Met signaling cascade is amplified by the recruitment of phosphorylated-Met to lipid rafts via CD24, and leads to drug resistance in endometrial cancer cell lines

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Abstract

Endometrial cancer is the most prevalent gynecological cancer in the Western world, and the number of advanced chemotherapy-resistant cancers is increasing with the absolute increase in patients. The development of resistance to chemotherapeutic drugs by cancer cells represents a major challenge in the clinical cure of advanced and metastatic cancers. CD24 has been reported to be a marker for a poor prognosis in several tumors, and we herein examined the functions of CD24 in human endometrioid adenocarcinoma cell lines, and evaluated how it contributes to cancer drug resistance. We demonstrated that CD24 was responsible for the recruitment of phosphorylated-Met to the lipid raft domain of the cell membrane, resulting in amplification of the Met signaling cascade, ultimately leading endometrial cancer cells to express higher levels of ATP binding cassette (ABC) transporters. Our findings suggest that CD24-mediated amplification of the Met cascade may contribute to the drug resistance of endometrial cancer.
Introduction

Endometrial cancer is the most prevalent gynecological cancer in the Western World, and is the third most common cancer in females (1). The major symptoms of endometrial cancer include dysfunctional uterine bleeding, hypermenorrhea, irregular menstruation and sterility. Endometrial cancer is a malignant epithelial tumor, which comprises endometrioid adenocarcinomas in approximately 80% of the newly diagnosed cases in the Western world (2,3). These tumors resemble the normal endometrium morphologically, and are frequently preceded by endometrial hyperplasia. The current treatment for endometrial cancer involves surgery, radiotherapy and chemotherapy in combination, and this approach has improved the prognosis for patients. However, the number of patients with advanced cancers is increasing with the absolute increase in patients, and advanced cancers recur and metastasize to other organs beyond the pelvic cavity. Surgery is not applicable in these patients, so radiotherapy or/and chemotherapy is administered, but the efficacy of these treatments is limited in advanced cases, and the prognosis is poor, indicating the need for a new type of therapy (4).

Numerous studies have indicated that alterations in diverse signaling elements may contribute to high levels of resistance to one or more chemotherapeutic drugs and radiation
(5-11). One of the underlying molecular mechanisms responsible for multiple drug resistance (MDR) is the upregulation of a family of ATP binding cassette (ABC) proteins that lead to chemotherapy resistance in cancer cells by allowing them to actively extrude a wide variety of therapeutic compounds. The same ABC transporters play an important protective function against toxic compounds in a variety of cells and tissues, especially in secretory organs, at the sites of absorption and at blood-tissue barriers.

The three major multidrug resistance ABC proteins are MDR1 (P-glycoprotein, ABCB1), multidrug resistance associated protein 1 (MRP1, ABCC1) and BCRP (ABCG2, placenta-specific ABC transporter). MDR1 and MRP1 can transport a variety of hydrophobic drugs, and MRP1 can also extrude anionic drugs or drug conjugates. The transport properties of BCRP overlap with those of both MDR1 and the MRP-type proteins. Thus, these three proteins form a special network involved in chemo-defense mechanisms. (12)

CD24 is a highly glycosylated protein that is linked to the membrane via a glycosyl-phosphatidyl-inositol (GPI) anchor. The mature protein is only 27–30 amino acids long, and most of the molecular weight of the protein is due to the extensive N- and O-linked glycosylation. CD24 is expressed in cells of the hematopoietic system, such as B-cell precursors
and neutrophils, in neuronal tissue and in certain epithelial cells, such as keratinocytes and the renal tubular epithelium. Various studies have identified CD24 as a marker for a poor prognosis in several tumors, including carcinomas of the ovary (13), breast (14) and pancreas (15), as well as non-small cell lung cancer (16), colorectal cancer (17) and diffuse-type gastric adenocarcinoma (18). In addition, high expressions of CD24 have also been reported to be associated with the poorer prognosis in endometrial cancer patients (19, 20). An expanding body of literature points to a role for CD24 in the initiation and progression of numerous types of cancer. Some investigators have suggested that CD24 interacts with the src kinases due to its localization in detergent-resistant membrane domains (DRM; lipid rafts), (21, 22). In fact, Zarn et al. showed the association of CD24 and src kinase families in a small cell lung cancer cell line, and Sammar et al. demonstrated that CD24 and src kinases are involved in the activation of cell aggregation in a lymphoma cell line. However, the cellular mechanism(s) involved in the activity of CD24 remain largely unclear.

