Mesothelin promotes the migration of endometrioid carcinoma and is associated with the MELF pattern

Shinichiro Tahara[1], Satoshi Nojima[1], Tsuyoshi Takashima[1], Daisuke Okuzaki[2], and Eiichi Morii[1]

[1]Department of Pathology, Osaka University Graduate School of Medicine, Osaka, Japan

[2]Single Cell Genomics, Human Immunology, WPI Immunology Frontier Research Center, Osaka University, Osaka, Japan

Correspondence

Professor Eiichi Morii, Department of Pathology, Graduate School of Medicine, Osaka University, Yamada-oka 2-2, Suita, Osaka 565-0871, Japan

Tel: 81-6-6879-3711 Fax: 81-6-6879-3719

E-mail addresses: morii@molpath.med.osaka-u.ac.jp

Key words

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Abstract

Mesothelin (MSLN) is expressed in the mesothelium in normal tissues but is overexpressed in various malignant tumors. In this study, we searched for genes that were more frequently expressed in cases of endometrioid carcinoma (EC) with the MELF (microcystic, elongated, and fragmented) pattern using laser microdissection and RNA sequencing, and found that MSLN was predominantly expressed in cases with the MELF pattern. The role of MSLN in

EC was analyzed by generating MSLN-knockout and -knockdown EC cell lines. MSLN promoted migration and epithelial–mesenchymal transition (EMT). Moreover, we found that cadherin-6 (CDH6) expression was regulated by MSLN. MSLN is known to bind to cancer antigen 125 (CA125), and we found that CA125 can regulate CDH6 expression via MSLN. Immunohistochemical investigations showed that MSLN, CA125, and CDH6 expression levels were considerably elevated in EC with the MELF pattern. The expression of CA125 was similar to that of MSLN not only in terms of immunohistochemical staining intensity but also the blood level of CA125. Our results showed that MSLN contributes to the migration and EMT of EC cells through upstream CA125 and downstream CDH6. Therefore, MSLN has potential as a therapeutic target for EC with the MELF pattern.

Abbreviations

ANOVA; analysis of variance

CA125; cancer antigen 125

CDH6; cadherin-6

EC; endometrioid carcinoma

EMT; epithelial-mesenchymal transition

EV; empty vector

FCS; fetal calf serum

FFPE; formalin-fixed paraffin-embedded

H-score; histological score

IPA; ingenuity pathway analysis

MELF; microcystic, elongated and fragmented

MSLN; mesothelin

NNMT; nicotinamide N-methyltransferase

PBS; phosphate-buffered saline

SDS; sodium dodecyl sulfate

SE; standard error

shRNA; short hairpin RNA

ZO-1; zonula occludens 1

Introduction

The most prevalent histological type of uterine corpus cancer is endometrioid carcinoma (EC). There are three grades of EC (G1–3), with the grade being based on the quantity of solid components. G1 has a comparatively favorable prognosis. However, some G1 cases show an aggressive histological pattern, known as MELF (microcystic, elongated, and fragmented) [1]. Previously, we used laser microdissection and RNA sequencing analyses to examine gene expression in the surface area and the invasive front area of EC with the MELF pattern, reporting that nicotinamide N-methyltransferase (NNMT) was related to MELF pattern invasion and epithelial–mesenchymal transition (EMT) [2]. However, histological findings of G1 with MELF pattern differ from G1 without MELF not only in the invasive front area but also in the surface area [3]. Actually, G1 with MELF pattern invasion is a different molecular subtype from G1 without MELF. G1 with MELF is associated with the "microsatellite instability hypermutated" group of The Cancer Genome Atlas (TCGA), whereas G1 without MELF is generally categorized as "copy-number low" [4]. In this study, we focused on features other than the morphology of the invasive front area of EC with the MELF pattern. Therefore, we compared G1 with and without MELF in terms of the gene expression profiles of the surface area, using laser microdissection and RNA sequencing analyses. We focused particularly on mesothelin (MSLN) expression.

MSLN is expressed in the mesothelium of normal tissue and is overexpressed in many types of cancer, such as pancreatic cancer and ovarian cancer [5]. In an immunohistochemical study, Kakimoto et al. reported that histological grade, myometrial invasion, and lymphovascular invasion in EC were related to the expression of MSLN [6]. However, the molecular mechanism of MSLN in EC, and the relationship between the MELF pattern and MSLN expression, remain to be elucidated. In the current investigation, we examined the molecular function of MSLN in EC using clinical samples and cell lines.

