Mesenchymal to epithelial transition in the human ovarian surface epithelium focusing on inclusion cysts

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Abstract. Most ovarian cancers arise from the mesothelial surface lining of the ovaries or from invaginations of this lining into the superficial ovarian cortex that form cortical inclusion cysts. Thus, these cysts are thought to be precursor lesions of ovarian carcinoma. Epithelial-mesenchymal transition, which is a transcriptional program for inducing maintenance of the mesenchymal phenotype, acts in tumor progression and metastasis. Little is known about the mechanisms involved in mesenchymal-epithelial transition (MET). We aimed to characterize the human ovarian surface epithelium (OSE) and inclusion cysts by immunohistochemical analysis to examine whether MET occurs during inclusion cyst formation in the OSE. We used specimens from 9 endometrial cancer patients who had undergone hysterectomy and bilateral salpingo-oophorectomy. Immunohistochemical analysis was performed in 10 normal ovaries containing 92 inclusion cysts and in 4 normal tubes to examine the expression of antigen markers including calretinin, podoplanin, D2-40, thrombomodulin, HBME-1, vimentin, EMA, WT1, CA125, MOC31, TAG-72, Ber-EP4 and E-cadherin. The positive staining rates for mesothelial markers in normal OSE were 100% (10/10) for calretinin, 80% (8/10) for podoplanin, 80% (8/10) for D2-40, 70% (7/10) for thrombomodulin, 100% (10/10) for HBME-1, 100% (10/10) for vimentin. The positive staining rates for epithelial markers in tubal epithelium were 100% (4/4) for HBME-1, 100% (4/4) for vimentin, 100% (4/4) for EMA, 75% (3/4) for TAG-72, 100% (4/4) for Ber-EP4. Inclusion cysts showed positive staining for both markers with an incidence of 51% (47/92) for HBME-1, 44% (41/92) for vimentin, 65% (60/92) for TAG-72, 88% (81/92) for Ber-EP4. The OSE showed the characteristics of both mesenchymal and epithelium cells. In contrast, inclusion cysts gained epithelial characteristics, but lost mesenchymal characteristics. These findings support that MET occurs during the inclusion cyst formation from OSE.

Introduction

The ovarian surface epithelium (OSE) is a modified pelvic mesothelium and originates from the mesoderm coelom. The OSE is a single layer of flat-to-cuboidal mesothelial cells (1,2). Despite its inconspicuous appearance *in vivo*, it is believed that OSE cells actively participate in the cyclical ovulatory rupture and repair process. The continuous rupturing of the OSE at ovulation and the subsequent proliferation required to repair the wound renders the cells susceptible to genetic damage and malignant transformation (3). There is evidence that the OSE surrounding the site of ovulatory rupture is exposed to inflammatory cytokines and other factors produced during follicle rupture such as reactive oxygen species, which increase the chance of DNA damage (4). The large majority of ovarian malignancies are of epithelial origin. However, the human OSE consists of mesothelial cells. It is generally accepted that ovarian epithelial cancers originate from the OSE cells that line the wall of inclusion cysts (5-7). Therefore, we hypothesize that mesenchymal to epithelial transition (MET) occurs during OSE inclusion cyst formation.

There is no published evidence concerning the occurrence of MET in ovaries; however, it has been reported that epithelial to mesenchymal transition (EMT) plays a role in carcinogenesis including ovarian cancers (7). The native ovarian surface mesothelium is of an 'uncommitted' phenotype and has the potential to change to the epithelial or mesenchymal phenotypes in response to signals such as those associated with ovulation (8). The exposure of the mesothelial lining of an inclusion cyst to the ovarian stromal microenvironment may be responsible for the phenotypic change to MET. Previously, Slot *et al* reported that the appearance of the Fas system and its related proteins in sequestered columnar OSE cells of irregularly shaped inclusion cysts might contribute to balancing cell growth with cell death (9).

The lack of epithelial differentiation markers including E-cadherin and CA125 and the expression of mesenchymal markers such as vimentin concurrently with epithelial markers including keratin and intermediate filaments suggest that the OSE is not as firmly determined as most other adult epithelia

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including epithelia of the oviduct and endometrium (10,11). Auersperg *et al* reported that OSE cells express both epithelial and mesenchymal intermediate filament proteins, i.e., keratin and vimentin (12,13).

The OSE is an inconspicuous single cell layer of flat-to-cuboidal cells on the ovary surface. Thus, a better understanding of the expression, regulation and activities of normal and neoplastic OSE cells is important for the identification of new clinical diagnostic and prognostic markers and for the treatment of ovarian cancer patients. In this study, we aimed to characterize the human OSE and inclusion cysts by immunohistochemical analysis with 13 epithelial and mesenchymal marker antibodies to examine MET during inclusion cyst formation.

