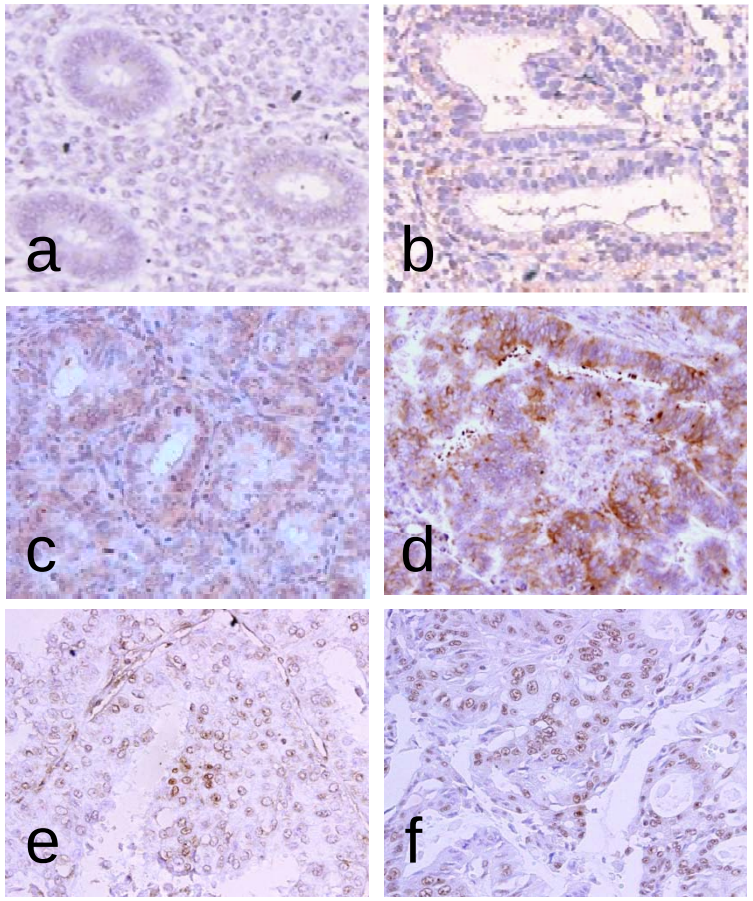
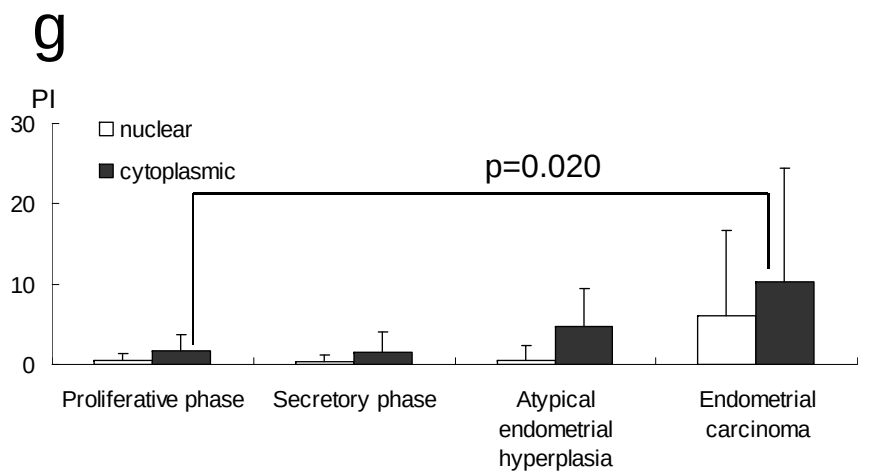


Fig. 3


a
b
c
d
e
f


g
PI
30
20
10
0
nuclear
cytoplasmic
p=0.020
Proliferative phase
Secretory phase
Atypical endometrial hyperplasia
Endometrial carcinoma