Materials and methods

Patients

We studied surgical specimens of EC taken from the uterine corpus of 120 patients between 2013 and 2021 at Osaka University Hospital. Four histological subtypes were identified: G1 with MELF pattern invasion (n = 30), G1 without MELF pattern invasion (n = 30), G2 (n = 30), and G3 (n = 30). The specimens were preserved in a 10% solution of formalin neutral buffer before being prepared for paraffin embedding. Subsequently, 4-μm-thick sections were cut from the specimens and stained with hematoxylin and eosin. Moreover, in cases of G1 with MELF pattern invasion and G1 without MELF, we obtained the blood level of cancer antigen 125 (CA125) from the medical records immediately before the surgery was performed. Of the 30 cases of G1 with MELF, 1 case was complicated by ovarian cancer, which may increase the level of CA125, while in another case the level of CA125 was not measured. Of the 30 cases of G1 without MELF, 1 case was complicated by ovarian cancer. Thus 28 cases of G1 with MELF and 29 of G1 without MELF were included in the final analysis. This research was approved by the Ethics Review Board of the Graduate School of Medicine, Osaka University (No. 15234). Written informed consent was obtained from all patients.

RNA sequencing analysis of formalin-fixed paraffin-embedded (FFPE) samples

RNA sequencing analysis of FFPE samples was performed as described previously [2]. We previously selected 2 of 30 cases of G1 with MELF pattern invasion, and 2 of 30 cases of G1 without MELF. Gene expression profiles of the surface area were compared between G1 with the MELF pattern (Gene Expression Omnibus [GEO] accession number for previously published raw data: GSE171033) and G1 without the MELF pattern (raw data newly

deposited in the NCBI GEO database; accession number: GSE269763) (Figure 1)

Cell lines

HEC1A, HEC1B, HEC116, and AN3CA cell lines of EC were acquired from Health Science Research Resources Bank (Osaka, Japan). HEC108 and SNG-M cell lines of EC were obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). During the cell culture process, 10% heat-inactivated fetal calf serum (FCS) was added to Dulbecco's modified Eagle's medium (DMEM).

Generation of MSLN-knockout HEC1B cells using the CRISPR/Cas9 system and generation of MSLN-knockdown SNG-M cells using short hairpin RNA (shRNA)

HEC1B cells were transfected with SIGMA CRISPR All-in-One-GFP plasmid DNA (Merck, Darmstadt, Germany) using Lipofectamine 3000 reagent (Thermo Fisher Scientific, Waltham, MA, USA) and incubated for 72 h. Green cell populations were sorted into 96-well plates using a cell sorter (SH800ZDP; Sony Imaging Products & Solutions Inc., Tokyo, Japan) operating in single-cell sorting mode. We constructed stable MSLN-knockout (ΔMSLN1 and ΔMSLN2) cells. Moreover, we transfected Crispr Universal Negative Control 1 (Merck) into HEC1B cells and selected stably transfected cells using the cell sorter to create control cells (empty vector [EV]).

We tried to construct MSLN-knockout SNG-M cells in a similar way but were unable to obtain knockout clone. Then, we constructed MSLN-knockdown cells using shRNA. MSLN/shRNA-encoding lentivirus was produced using Lenti-X 293T cells (Takara, Shiga, Japan). Cells were plated on 10-cm dishes at 80% confluency, and the media was then removed. We mixed Lentiviral High Titer Packaging Mix (Takara), MISSION TRC-Hs1.0 (Merck), Opti-MEM (Thermo Fisher Scientific), and TransIT-293 Transfection Reagent

(Takara). Cells were then transfected and, 24 h after transfection, the media was changed to fresh DMEM containing 10% FCS. The media was collected and filtered through a 0.22-μm filter after an additional 48 h, and it was then stored at -80℃. SNG-M cells were plated in 6-well plates and incubated for 24 h with 3 ml of the preserved lentivirus in media containing 10 μg/ml polybrene. The media was then changed, and stable MSLN-knockdown SNG-M cells were constructed. We constructed control SNG-M cells in a similar way using SHC002 MISSION pLKO.1-puro plasmid (Merck).