In the present study, we evaluated this mechanism, and how CD24 it contributes to drug resistance in endometrial cancer (EC) cell lines.
Materials and Methods

Materials

The rabbit polyclonal anti-human MDR1 antibody, rabbit polyclonal anti-human MRP1 antibody and mouse monoclonal anti-human caveolin-1 antibody used for immunoblotting were purchased from Abcam (Cambridge, MA, USA). The rabbit polyclonal anti-human CD24 antibody used for immunoblotting and rabbit monoclonal anti-human Met antibody used for immunoblotting and immunocytochemistry were purchased from LifeSpan BioSciences, Inc (USA). The rabbit monoclonal anti-human phospho-Met antibody, rabbit polyclonal anti-human Akt antibody, rabbit polyclonal anti-human phospho-Akt antibody, rabbit polyclonal anti-human ERK antibody, rabbit polyclonal anti-human phospho-ERK antibody and rabbit monoclonal anti-human beta-actin antibody used for immunoblotting were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cisplatin was purchased from Selleck Chemicals (Houston, TX, USA). The BD Falcon™ 96-well microplates used for the cell proliferation assays were purchased from BD Biosciences (Franklin Lakes, NJ, USA).
Cell lines

We used two human cell lines of endometrioid adenocarcinoma. The hec-1A and hec-108 cells were obtained from the American Type Culture Collection (Rockville, MD) in 2013 and authenticated by Short Tandem Repeat (STR) polymorphism profiling analysis. Both cell lines were grown in dishes (Becton Drive, Franklin Lakes, NJ) in DMEM supplemented with 10% fetal bovine serum (Equitec-Bio, Kerrville, TX) (growth media) in an atmosphere of 5% CO₂ at 37 °C. Serum-free DMEM was used for cell starvation. The hec-1A and hec-108 cells were derived from grade 2 and grade 3 endometrioid adenocarcinoma of the uterus, respectively, and have been reported to be cisplatin resistant (23).

RNA extraction and semi-quantitative reverse transcription-polymerase chain reaction

(RT-PCR)

Total RNA from homogenized tissue or from cultured cells was obtained using the RNaseasy Mini kit (Qiagen, Germantown, MD, USA), and 2 μg were reverse-transcribed with Superscript II RNase H-reverse transcriptase (Invitrogen, Carlsbad, CA, USA) using random primers according to the manufacturer’s instructions. The cDNA (1 μl) was amplified using 0.1
μM of each primer, 1 U of Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany),

PCR buffer with 1.5 mM MgCl2 and 0.25 mM dNTPs in a 20 μl reaction volume in a PTC200

Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). The amplification conditions were

as follows: initial denaturation at 94°C for 3 min, followed by 33 cycles comprising

denaturation at 94°C for 30 sec, annealing at the optimized temperature for each set of primers

for 30 sec and extension at 72°C for 30 sec. The final extension was carried out for 5 min at

72°C. The products were analyzed on 2.0% (w/v) agarose gels stained with 0.5 mg/ml ethidium bromide (Sigma-Aldrich) and were visualized under an ultraviolet transilluminator. The product size was approximated using a 100-bp DNA ladder (Bangalore Genei, Bangalore, India). The negative control did not contain reverse transcriptase (RT) enzyme in the reaction mixture. The primers used were as follows: CD24-F: 5’-ACCCACGCAGATTTATTCCA-3’ and CD24-R:

5’-ACCACGAAGAGACTGGCTGT-3’, ABCB1-F: 5’-TGATTGCATTTGGAGCAAA-3’ and

ABCB1-R: 5’-CCAGAAGGCCAGAGCATAAG-3’, ABCC1-F:

5’-ACCATGCTGCTTGCTACCTT-3’ and ABCC1-R: 5’-ACTTGTTCCGACGTGTCCTC-3’,

ABCG2-F: 5’-AGGCAGATGCCTTCTTCGTT-3’ and ABCG2-R:

5’-ATTCTTCCACAAGGGCGG-3’, GAPDH-F: 5’- AGCCACATCGCTCAGACA -3’ and
GAPDH -R: 5’-GCCCAATACGACCAAATCC-3’

The experiments were repeated at least three times and yielded similar results.

**Proliferation assay**

The cells in 10% growth media were seeded in 96-well plates at a density of $2 \times 10^4$ cells per well. After 16 hours of starvation, the cells were incubated for 48 hours in the absence or presence of 0.1, 1, 10, 100 or 500 μM of cisplatin. Then, CellTiter 96 AQueous (MTS) One Solution reagent (Promega, Tokyo, Japan) was added to each well, and the absorbance was recorded at 490 nm using a Corona SH-1000 lab absorbance microplate reader (Corona Electric Co. Inc, Ibaraki, Japan). The cell numbers were then calculated using a standard curve correlating the absorbance to the cell number counted under a microscope. All experiments were carried out in quadruplicate, and the cell viability was expressed as the ratio of the number of viable cells with cisplatin treatment to that of cells without treatment.