Materials and methods

Patient materials. The Jikei University School of Medicine Ethics Review Committee approved the study protocol and informed consent was obtained from all patients. Formalin-fixed paraffin-embedded sections were obtained from 9 endometrial cancer patients who had undergone hysterectomy and bilateral salpingo-oophorectomy. The specimens included 10 normal ovaries containing 92 inclusion cysts and 4 normal tubes. We defined inclusion cysts as cysts of <1 cm in size that lacked OSE sequences and displayed different endosalpingiosis phenotypes. The histological subtypes of the inclusion cysts were 29 columnar/ciliated type, 15 mixed epithelial type and 48 cuboidal/flat type (Table I, Fig. 1).

IHC assay. Immunohistochemical analysis was performed using the labeled streptavidin-biotin peroxidase complex method with the Ventana auto-immunostaining system (Ventana Japan, Yokohama, Japan). Monoclonal antibodies against calretinin, podoplanin, D2-40, thrombomodulin,

Table I. Histological subtypes of the inclusion cysts.

	Number of cysts
Columnar/ciliated type (endosalpingeal type)	29
Mixed epithelial type	15
Cuboidal/flat type	48
Endometrioid type	0
Mucinous type	0
Transitional cell type	0

vimentin, EMA, HBME-1, WT1, CA125, MOC31, TAG-72, Ber-EP4 and E-cadherin were used for the immunohistochemical study (Table II). Calretinin, podoplanin, D2-40 and thrombomodulin were used as mesothelial markers. MOC31, TAG-72, Ber-EP4 and E-cadherin were used as epithelial markers. HBME-1, vimentin, EMA, WT1 and CA125 were used as both mesothelial and epithelial markers.

The specimens were fixed in formalin and paraffin-embedded according to standard procedures. The antigen retrieval procedure was carried out in a microwave oven in antigen retrieval solution for 10 min at 95°C in order to efficient staining. The sections were developed with 3,3'-diaminobenzidine mixed with 0.3% H_2O_2 and counterstained with hematoxylin. Slides from all inclusion cysts studied were then simultaneously processed for immunohistochemistry on the TechMate Horizon automated staining system (Dako, Glostrup, Denmark) using the Vectastain ABC perroxidase kit (Vector Laboratories, Burlingame, CA, USA). Endogenous biotin was saturated with a biotin blocking kit (Vector
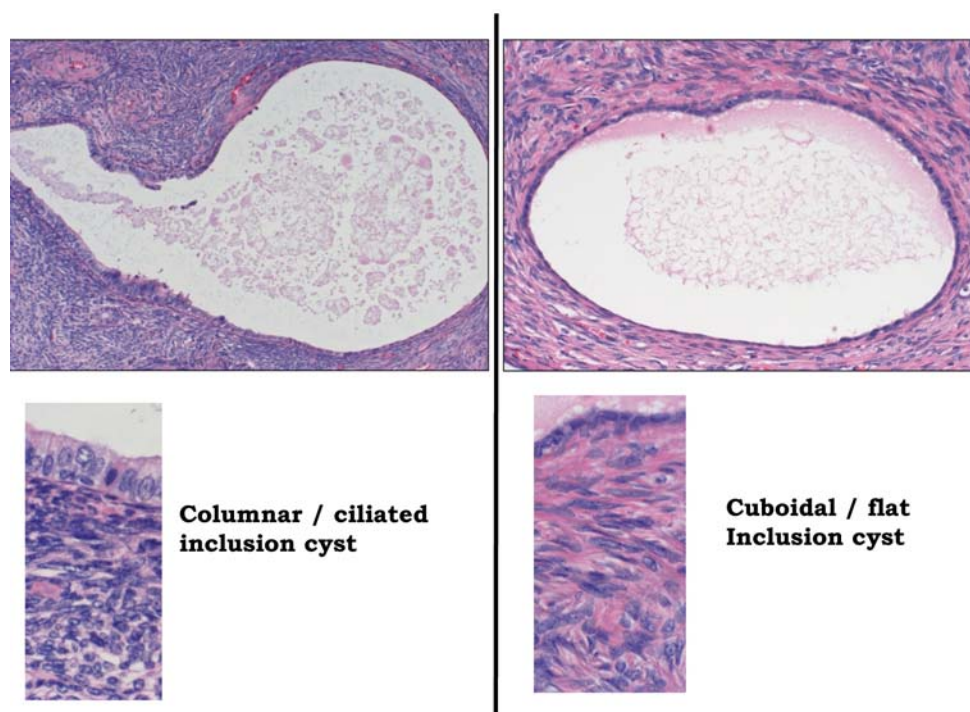

Columnar / ciliated inclusion cyst

Cuboidal / flat Inclusion cyst

Figure 1. Microscopic findings of columnar/ciliated type inclusion cysts and cuboidal/flat type inclusion cysts stained with hematoxin-eosin.