Generation of MSLN knockout-rescue HEC1B cells

MSLN-GFP lentiviral vector plasmid was purchased from VectorBuilder (Chicago, IL, USA). MSLN-GFP lentivirus was produced in the manner described above. We concentrated the MSLN-GFP lentivirus in media with Lenti-X Concentrator (Takara). Then, ΔMSLN1 and ΔMSLN2 HEC1B cells were plated in 48-well plates, the media was removed, and 400 μl of the lentivirus-containing concentrated medium was added. Cells were incubated and placed in 6-well plates. We then removed confluent cells from 6-well plates, and green cell populations were sorted into 24-well plates with SH800ZDP. Constructed cells were subjected to immunoblotting, migration assay, invasion assay, proliferation assay, and RNA sequencing.

Antibodies

For immunoblotting (1:1,000), immunohistochemical (1:250) and immunofluorescence (1:250) analyses, an antibody against MSLN (cat. no. 99966; Cell Signaling Technology, Beverly, MA, USA) was used. For immunoblotting, Epithelial-Mesenchymal Transition (EMT) Antibody Sampler Kit, including zonula occludens-1 (ZO-1), N-cadherin, E-cadherin, β-catenin, vimentin, claudin-1, snail, slug, and ZEB1 antibodies (1:1,000; cat. no. 9782; Cell Signaling Technology) was used. For immunoblotting (1:1,000), immunohistochemistry

(1:800), and immunofluorescence (1:400) analyses, an antibody against cadherin-6 (CDH6) (cat. no. 48111; Cell Signaling Technology) was used. Immunohistochemical (1:100) and immunofluorescence (1:100) analyses were performed using an antibody against CA125 (clone M11; Agilent, Santa Clara, CA, USA). For immunoblotting loading controls, an antibody against β-actin was used (1:1,000; 13E5, HRP conjugate, cat. no. 5125; Cell Signaling Technology).

Immunoblotting

Cells seeded in dishes were washed twice with phosphate-buffered saline (PBS) and lysed in buffer containing 20 mM tris hydrochloride, 150 mM sodium chloride, 1 mM ethylenediaminetetraacetic acid, 1% Nonidet P-40, and protease inhibitor cocktail (Roche, Basel, Switzerland). Lysed cells were scraped with a cell lifter (AS ONE, Osaka, Japan) and sonicated. They were then centrifuged at 13,000 rpm for 10 min. After collecting the supernatant, an equivalent volume of 2× sodium dodecyl sulfate (SDS) buffer (7% SDS, 140 mM Tris–HCl, 20% glycerol, 0.01% bromophenol blue, and 10% 2-mercaptoethanol) was added. Subsequently, protein samples were heated for 4 min at 98°C. We used 5%–20% gradient SDS–polyacrylamide gels (ATTO, Tokyo, Japan) for electrophoresis. Proteins were semi-dry transferred onto polyvinylidene fluoride membranes. Anti-rabbit IgG (H+L chain) coupled with horseradish peroxidase (1:5,000; MBL, Nagoya, Japan) was used to identify primary antibodies. Images were obtained with an imaging system (ChemiDoc Touch; Bio-Rad, Hercules, CA, USA). ImageJ (NIH, Bethesda, MD, USA) was used to quantify the results.

In the CA125 experiment, DMEM with 0.4 μg/ml recombinant CA125 (R&D Systems, Minneapolis, MN, USA) was used as a substitute medium 10 h after 2×10^5 cells were seeded into 6-well plates. The cells were lysed and subjected to immunoblotting analysis after

72 h.

Wound-healing assay

Using sterile 200-µl pipette tips (Watson, Tokyo, Japan), confluent cells were perforated on 6-well plates and incubated for 17 h (HEC1B) or 22 h (SNG-M). The migration distance was calculated by subtracting the wound width at 17 h (HEC1B) or 22 h (SNG-M) from that at 0 h.

Matrigel invasion assay

A Matrigel Invasion Chamber (Corning Inc., Corning, NY, USA) was used to investigate tumor cell invasion. Cells that had been incubated in DMEM without FCS for 9 h were placed in the upper chamber in DMEM without FCS and incubated at 37°C for 24 h. DMEM with 10% FCS was present in the lower chamber. Diff-Quik (Sysmex, Hyogo, Japan) was used to label invasive cells and those that moved to the upper side of the lower chamber. Invading cells were counted in five arbitrary fields per chamber under high magnification.