**Western blot analysis**

The cells were washed twice with ice-cold phosphate-buffered saline and lysed using
Pierce RIPA Buffer (Thermo Fisher Scientific, MA, USA). Equal amounts of whole cell protein lysates were separated by SDS polyacrylamide gel electrophoresis and were electrotransferred to nitrocellulose membranes. Nonspecific antigen sites were blocked with 10% bovine serum albumin in 1x Tris-buffered saline for 1h. The Western blot analyses were performed with primary antibody of CD24 (1:100 dilution), MDR-1 (1:1000 dilution), MRP (1:500 dilution), BCRP (1:500 dilution), phosphorylated-Met (1:1000 dilution), Met (1:1000 dilution), phosphorylated-Akt (1:1000 dilution), Akt (1:1000 dilution), phosphorylated-ERK (1:1000 dilution), ERK (1:1000 dilution), ACTB (1:1000 dilution) overnight at 4°C.

The immunoreactive bands in the immunoblots were visualized with horseradish peroxidase-coupled goat anti-rabbit immunoglobulin using an enhanced chemiluminescence Western blotting system (ECL Plus, GE healthcare Life Sciences, Pittsburgh, PA, USA).

**Flow cytometry**

The cells were plated in six-well plates at a density of $2 \times 10^5$ per well and were cultured in growth media until they reached 70% to 80% confluence. Then the cells were starved for 16 hours before being harvested after a 24-h incubation with vehicle (PBS) or 10 μM
of cisplatin. The cells were then resuspended in PBS/2% FBS/1mM EDTA and stained with 0.5 μg anti-CD24 (FITC-labeled; Thermo Fisher Scientific). Respective IgG isotype antibodies were included as negative controls. Non-viable cells were excluded using the LIVE/DEAD Fixable Dead Cell Stain Kit (Invitrogen, Carlsbad, CA) per the manufacturer’s recommendations. After being washed in PBS/2% FBS/1 mM EDTA, the cells were fixed by incubation in 4% paraformaldehyde for 60 minutes and analyzed using EC800 (BD Biosciences) within 24 hours. The data were analyzed using the FlowJo version 8.2 software program. The experiment was performed three times with similar results.

**Transfection experiments**

The cell transfection with small interfering RNA (siRNA) and/or DNA construct was carried out as described in the manufacturer’s protocol. Briefly, 75 pmol of siRNA or 2.5 micrograms of DNA construct per well were transiently transfected into hec-1A or hec-108 cells grown in 6-well plate with Lipofectamine 2000 (Invitrogen). The CD24 knockdown or CD24 overexpression was confirmed by CD24 immunoblotting. CD24 siRNA #1, #2 and scrambled siRNA were purchased from Invitrogen. To create the pcDNA3.1-CD24 expression construct,
the cDNA of the full length CD24 was amplified by PCR using a human mammary gland cDNA library as the template.

**Immunofluorescence**

The cells were plated on fibronectin-coated chamber slides. After 24 h, the cells were rinsed with phosphate-buffered saline (PBS), fixed with paraformaldehyde (PFA) for 10 min and permeabilized with 0.1% NP-40 for 10 min. The slides were then blocked with 10% fetal calf serum in PBS and incubated with primary antibodies against CD24 (1:20 dilution), phosphorylated-Met (1:100 dilution) or caveolin-1 (1:400 dilution) for overnight at 4°C. After being washed with PBS, the cells were incubated with goat anti-rabbit antibodies coupled to an Alexa Fluor 546 or goat anti-mouse antibodies coupled to an Alexa Fluor 488 secondary antibody for 30 min. The cells were then rinsed in PBS, mounted in Immuno Mount mounting medium (Life Sciences International), imaged at room temperature with a BZ-8000 fluorescence microscope (Keyence, Osaka, Japan) and were finally analyzed using the BZ Viewer 2.5 software program (Keyence, Osaka, Japan).
**MDR assay (dye uptake protocol)**

The detailed protocol for the MDR assay is available in the Instruction Manual for the eFluxx-IDH Green Multi-Drug Resistance Assay Kit (ENZO Life Sciences, Inc., Farmingdale, NY). Briefly, on the day of the assay, cells were collected, washed with PBS and incubated with or without MDR inhibitors in the presence of the eFluxx-IDH Green probes for 30 min at 37°C, then were analyzed immediately by flow cytometry. eFluxx-IDH Green probes are novel xanthene-based small molecule dyes developed for the detection of MDR activity in living cells. In order to exclude dead cells from the analyses, propidium iodide (PI) solution was added to the cells during the last 5 min of incubation. The multidrug resistance activity factor (MAF) was calculated according to the manufacturer’s protocol.