Table II. Antibodies and conditions for immunohistochemical analysis.

Antibodies	Clone	Source		Dilution for IHC[a]	AR[b] procedure	Specificity
Calretinin	Z11-E3	Zymed	Mouse IgG	Ready-to-use	MWO[c]	Mesothelial
Podoplanin	18H5	AngioBio	Mouse IgG	1:200	MWO[c]	Mesothelial
D2-40	D2-40	Nichirei	Mouse IgG	Ready-to-use	MWO[c]	Mesothelial
Thrombomodulin	1009	Dako	Mouse IgG	1:100	NR[d]	Mesothelial
HBME-1	HBME1	Dako	Mouse IgG	1:500	NR[d]	Both
Vimentin	-	Novocastra	Mouse IgG	1:50	MWO[c]	Both
EMA	E29	Dako	Mouse IgG	1:200	NR[d]	Both
Wilms' tumor 1 (WT1) protein	6F-H2	Dako	Mouse IgG	1:500	Protease	Both
CA125	-	Turner	Mouse IgG	1:40	NR[d]	Both
Epithelial-related antigen	MOC31	Dako	Mouse IgG	1:50	MWO[c]	Epithelium
TAG-72	B72.3	BioGenex	Mouse IgG	1:50	MWO[c]	Epithelium
Epithelial antigen, Ber-EP4	Ber-EP4	Dako	Mouse IgG	1:100	Protease	Epithelium
E-cadherin	-	Novocastra	Mouse IgG	1:50	MWO[c]	Epithelium

[a]IHC, immunohistochmistry; [b]AR, antigen retrieval; [c]MWO, microwave oven and [d]NR, not required.

Table III. Positive expression rate in OSE, tube and inclusion cyst.

| | OSE | Tube | Inclusion cyst | | | |
			Columnar/ciliated type	Mixed epithelial type	Cuboidal/flat type	Total
Calretinin	100% (10/10)	0% (0/4)	0% (0/29)	13% (2/15)	2% (1/48)	3% (3/92)
Podoplanin	80% (8/10)	0% (0/4)	0% (0/29)	0% (0/15)	2% (1/48)	1% (1/92)
D2-40	80% (8/10)	0% (0/4)	3% (1/29)	0% (0/15)	4% (2/48)	3% (3/92)
Thrombomudulin	70% (7/10)	0% (0/4)	0% (0/29)	0% (0/15)	2% (1/48)	1% (1/92)
HBME-1	100% (10/10)	100% (4/4)	72% (21/29)	73% (11/15)	31% (15/48)	51% (47/92)
Vimentin	100% (10/10)	100% (4/4)	72% (21/29)	60% (9/15)	23% (11/48)	44% (41/92)
WT 1	40% (4/10)	50% (2/4)	3% (1/29)	13% (2/15)	2% (1/48)	4% (4/92)
CA125	20% (2/10)	25% (1/4)	0% (0/29)	0% (0/15)	0% (0/48)	0% (0/92)
EMA	0% (0/10)	100% (4/4)	100% (29/29)	47% (7/15)	0% (0/48)	39% (36/92)
MOC 31	0% (0/10)	25% (1/4)	0% (0/29)	7% (1/15)	2% (1/48)	2% (2/92)
E-cadherin	0% (0/10)	75% (3/4)	3% (1/29)	27% (4/15)	4% (2/48)	7% (7/92)
TAG 72	0% (0/10)	75% (3/4)	55% (16/29)	60% (9/15)	73% (35/48)	65% (60/92)
Ber-EP-4	0% (0/10)	100% (4/4)	93% (27/29)	93% (14/15)	83% (40/48)	88% (81/92)