Proliferation assay

In 6-well plates, we seeded 1×10^5 cells per well and then cultivated the cells for 5 days at 37°C in an atmosphere with 5% CO_2. The Muse Cell Analyzer (Merck) was used to count the cells on days 3 and 5.

Cell shape analyses

A BZ-X800L microscope (Keyence, Osaka, Japan) was used to photograph the cells. Using ImageJ, we evaluated the circularity of 30 randomly selected cells. A circularity value of 1.0 denotes a perfect circle, whereas values close to 0.0 denote an increasingly elongated

polygon.

RNA sequencing analyses of HEC1B cells and ingenuity pathway analysis

To compare gene expression profiles between MSLN-knockout HEC1B cells (ΔMSLN1, ΔMSLN2), MSLN knockout-rescue cells (ΔMSLN1 rescue, ΔMSLN2 rescue), and control cells (EV), total RNA was extracted from the cells. Library preparation was performed using a TruSeq stranded mRNA sample prep kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. The subsequent procedures were described previously [2]. Raw data were deposited in the NCBI GEO database (accession number: GSE269766).

The genes identified by RNA sequencing were subjected to ingenuity pathway analysis (IPA) (QIAGEN IPA; Qiagen, Hilden, Germany), to identify pathways related to MSLN. We identified 130 genes with significantly lower expression in ΔMSLN than EV (fold change < –2) and higher expression in ΔMSLN rescue than ΔMSLN (fold change > 2) (Table S1), along with 251 genes with significantly higher expression in ΔMSLN than EV (fold change > 2) and lower expression in ΔMSLN rescue than ΔMSLN (fold change < -2) (Table S2). ΔMSLN is the average of ΔMSLN1 and ΔMSLN2, and ΔMSLN rescue is the average of ΔMSLN1 rescue and ΔMSLN2 rescue. The list of 381 genes was subjected to core analyses, in which we focused on molecular and cellular functions and canonical pathways.

Immunofluorescence analysis of CDH6

Eight-well chamber slides (Matsunami Glass, Osaka, Japan) were used to seed cells. Following removal of the chambers, the cells were fixed for 10 min using a 4% paraformaldehyde phosphate buffer solution (Nacalai Tesque Inc., Kyoto, Japan) and then rinsed with PBS. Next, 0.25% Triton X-100 in PBS was used to permeabilize the cells. Bovine serum albumin (0.15%) in PBS was used as a blocking agent. Subsequently, the

slides were treated at 4°C overnight with an antibody against CDH6. On the following day, the slides were incubated with Opal Polymer HRP Ms + Rb (Akoya Biosciences, Marlborough, MA, USA) for 30 min at room temperature and incubated with Opal fluorophores including Opal 570. Using a BZ-X800L microscope, they were photographed after being coverslipped with Fluoro-KEEPER Antifade Reagent, Non-Hardening Type with DAPI (Nacalai Tesque Inc.).

Immunohistochemical and immunofluorescence analyses of clinical samples

Ventana BenchMark GX Autostainer (Roche) was used for MSLN immunostaining, whereas Dako Autostainer Link 48 (Agilent) was used for CA125 and CDH6 immunostaining. After determining the staining intensity (0, 1+, 2+, or 3+), the histological score (H-score) was computed using the formula [1 × (% tumor cells of 1+) + 2 × (% tumor cells of 2+) + 3 × (% tumor cells of 3+)]. An Opal 4-Color anti-Rabbit Manual IHC Kit (Akoya Biosciences) was used for fluorescence immunostaining of MSLN, CA125, and CDH6, according to the vendor's protocol.

Statistical analyses

We used JMP Pro 17 software (SAS Institute, Cary, NC, USA) to conduct statistical analyses. The results are presented as mean ± SE. Student's t-test, analysis of variance, the Wilcoxon signed-rank test, and Dunnett's test were used for the analyses, in which $P < 0.05$ was considered statistically significant.

Results

MSLN expression was high in cases with the MELF pattern

We performed RNA sequencing analyses of EC with and without the MELF pattern to

identify the mechanism underlying the carcinogenesis associated with the MELF pattern (Table 1). S100A9 is known to be associated with S100A4 (see top part of Table 1) [7]. We previously demonstrated the relevance of S100A4 to the MELF pattern invasion [8]. Therefore, we focused on MSLN.