**Detection of apoptotic cells**

The apoptosis of EC cells was assessed under various conditions according to a caspase activity assay using the CspACETM FITC-VAD-FMK In Situ Marker (Promega, WI, USA), in accordance with the manufacturer’s instructions. The slides were analyzed with a confocal laser scanning microscope (Zeiss 410, Carl Zeiss GmbH, Gottingen Germany), and a
flow cytometric analysis was conducted using the FACScan device in order to quantify the level of apoptosis. Approximately 10,000 gated events were analyzed per sample. The results were analyzed using the Windows Multiple Document Interface flow cytometry applications software program, and the rate of apoptosis was calculated.

**CD24 isolation via magnetic beads**

Hec-1A and hec-108 cells were separated into CD24-positive and CD24-negative fractions using CD24 microbeads (Miltenyi Biotec Inc.) and MACS® LD separation columns according to the manufacturer’s recommendations.

**Subcellular fractionation and protein precipitation**

Scrambled or siRNA-CD24-treated hec-1A and hec-108 cells (1×10^7 cells) were washed twice with PBS at 4°C. The cells were gently scraped from culture plates and collected by centrifugation. They were then homogenized in 200 μl of buffer (78 mM KCl, 4 mM MgCl₂, 8.37 mM CaCl₂, 10 mM EGTA, 50 mM HEPES/KOH, pH 7.0) containing 250 mM of sucrose, and were centrifuged at 1000 × g for 5 min. The supernatants were placed on a 5–20% linear
Optiprep™ (Nycomed, Amersham Biosciences) gradient, formed in 12 ml of the above buffer, and centrifuged at 4°C for 20 h at 100,000 × g in a BECKMAN XL-100 centrifuge (Beckman, Germany). Following the centrifugation, the total volume gradient was separated into 1-ml fractions, and collected from top to bottom (from 5% to 20% Optiprep™ concentration, respectively).

The protein precipitation was as follows: the volume of each collected fraction (1 ml) was duplicated with cool acetone (1 ml, -20°C) in acetone-compatible tubes. The samples were then vortexed and incubated for 1 h at -20°C, and further centrifuged for 10 min at 13,000 × g. Samples were decanted, and the protein pellets were resuspended in Laemmli buffer before being resolved by SDS-PAGE (12%) and Western blotting analyses using specific antibodies.

Xenograft study

Female six-week-old athymic nude mice (BALB/c Slc-nu/nu) were used for the xenograft study. All mice were purchased from Japan SLC, Int. and were housed three mice per cage. The mice had access to sterile food pellets and water ad libitum. The institutional guidelines for animal welfare and experimental conduct were followed. Twelve mice were injected with 10⁶ hec-1A
cells into the upper right fat pad, and twelve other mice were injected with $10^6$ hec-108 cells into the upper left fat pad. Two weeks after inoculation, tumor-bearing mice were randomly assigned to two groups to be treated with either vehicle (PBS) or cisplatin (3 mg/kg) once a week for two weeks. The tumor volumes were determined as described previously (24). Animals were sacrificed three weeks after initiating treatment, and tumors were collected, snap-frozen, or fixed as described below.

**Immunohistochemistry**

The tumors tissue samples collected from the xenograft study were fixed in formalin and embedded in paraffin. Deparaffinized and rehydrated sections (4 μm) were then autoclaved in 0.01 mol/l of citrate buffer (pH 6.0) for 15 minutes at 121°C for antigen retrieval. The endogenous peroxidase activity was blocked with 0.3% solution hydrogen peroxide in methanol for 30 minutes, and the sections were subsequently incubated at 4°C for 12 hours with anti-CD24 rabbit antibodies (1:50 dilution; LifeSpan BioSciences), anti-phospho-Met antibodies (1:50 dilution; Abcam), anti-MDR-1 antibodies (1:50 dilution; Abcam) or anti-MRP antibodies (1:100 dilution; Abcam). The sections were washed with 1X phosphate-buffered saline.
saline (PBS) and incubated with Histofine simple stain MAX PO (multi; Nichirei) for 30 minutes at room temperature. Finally, the sections were washed with 1X PBS, and the signals were visualized via incubation with H₂O₂/diaminobenzidine substrate solution for five minutes. Additionally, the sections were subjected to hematoxylin and eosin staining for a histological examination with light microscopy. The slides were assessed by two experienced pathologists, and the findings were evaluated with the percentage of the sample that was stained. In cases where there was a disagreement between the examiners, the scores were reviewed together and a consensus was reached.

**Statistical analyses**

All experiments were performed in triplicate, except for the cell proliferation assay. The statistical calculations were performed using the StatView statistical software package (SAS Institute, Cary, NC), and the statistical significance of each difference was determined using the Kruskal-Wallis and Mann-Whitney U test, or a paired t-test, as appropriate. A value of \( p < 0.05 \) was considered to be statistically significant.
Results

The CD24-positive phenotype is associated with cisplatin resistance in endometrial cancer cell lines.