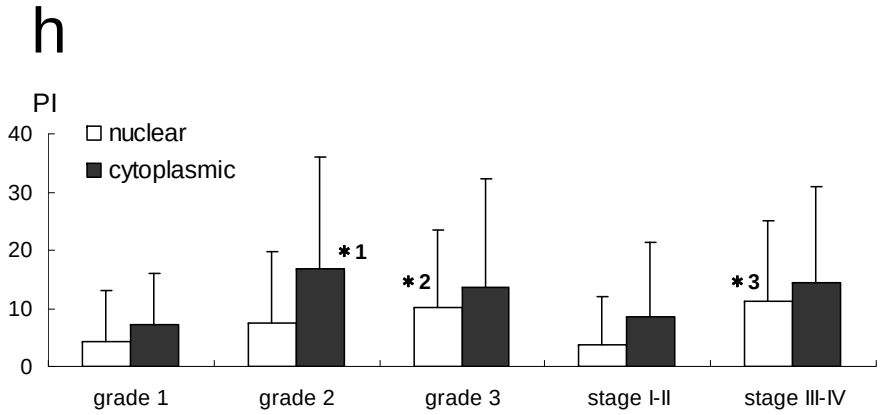

h
PI
40
30
20
10
0
nuclear
cytoplasmic
*1
*2
*3
grade 1
grade 2
grade 3
stage I-II
stage III-IV