MSLN expression in EC cell lines

We evaluated MSLN expression in HEC1A, HEC1B, HEC108, HEC116, SNG-M, and AN3CA cells. Immunoblotting revealed that MSLN was expressed in HEC1B, HEC108, and SNG-M cells (Figure 2a). We referred to CellMinerCDB (version 2.0; NIH), to examine MSLN mRNA expression (Table S3). The level of MSLN mRNA expression was high in HEC1B and SNG-M cells. Then, we used HEC1B and SNG-M to evaluate the function of MSLN. We constructed MSLN-knockout HEC1B cells, MSLN knockout-rescue HEC1B cells, and MSLN-knockdown SNG-M cells and confirmed the expression of MSLN by immunoblotting (Figure 2b).

Involvement of MSLN in the migration of EC cells

MSLN depletion attenuated EC cell migration and vice versa (Figure 3a). However, MSLN depletion induced no significant changes in invasion (Figure 3b). Furthermore, MSLN depletion improved proliferation in SNG-M cells but not in HEC1B cells (Figure 3c).

Involvement of MSLN in EMT

EMT, the process by which epithelial cells transdifferentiate into motile mesenchymal cells, contributes to cancer progression [9]. MSLN-knockout and -knockdown cells had a more rounded shape than control cells (Figure 3d). During EMT, the expression of ZO-1, a tight junction protein, is decreased [9]. Our immunoblotting analyses revealed that MSLN

depletion elevated the expression of ZO-1, suggesting that MSLN promotes EMT (Figure 3e). No significant differences were found for N-cadherin, E-cadherin, β-catenin, vimentin, claudin-1, snail, slug, and ZEB1, which are also associated with EMT (Figure 3e).

IPA revealed the involvement of MSLN in cellular movements

We performed RNA sequencing analyses of HEC1B cells (EV, ΔMSLN1, ΔMSLN2, ΔMSLN1 rescue, ΔMSLN2 rescue). We subjected 130 genes whose expression was upregulated by MSLN and 251 genes whose expression was downregulated by MSLN to IPA (Figure 4a). IPA revealed that MSLN was most involved in cellular movement among molecular and cellular functions (Table 2). The results were consistent with the migration assay.

MSLN altered the expression of CDH6

Through RNA sequencing analyses of HEC1B cells, we identified and listed 92 genes whose expression was significantly downregulated by MSLN depletion and 43 whose expression was elevated by forced expression of MSLN (Table S4, S5). We focused on 13 genes in both lists (Figure 4b, Table 3). Of those 13 genes, the top 8 were non-coding RNAs. Focusing on CDH6, immunoblotting analyses revealed that changes in CDH6 expression corresponded to changes in MSLN expression (Figure 4c). Moreover, immunofluorescence confirmed that depletion of MSLN resulted in reduced expression of CDH6 at the plasma membrane (Figure 4d). These results suggested that MSLN altered the expression of CDH6.

CA125–MSLN–CDH6 regulation axis

MSLN binds to CA125, and MSLN–CA125 binding facilitates tumorigenesis [10,11]. Applying recombinant CA125 to the medium of control cells elevated the expression of both

MSLN and CDH6. However, in MSLN-knockout cells, applying CA125 induced no remarkable change in the expression of CDH6 (Figure 4e). Therefore, MSLN was regulated by CA125 and, in turn, regulated CDH6.

MSLN, CA125, and CDH6 expression in clinical samples

Immunohistochemical staining of surgical specimens with anti-MSLN antibody was strongest for G1 EC with the MELF pattern, followed by G1 without the MELF pattern, G2, and G3 (Figure 5a). The expression of CA125 was higher in G1 EC specimens with versus without the MELF pattern. In individual cases of G1 with the MELF pattern, the H-score of CA125 was correlated with that of MSLN (Figure 5b). Moreover, we examined the blood level of CA125 immediately before surgery was performed, and found that it was higher in G1 with versus without the MELF pattern. In individual cases of G1 with the MELF pattern, the blood level of CA125 was weakly correlated with the H-score of MSLN (Figure 5c). Finally, we confirmed that the expression of CDH6 was higher in G1 with versus without the MELF pattern (Figure 5d). In cases of G1 with the MELF pattern, MSLN, CA125 and CDH6 were all expressed throughout the tumor, not confined to the invasive front area. Co-expression of MSLN, CA125 and CDH6 was observed in some tumor cells (Figure 5e).