In order to clarify whether the CD24-positive cells are more resistant to cisplatin than their CD24-negative counterparts, the CD24 status of cisplatin-treated endometrial cell lines (EC cells) was analyzed by flow cytometry. As shown in Figure 1A, almost half of the cell population was CD24-positive in both the hec-1A and hec-108 cells. Moreover, cisplatin treatment decreased the CD24-negative cell population in both types of cells, suggesting that the CD24-positive cells are more resistant to cisplatin treatment compared to CD24-negative cells.

To confirm whether the CD24-positive cells are more resistant to cisplatin treatment than the CD24-negative cells, we divided the EC cells into CD24-positive and negative fractions using MACS, and performed a MTS assay with various concentrations of cisplatin. As shown in Figure 1B, the CD24-negative cells were more sensitive to cisplatin treatment in both cell lines.

Next, to assess whether the decrease in cell viability in the cisplatin-treated cells was the result of cell apoptosis, and to determine whether CD24 affects the apoptosis status of cells,
an activated caspase-3 in situ detection assay was performed on vehicle (PBS)- or
cisplatin-treated scrambled or CD24 siRNA-transfected EC cells. The CD24 knockdown was
confirmed by using a semi-RT-PCR analysis and Western blot analysis (supplemental figure
1AB). As shown in Figure 1C and supplemental figure 2, cisplatin treatment induced
significantly more apoptosis in CD24 siRNA-transfected cells than in the control cells.

**MDR-1 and MRP are strongly expressed in CD24-positive endometrioid adenocarcinoma

We next examined whether CD24 expression affected the expression of major
multidrug resistance ABC proteins, MDR1 (ABCB1), MRP (ABCC1) and BCRP (ABCG2), in
EC cells using a semi-RT-PCR analysis and Western blot analysis.

As shown in Figure 2A, the mRNA expression of MDR-1 and MRP was stronger in
the CD24-positive cells compared to the CD24-negative cells, but the mRNA expression of
BCRP did not differ significantly between the cells. Similar results were obtained for the protein
expression level; both MDR-1 and MRP were expressed at significantly higher levels in
CD24-positive cells compared to CD24-negative cells. These data suggest that CD24 is
associated with MDR-1 and MRP expression, which may explain why the CD24-positive cells are more resistant to cisplatin treatment.

As ABC transporters actively transport compounds against the concentration gradient across the cell membrane, we assessed the ability of EC cells to efflux chemical compounds using a fluorescent imaging-based efflux assay.

As shown in Figure 2B, the CD24-positive EC cells possessed a stronger efflux capacity than the CD24-negative cells (CD24-MAF; hec-1A: 77.5, hec-108: 87.7). In order to assess which of the major ABC transporters was responsible for the CD24-related efflux activity, EC cells were treated with an inhibitor of MDR-1 (Verapamil), MRP (MK-571) or BCRP (Novobiocin). As shown in Figure 2C, treatment with the MDR-1 or MRP inhibitors decreased the ability of the EC cells the efflux chemicals, but this was not observed with the BCRP inhibitor, suggesting that MDR-1 and MRP are actively functioning in EC cells, while BCRP is not (MDR-1-MAF: hec-1A, 84.7; hec-108, 83.6; MRP-MAF: hec-1A, 82.7; hec-108, 80.2; BCRP-MAF: hec-1A, 11.1; hec-108, 13.5, respectively).

CD24 differentially promotes the expression of MDR and MRP via the Met signaling.
cascade in endometrial cancer cell lines

It has been reported that transcriptional activator complexes for the ABC transporter genes are likely shared by a large set of transcription factors that are involved in conferring drug resistance and cell survival (25). Because the PI3K/Akt and Ras-based MAP kinase (MAPK) pathways are well known for increasing drug resistance (26, 27), we assessed the PI3K/Akt and MAPK pathways using a Western blot analysis.

As shown in Figure 3A, Akt and ERK were significantly more phosphorylated in CD24-positive cells compared to CD24-negative cells. Moreover, as shown in Figure 3B, the Akt and ERK phosphorylation were significantly decreased in CD24 siRNA-transfected cells. These data suggest that CD24 influences the PI3K/Akt and MAPK pathways.