Fig. 4

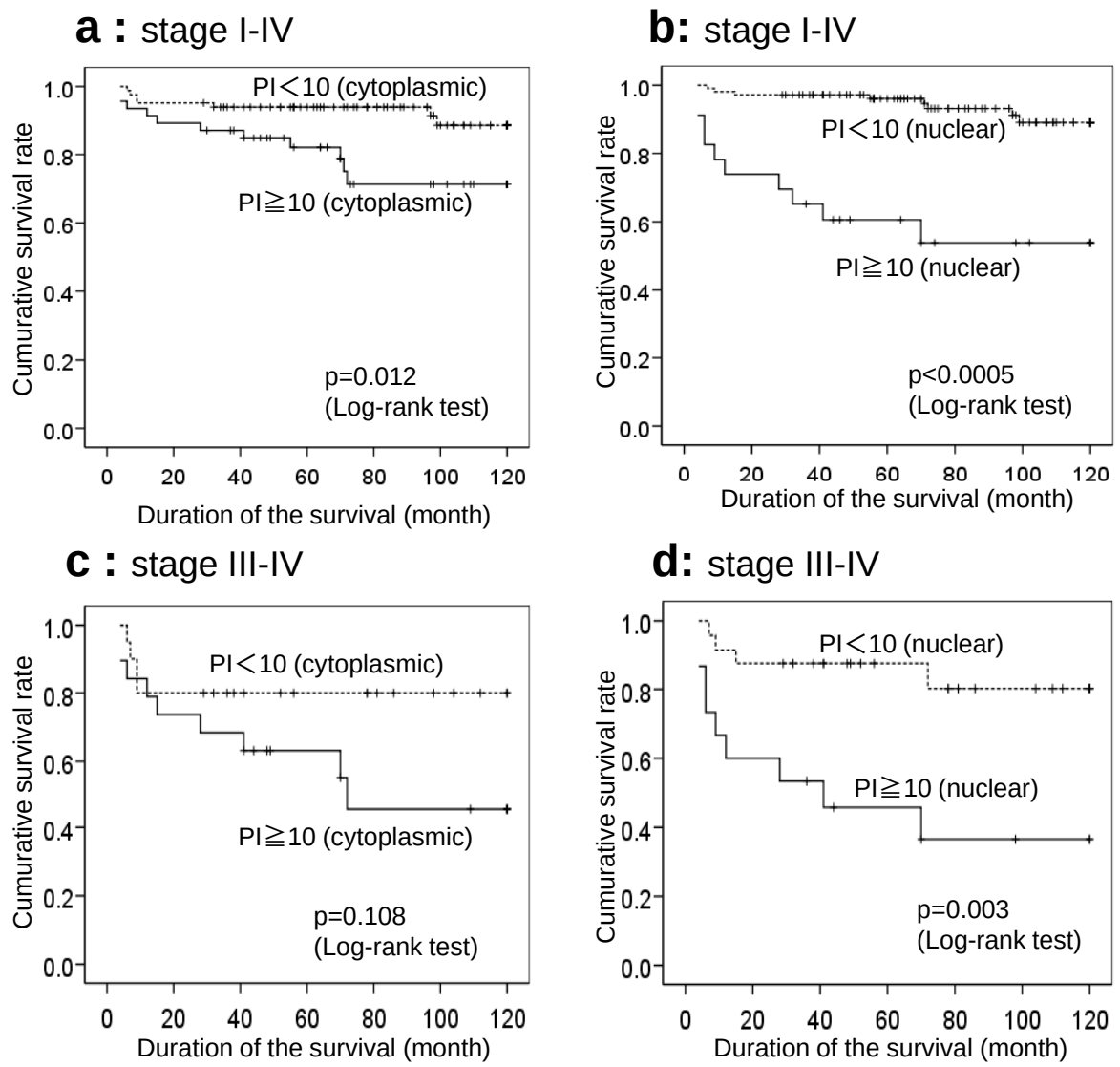
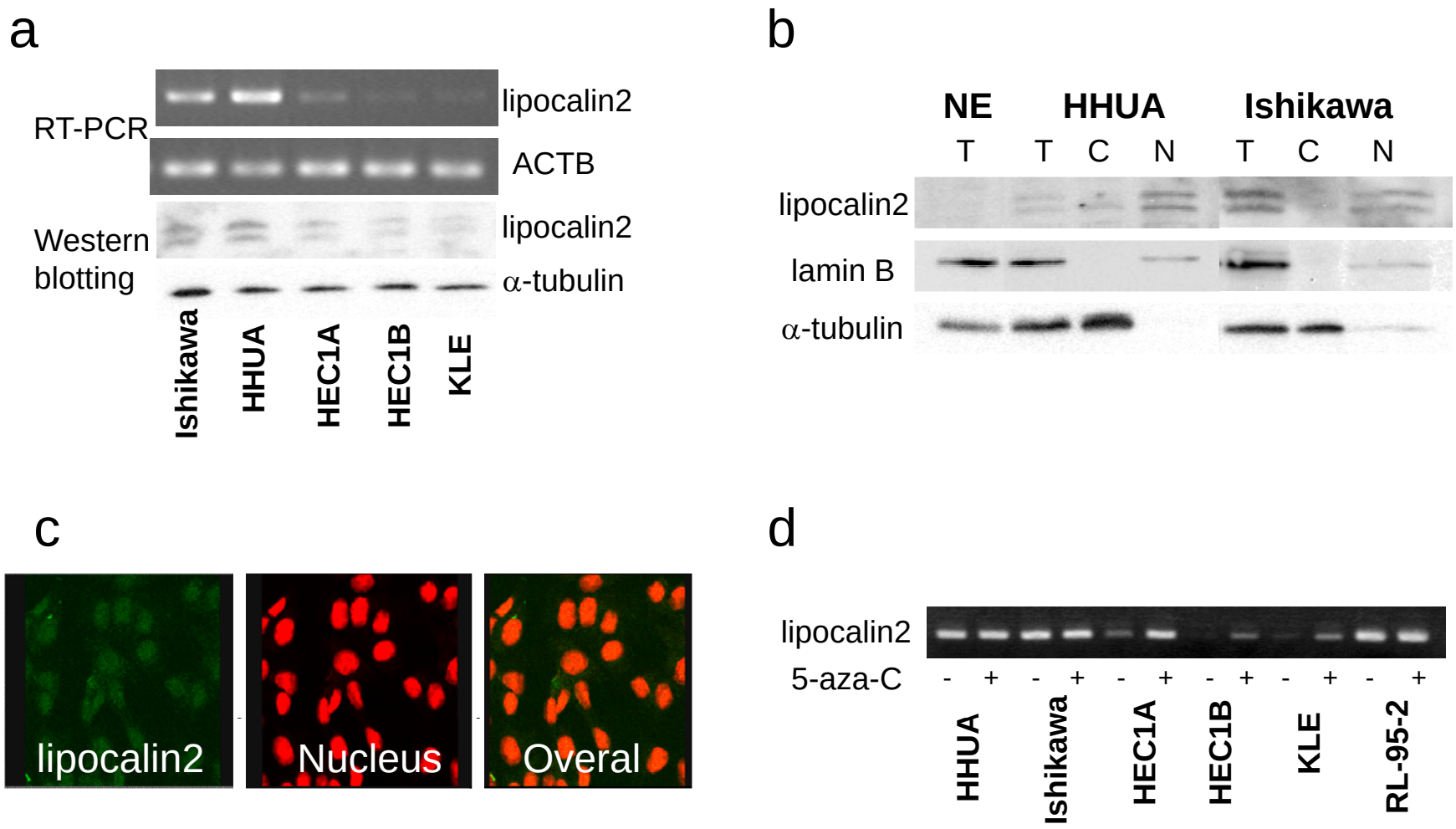

a : stage I-IV
PI＜10 (cytoplasmic)
PI≧10 (cytoplasmic)
p=0.012
(Log-rank test)
Cumurative survival rate
Duration of the survival (month)
b: stage I-IV
PI＜10 (nuclear)
PI≧10 (nuclear)
p<0.0005
(Log-rank test)
Cumurative survival rate
Duration of the survival (month)
c : stage III-IV
PI＜10 (cytoplasmic)
PI≧10 (cytoplasmic)
p=0.108
(Log-rank test)
Cumurative survival rate
Duration of the survival (month)
d: stage III-IV
PI＜10 (nuclear)
PI≧10 (nuclear)
p=0.003
(Log-rank test)
Cumurative survival rate
Duration of the survival (month)