Discussion

In our experiments, we showed that in EC cell lines, MSLN promoted migration and EMT. MSLN was regulated by CA125 and, in turn, regulated CDH6. Immunohistochemical analyses revealed that in cases of G1 with MELF pattern, the expression levels of MSLN, CA125, and CDH6 were significantly higher than in G1 without MELF.

In cases of G1 with the MELF pattern, MSLN was expressed throughout the tumor, not confined to the invasive front area. We have previously reported that NNMT and S100A4

are associated with the MELF pattern [2,8]. NNMT was expressed preferentially in the invasive front area, whereas S100A4 was expressed throughout the tumor. The pattern of MSLN expression in cases of G1 with the MELF pattern was similar to S100A4. This is consistent with that MSLN and S100A4 were both found in gene expression profiles of the surface area of EC with the MELF pattern (Table 1).

The expression of CA125 was correlated with that of MSLN, indicated not only by the H-score but also by the blood level of CA125. Song et al. reported that the blood level of CA125 was related to the MELF pattern [12]. However, the mechanism underlying this association is unclear. CA125 is known to bind MSLN, and MSLN–CA125 binding enhances tumorigenesis in ovarian cancer [10,11]. In our experiments, applying recombinant CA125 elevated the expression of MSLN. Liu et al. reported that, in cancer cells, many types of proteases are involved in MSLN shedding [13]. We posited that MSLN–CA125 binding suppresses MSLN shedding, and the results showed increased expression of MSLN. The increase in the expression of MSLN in HEC1B cells associated with recombinant CA125 was consistent with the fact that blood CA125 levels correlated with MSLN expression in clinical samples. Moreover, we confirmed the co-expression of MSLN and CA125 (Figure 4e). The co-expression of MSLN and CA125 suggests that CA125 produced by the tumor cells themselves regulates MSLN. The fact that CA125 in the blood correlates with the expression of MSLN suggests that CA125 produced by other tumor cells and flowing in the blood also regulates MSLN. The mechanism via which CA125 increased the expression of MSLN suggested to involve these two pathways.

MSLN expression was upregulated in the CA125-applied HEC1B cells, and CDH6 expression was also upregulated. In addition, ΔMSLN Rescue resulted in stronger MSLN expression than EV, suggesting that ΔMSLN Rescue is a condition similar to MSLN overexpression (Figure 2b). The expression of CDH6 was also upregulated in ΔMSLN

rescue, although not significantly so (Figure 4c). These results suggest that the CA125/MSLN and MSLN-over expressed pathways are identical and both upregulate the expression of CDH6.

CDH6, also known as K-cadherin, is a member of the cadherin family involved in the morphogenesis of the central nervous system and the fetal kidney [14]. Gugnoni et al. reported that in thyroid papillary carcinoma, CDH6 is a target of TGF-β and promotes EMT and metastasis by suppressing autophagy [15]. In our experiments, fluorescence immunostaining showed reduced expression of CDH6 in the plasma membrane in MSLN-knockout HEC1B cells. The expression of CDH6 on the plasma membrane is thought to promote migration and EMT of EC cells.

It has already been said in previous papers that MELF is associated with EMT in clinical samples. Stewart et al. reported that in cases of EC with MELF pattern, the expression of E-cadherin in invasive front area was attenuated and vimentin showed variable expression [16]. The results from our in vitro experiments about EMT showed that only one epithelial marker, ZO-1, was altered (Figure 3e). We supposed that this is due to the characteristics of EC cells. They differ from other cancers with respect to EMT in that they often lack the typical epithelial marker E-cadherin and express the essentially mesenchymal marker vimentin. Since there are fewer markers in EC than in other cancers that can be properly evaluated as EMT, we thought it worthwhile to point out changes in ZO-1 alone. In gene expression profiles of the surface area of FFPE samples, the expression of ZO-1 tended to be slightly weaker in G1 with the MELF than without MELF. Moreover, IPA analysis using RNA sequencing analyses of HEC1B cells revealed that S100 family signaling pathway is related in canonical pathways (Table S6). Low et al. reported that S100 family proteins are link to EMT in pancreatic cancer [17]. It was suggested that EMT induced by MSLN involves S100 family signaling as well as CDH6.