Because the hepatocyte growth factor (HGF)/Met signaling pathway is located at upstream of both the PI3K/Akt and MAPK pathways, we anticipated that there would be a connection between CD24 and the HGF/Met signaling pathway. To determine whether CD24 has any effect on the HGF/Met signaling pathway, the Met phosphorylation status was assessed using a Western bolt analysis. As shown in Figure 3A, the Met were significantly more phosphorylated in CD24-positive cells compared to CD24-negative cells. Moreover, the Met
phosphorylation was significantly decreased in the CD24 siRNA-transfected cells, and significantly increased in CD24 overexpressed cells compared to the scrambled siRNA transfected cells (Figure 3B). The effect of the CD24 overexpression was confirmed by using a Western blot analysis (supplemental figure 3). These data suggest that CD24 somehow enhances the HGF/Met signaling pathway, and that the PI3K/Akt and MAPK pathways may be promoted via this enhanced HGF/Met signaling.

To explore this question, EC cells were treated with vehicle (PBS), foretinib, a Met kinase inhibitor, PD98059, a selective inhibitor of MAPK/ERK kinase or LY294002, an inhibitor of PI3K, and the phosphorylation of proteins in each signaling pathway and the ABC transporter expression was assessed by using a Western blot analysis. As shown in Figure 3C, the levels of Met, Akt and ERK phosphorylation were significantly decreased with foretinib treatment, suggesting that the PI3K/Akt and MAPK pathways are promoted via the HGF/Met signaling pathway in EC cell lines. Moreover, the expression levels of MDR-1 and MRP were also significantly decreased by foretinib treatment. Interestingly, specifically inhibiting the PI3K/Akt pathway by LY294002 treatment significantly decreased MDR-1, but not MRP, and specifically inhibiting the MAPK pathway by PD98059 treatment significantly decreased MRP.
but not MDR-1, suggesting that MDR-1 is controlled mainly by the PI3K/Akt pathway and
MRP by the MAPK pathway.

**CD24 is essential for the formation of caveolin-1 enriched lipid rafts and the accumulation
of p-Met**

GPI-linked proteins, including CD24, are often associated with cholesterol-rich lipid
raft microdomains in the plasma membrane (28), and CD24 has been reported to recruit β1
integrin to lipid raft domains (29), or to interact with c-src within lipid rafts (30).

To determine whether CD24 interacts with phosphorylated Met (p-Met) at lipid rafts,
we performed a sucrose gradient assay and immunocytochemistry (ICC) using antibodies
against p-Met or caveolin-1, a protein that acts as a scaffolding protein within caveolar
membranes by compartmentalizing and concentrating signaling molecules. As shown in Figure
4A and supplemental figure 4, p-Met, CD24 and caveolin-1 were clustered in the same lipid raft
fraction in EC cells, but the CD24 siRNA-transfected cells showed a scattered pattern of
distribution compared to scramble siRNA-transfected cells, suggesting that CD24 is recruiting
p-Met and caveolin-1 to the lipid raft domain. Furthermore, CD24 and p-Met, and p-Met and
caveolin-1 colocalization were confirmed by ICC as shown in Figures 4B and C. In addition, the colocalization of p-Met and caveolin-1 was diminished in CD24 siRNA-transfected cells.

**The CD24-positive phenotype is associated with cisplatin resistance in endometrial cancer tumor xenografts, and is accompanied by high expression of ABC transporters**

In order to confirm the results obtained in the *in vitro* experiments, we conducted an *in vivo* experiment using established hec-1A and hec-108 tumor xenografts. The results showed that cisplatin at a dose of 3 mg/kg did not inhibit the growth of either of the tumor xenografts throughout the experiment (Figure 5A). On the twenty-eighth day after the cell injection, all animals were sacrificed, and tumors were collected, snap-frozen or fixed. In order to evaluate the differences in the CD24 and ABC transporter expression, we performed a histological analysis of the fixed tumors. As shown in Figure 5B and supplemental figure 5AB, staining for immunoreactive CD24 was strongly increased in the cisplatin-treated tumors compared to the vehicle-treated tumors. Moreover, phosphorylated Met, MDR-1 and MRP were all more strongly expressed in the cisplatin-treated tumors compared to the vehicle-treated tumors.

These data support the *in vitro* data that CD24-positive EC cells express higher levels
of ABC transporters and are more resistant to cisplatin treatment compared to CD24-negative EC cells.
Discussion

In the present study, we used hec-1A and hec-108 human endometrial cancer cells to examine the relationship between CD24 and drug resistance, and revealed that CD24 was responsible for the recruitment of phosphorylated Met to the lipid raft domain of the cell membrane, resulting in amplified HGF/Met signaling and ultimately leading EC cells to express higher levels of ABC transporters.