Fig. 5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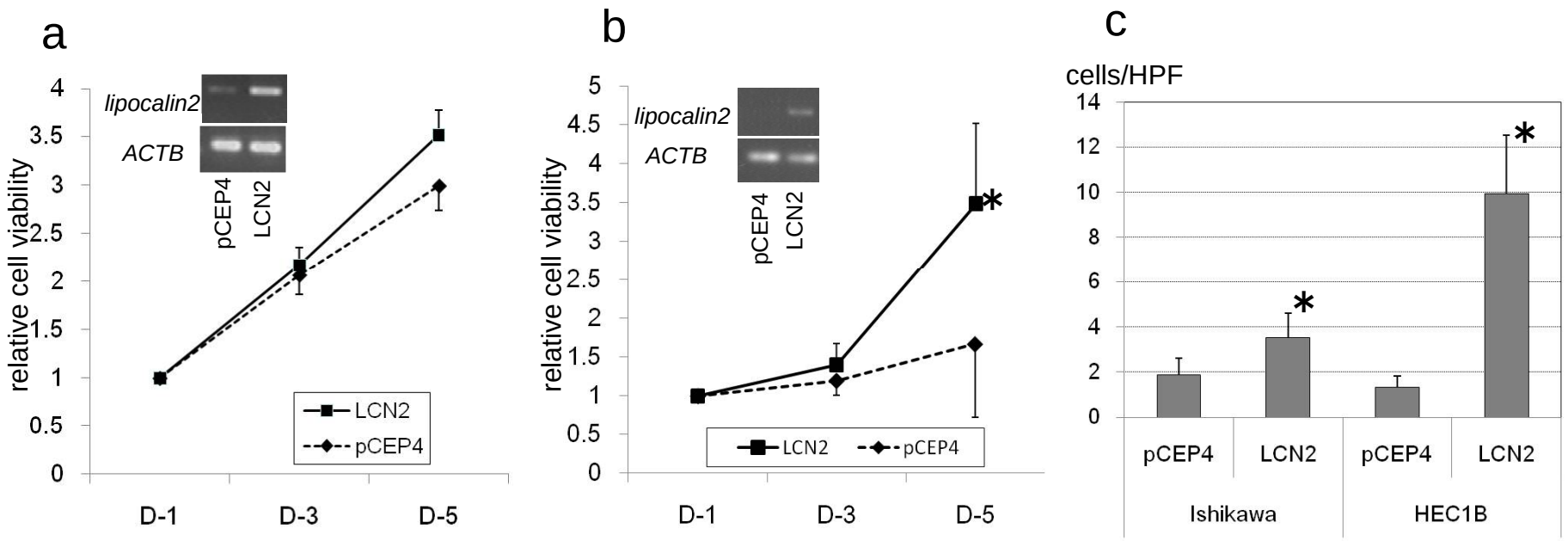
ACTB
Western blotting
lipocalin2
α-tubulin
Ishikawa
HHUA
HEC1A
HEC1B
KLE
b
NE
HHUA
Ishikawa
T T C N T C N
lipocalin2
lamin B
α-tubulin
c
lipocalin2
Nucleus
Overal
d
lipocalin2
5-aza-C
- + - + - + - + - + - +
HHUA
Ishikawa
HEC1A
HEC1B
KLE
RL-95-2

Fig. 6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ACTB
pCEP4
LCN2
relative cell viability
4
3.5
3
2.5
2
1.5
1
0.5
0
LCN2
pCEP4
D-1
D-3
D-5
b
lipocalin2
ACTB
pCEP4
LCN2
relative cell viability
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
LCN2
pCEP4
D-1
D-3
D-5
c
cells/HPF
14
12
10
8
6
4
2
0
*
*
pCEP4
LCN2
pCEP4
LCN2
Ishikawa
HEC1B