We have considered using nude mice for this study to validate the results obtained in clinical samples. However, in the most common xenograft models of subcutaneous inoculation of cancer cells, studies about tumor spread and the formation of metastases are restricted because the blood and lymphatic vasculature in subcutaneous tumors is often incompletely formed [18]. Moreover, in a previous paper in which HEC1B cells were injected into nude mice, they don't form a clear invasive area at the margins and often form a solid mass [19]. Therefore, we considered it difficult to reproduce the morphological features of MELF with xenografts, so we investigated it in vitro experiments.

In clinical samples, we found that MSLN was related to MELF. However, there was no association between MSLN and invasion in experiments with EC cell lines; we attributed this to MSLN being identified in specimens taken from the surface area of EC with the MELF pattern. Previously, we reported that NNMT in the invasive front area of EC with the MELF pattern promoted invasion [2]. We posit that multiple molecules are involved in the tumorigenesis of MELF, with MSLN making a contribution in the stage prior to deep invasion.

Our results showed that MSLN contributes to the migration and EMT of EC cells through upstream CA125 and downstream CDH6. Moreover, we found that MSLN, CA125, and CDH6 are related to MELF pattern invasion. Therefore, MSLN has potential as a therapeutic target for EC with the MELF pattern.

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Disclosure

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Conflict of Interest

All authors have no conflict of interest.

Ethics Statement

Approval of the research protocol by an Institutional Reviewer Board: This research was approved by the Ethics Review Board of the Graduate School of Medicine, Osaka University (No. 15234).

Informed Consent: N/A.

Registry and the Registration No. of the study/trial: N/A.

Animal Studies: N/A.

Author Contributions

ST: Conceptualization; data curation; funding acquisition; investigation; visualization; writing - original draft. **SN:** Resources; supervision. **TT:** Resources; validation. **DO:** Data curation; formal analysis. **EM:** Project administration; supervision; writing - review & editing.

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Figure legends

Figure 1. Schematic of the experiment. We microdissected the surface area of grade 1 (G1) endometrioid carcinoma (EC) with and without the MELF (microcystic, elongated, and fragmented) pattern. Then, we extracted RNA and performed transcriptome analyses. Scale bars: 100 μm.

Figure 2. Immunoblotting of mesothelin (MSLN) in endometrioid carcinoma (EC) cells and generation of MSLN-knockout cells, MSLN knockout-rescue HEC1B cells, and MSLN-knockdown SNG-M cells. (a) MSLN expression in HEC1A, HEC1B, HEC108, HEC116, SNG-M, and AN3CA cells. (b) Confirmation of loss of MSLN expression in MSLN-knockout HEC1B cells (ΔMSLN1 and ΔMSLN2), restoration of MSLN expression in MSLN knockout-rescue HEC1B cells (ΔMSLN1 rescue and ΔMSLN2 rescue), and reduced protein expression in MSLN-knockdown SNG-M cells (shMSLN) by immunoblotting. Equal protein loading was confirmed by quantifying β-actin (input control). EV, empty vector control cells.

Figure 3. Functional analyses of mesothelin (MSLN). (a) Wound-healing assay. Cells were wounded, and the distance between areas without cells was monitored. The migration distance was calculated by subtracting the width of the wound at 17 h (HEC1B) or 22 h (SNG-M) from that at 0 h. The migration distance of control cells (EV) is expressed as 1. The relative migration distance is presented as a ratio to that of control cells. (b) Matrigel invasion assay. Representative images of invading cells are shown. Invading cells were counted in five random fields per well. (c) Cell proliferation. (d) Cell shape analyses. Representative images of cells are shown. We calculated the circularity of 30 cells using ImageJ. (e) Immunoblotting of zonula occludens-1, N-cadherin, E-cadherin, β-catenin, vimentin, claudin-1, snail, slug, and ZEB1 in HEC1B and SNG-M. Equal protein loading was confirmed by quantifying β-actin (input control). Data are representative of three independent experiments and are shown as mean ± SE. Asterisks indicate significant differences as determined using Dunnett's test

(HEC1B) and Student's *t*-test (SNG-M) (*$P < 0.05$, **$P < 0.01$). Scale bars: 200 μm (a), 50 μm (b), and 20 μm (d). N.S., not significant.