CD24 can function as an adhesion molecule that interacts with ligands like P-selectin (31, 32) and the cell adhesion molecule, L1-CAM (22). Although the binding to P-selectin supports the interaction of CD24-expressing tumor cells with activated platelets or endothelial cells (31), it was shown that its association with L1-CAM has regulatory functions in neurite outgrowth (33). Additionally, CD24 was reported to be involved in doxorubicin and paclitaxel sensitivity in endometrial carcinoma cells (34). Furthermore, a recent study demonstrated that the expression of CD24 in a rat carcinoma system stimulated cell adhesion, migration and proliferation (35). Moreover, CD24 expression supports rapid cell spreading and strongly induces cell motility (36).

We herein demonstrated for the first time that CD24 amplified cell growth-related
intracellular signaling via the PI3K/Akt and MAPK pathways by affecting receptor tyrosine kinase (RTK) activity. Previous reports have indicated that CD24 is related to rapid cell spreading, strong cell motility, stimulated cell adhesion, migration and proliferation, which may be the results of amplified cell growth-related intracellular signaling. Moreover, the amplification of these signals resulted in a stronger ability to efflux chemical compounds in the EC cell lines via induced ABC transporter expression, suggesting that the poor prognosis of CD24-positive carcinomas may be at least partly the result of ABC transporter-related drug resistance.

HGF activates the Met signaling pathway, which is known to be a very powerful stimulator of the invasive and metastatic potential of cells, which allows the survival of cancer cells in the bloodstream in the absence of anchorage (36). HGF is also well known as a potent angiogenic cytokine, and Met signal activation can modify the microenvironment to facilitate cancer progression (36).

The development of resistance by cancer cells to chemotherapeutic drugs represents a major challenge in the clinical cure of advanced and metastatic cancers. Therefore, it is crucially important to establish the molecular mechanisms involved in the development of
multidrug resistance by cancer cells to improve the current therapies against aggressive cancers, and to identify novel targets to prevent or circumvent resistance. It is obvious that subsequent studies will be needed, but understanding CD24-mediated drug resistance might be important for the development of new therapeutic approaches for advanced and metastatic cancers.

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References


33. Kleene R, Yang H, Kutsche M, Schachner M. The neural recognition molecule L1 is a


Figure legends

Figure 1. CD24-positive EC cells are more resistant to cisplatin treatment than CD24-negative cells

(A) Cisplatin treatment and the expression of CD24 in EC cells

Representative examples of histograms from the FACS analysis are shown on the left and the percentage of CD24 positive cells is shown on the right. The data are representative of three independent experiments. Data are shown as the means ± SD (N = 3), * P < 0.05.

(B, C) CD24-positive EC cells are more cisplatin-resistant than CD24-negative cells

(B) Hele-1A and hec-108 cells were treated with cisplatin at various concentrations for 48 hours, then proliferation was measured by the MTS assay. (C) Apoptotic cells were assessed by caspase-3 activity assay. Representative examples of immunostaining are shown on the left. FITC-labeled cells indicated the presence of apoptosis, and DAPI was used for counterstaining. Percentage of apoptotic cells was counted using the FACScan device. The data on the left are representative of five independent experiments. Data are shown as the means ± SD (N = 5), * P < 0.05, ** P < 0.01.
Figure 2. CD24-positive EC cells possess a greater potential to efflux chemical compounds via ABC transporters, MDR-1 and MRP, than do CD-24 negative cells

(A) MDR-1 and MRP are strongly expressed in CD24-positive EC cell lines

The mRNA and protein expression of MDR-1, MRP and BCRP were determined by RT-PCR and Western blot analyses. Representative examples of bands shown on the left, and densitometric quantification of the Western blot bands expressed as percentage of the band intensity of CD24-positive cells is shown on the right. The means ± SD of three determinations are shown. * P < 0.05, ** P < 0.01.

(B, C) CD24-positive EC cells possess a greater potential to efflux chemical compounds than CD24-negative cells

The ability to efflux chemical compounds was assessed using the MDR assay for cells separated into CD24-positive and negative fractions (B) or cells pretreated with vehicle (PBS), MDR-1 inhibitor, MRP inhibitor or BCRP inhibitor (C). Representative examples of histograms from the FACS analysis are shown, and the multidrug resistance activity factor (MAF) is shown in the figures.
Figure 3. The HGF/Met, PI3K/Akt and MAPK pathways play a crucial role in CD24-mediated drug resistance in EC cell lines

(A) The PI3K/Akt, MAPK pathways and CD24

The Western blot analyses were performed with primary antibodies against phosphorylated-Met (p-Met), Met, phosphorylated-Akt (p-Akt), Akt, phosphorylated-ERK (p-ERK) or ERK. Representative examples of bands are shown on the left, and the densitometric quantification of the Western blot bands expressed as percentage of the band intensity of CD24-positive cells is shown on the right. The means ± SD of three determinations are shown. * P < 0.05.

(B) The HGF/Met, PI3K/Akt, MAPK pathways and CD24

The cell lysates from hec-1A and hec-108 cells transfected with scrambled siRNA, CD24-specific siRNA or DNA construct designed for CD24 overexpression were analyzed by Western blotting using antibodies against phosphorylated-Met (p-Met), Met, phosphorylated-Akt (p-Akt), Akt, phosphorylated-ERK (p-ERK) or ERK. Representative examples of bands from are shown on the left, and the densitometric quantification expressed as
a percentage of the band intensity of scrambles siRNA-transfected cells is shown on the right. The means ± SD (N=3) of three determinations are shown. * P < 0.05.

(C) The HGF/Met, PI3K/Akt and MAPK pathways and ABC transporters in EC cell lines

The cell lysates from hec-1A and hec-108 cells treated with vehicle (PBS), foretinib, PD98059 or LY294002 were analyzed by Western blotting using antibodies against phosphorylated-Met (p-Met), Met, phosphorylated-Akt (p-Akt), Akt, phosphorylated-ERK (p-ERK) or ERK, MDR-1, MRP or ACTB. Representative examples of bands from are shown on the left, and the densitometric quantification expressed as a percentage of the band intensity of vehicle treated cells is shown on the right. The means ± SD of three determinations are shown. * P < 0.05.

Figure 4. CD24 is essential for caveolin-1 and p-Met lipid-raft co-localization in EC cell lines

(A) Phosphorylated-Met is recruited to lipid rafts in CD24-positive EC cells

DRMs (lipid raft fraction) were isolated by sucrose density gradient centrifugation of
Triton X-100 lysates, and the fractions were analyzed by Western blotting using antibodies against phosphorylated-Met (p-Met), caveolin-1 and CD24. Representative examples of bands are shown.

(B) **Phosphorylated-Met and CD24 colocalize at the cell membrane in EC cell lines**

CD24 and phosphorylated-Met (p-Met) colocalization in EC cells was assessed by confocal microscopy. Representative examples of immunostaining with an anti-CD24 antibody (green) and an anti-phosphorylated-Met antibody (red) in hec-1A and hec-108 cells are shown. The arrows indicate areas of colocalization. *Bar* 10 μm.

(C) **Caveolin-1 (Cav-1) and phosphorylated-Met partially colocalize in EC cells via CD24**

The colocalization of caveolin-1 (Cav-1) and phosphorylated-Met in EC cells was assessed by confocal microscopy. Representative examples of immunostaining with an anti-phosphorylated-Met antibody (p-Met, red) and an anti-caveolin-1 antibody (Cav-1, green) in scrambled or CD24 siRNA-transfected hec-1A and hec-108 cells are shown. The arrows indicate areas of colocalization. *Bar* 10 μm.
Figure 5. The CD24-positive phenotype is associated with cisplatin resistance in endometrial cancer tumor xenografts, which is accompanied by high expression of ABC transporters.

(A) Cisplatin treatment did not inhibit the growth of the tumor xenografts

The growth of hec-1A and hec-108 tumor xenografts in nude mice treated with cisplatin (3 mg/kg) or vehicle. The data were collected from six independent experiments, and are shown as the means ± SD.

(B) High expression of the ABC transporter in cisplatin-treated endometrial cancer xenograft tumors

Immunohistochemistry of the tumor xenografts with primary antibody against CD24, phospho-Met (p-Met), MDR-1 and MRP was performed. Representative histological images of HE and immunohistochemical staining are shown. Scale bars = 100 μm.
Figure 1

A

CD24 positive cells (%)

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<tr>
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<th>hec-1A</th>
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B

Cell Viability (% Control)

- hec-1A CD24+
- hec-1A CD24-

C

Activated caspase

DAPI

Apoptosis (%)

- hec-1A
- hec-108
Figure 2

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B

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Figure 3

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Figure 4

A  hec-1A Scramble
    p-Met
    CD24
    Caveorin-1

hec-1A si-CD24
    p-Met
    CD24
    Caveorin-1

hec-108 Scramble
    p-Met
    CD24
    Caveorin-1

hec-108 si-CD24
    p-Met
    CD24
    Caveorin-1

B  p-Met  CD24  DAPI  Merge
    hec-1A
    hec-108

C  p-Met  Cav-1  DAPI  Merge
   hec-1A Scramble
   hec-1A si-CD24
   hec-108 Scramble
   hec-108 si-CD24
Figure 5

A

![Graph showing tumor volume (mm^3) over time for hec-1A and hec-108 with Vehicle and Cisplatin treatments.](image)

B

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Molecular Cancer Therapeutics

Met signaling cascade is amplified by the recruitment of phosphorylated-Met to lipid rafts via CD24, and leads to drug resistance in endometrial cancer cell lines

Yoshihiro Joshua Ono, Akiko Tanabe, Tomohito Tanaka, et al.

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