Figure 4. Cancer antigen 125 (CA125) regulates mesothelin (MSLN), and MSLN regulates cadherin-6 (CDH6). (a) Schematic of the selection of genes for ingenuity pathway analysis. (b) Schematic of the selection of genes closely related to MSLN. (c) Immunoblotting of CDH6 in HEC1B and SNG-M cells. Equal protein loading was confirmed by quantifying β-actin (input control). (d) Immunofluorescence of CDH6 in HEC1B cells. In EV cells, CDH6 (red signal) was clearly expressed in the plasma membrane. In contrast, in ΔMSLN1 and ΔMSLN2, the expression of CDH6 was reduced. Blue signal indicates DAPI. All fluorescence exposure times were the same. (e) Immunoblotting of MSLN and CDH6 in HEC1B cells. We used 0.4 μg/ml recombinant CA125. Equal protein loading was confirmed by quantifying β-actin (input control). Data are representative of three independent experiments and are shown as mean ± SE. Asterisks indicate significant differences as determined by Dunnett's test or Student's *t*-test (*$P < 0.05$, **$P < 0.01$). Scale bars: 20 μm (d).

Figure 5. Immunohistochemistry of mesothelin (MSLN), cancer antigen 125 (CA125), and cadherin-6 (CDH6) in endometrioid carcinoma (EC). (a) Representative immunohistochemical images of MSLN are shown with intensity classified as weak (1+), moderate (2+), or strong (3+) (upper panels). Representative images and comparison of histological scores (H-scores): G1 with MELF (microcystic, elongated, and fragmented) pattern invasion (n = 30), G1 without MELF pattern invasion (n = 30), grade 2 (n = 30), and grade 3 (n = 30) (lower four panels and graph). (b) Representative images of immunohistochemical analyses of CA125 are shown, with intensity classified as weak (1+), moderate (2+), or strong (3+) (upper panels). Representative images and comparison of H-scores between G1 with MELF pattern invasion (n = 30), and G1 without MELF (n= 30) (lower left panels and upper right graph). Correlation between the H-scores of CA125 and

MSLN in cases of G1 with MELF pattern invasion (n = 30) (right lower graph). (c) Comparison of the blood level of CA125 between G1 with MELF pattern invasion (n = 28), and G1 without MELF (n = 29) (left graph). Correlation between the blood level of CA125 and the H-score of MSLN in cases of G1 with MELF pattern invasion (n = 28) (right graph). (d) Representative immunohistochemical images of CDH6 are shown, with intensity classified as weak (1+), moderate (2+), or strong (3+) (upper panels). Representative images and comparison of H-scores between G1 with MELF pattern invasion (n = 30) and G1 without MELF (n = 30) (lower left panels and upper right graph). (e) Immunofluorescence of MSLN, CA125, and CDH6 in cases of G1 with MELF pattern invasion. MSLN (green signal), CA125 (red signal), and CDH6 (yellow signal) were co-expressed in some of the tumor cells. Blue signal indicates DAPI. Asterisks indicate significant differences as determined by Dunnett's test or Student's *t*-test (*$P < 0.05$, **$P < 0.01$). Scale bars: 50 μm in upper panels (a, b, d), 200 μm in lower panels (a, b, d), and 20 μm (e).

Supporting information

Table S1. List of 130 genes showing significantly lower expression in ΔMSLN than in empty vector (EV) control cells (fold change < –2) and higher expression in ΔMSLN rescue than in ΔMSLN (fold change >2)

Table S2. List of 251 genes showing significantly higher expression in ΔMSLN than empty vector (EV) control cells (fold change > 2) and lower expression in ΔMSLN rescue than in ΔMSLN (fold change < -2)

Table S3. Expression of mesothelin (MSLN) mRNA in endometrioid carcinoma (EC) cells obtained from CellMinerCDB

Table S4. List of 92 genes showing significantly lower expression in ΔMSLN than in empty vector (EV) control cells (fold change < –5)

Table S5. List of 43 genes showing significantly higher expression in ΔMSLN rescue than in ΔMSLN (fold change > 5)

Table S6. List of canonical pathways analyzed by IPA