Laboratories). The sections were incubated with normal mouse serum for 10 min and then with mouse monoclonal antiserum against antigen (Cayman, Ann Arbor, MI, USA) by for 1 h. Negative controls were performed using non-immunized mouse serum or omitting the primary antiserum. Expression was evaluated in the ovarian surface epithelium, tubal epithelium and inclusion cysts. The intensity of staining was evaluated subjectively using the following categories: 0-20% (absent), 20-80% (focal present) and 80-100% (present). Positive and negative controls were run in parallel for each stain. We judged positive the specimens showing either focal present, or present staining.
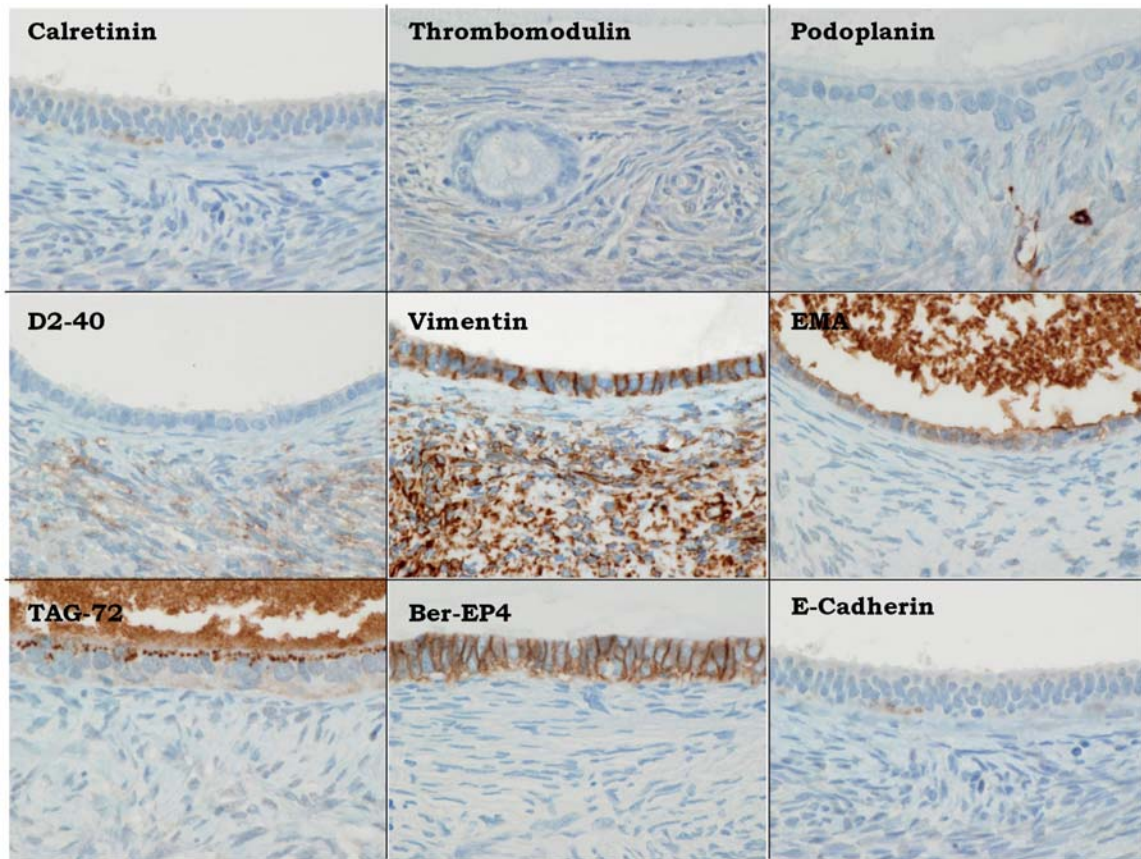
Figure 2. Immumohistochemical expression of calretinin, podoplanin, D2-40, thrombomodulin, vimentin, EMA, TAG-72, Ber-EP4 and E-cadherin in inclusion cysts. Immunohistochemical analysis was performed using the labeled streptavidin-biotin peroxidase complex method with the Ventana auto-immunostaining system. Monoclonal antibodies against calretinin, podoplanin, D2-40, thrombomodulin, vimentin, EMA, HBME-1, WT1, CA125, MOC31, TAG-72, Ber-EP4 and E-cadherin were used for the immunohistochemical study.

Results

OSE. The positive staining rates for mesothelial markers in normal OSE were 100% (10/10) for calretinin, 80% (8/10) for podoplanin, 80% (8/10) for D2-40 and 70% (7/10) for thrombomodulin. The positive staining rates for both markers were 100% (10/10) for vimentin, 0% (0/10) for EMA, 100% (10/10) for HBME-1, 40% (4/10) for WT1 and 20% (2/10) for CA125. As for the epithelial markers, no staining was detected for MOC31, TAG-72, Ber-EP4, or E-cadherin (Table III).

Tubal epithelium. The positive staining rates for mesothelial markers were 0% (0/29) for calretinin, 0% (0/29) for podoplanin, 3% (1/29) for D2-40, and 0% (0/29) for thrombomodulin. The positive staining rates for both markers were 100% (29/29) for vimentin, 100% (29/29) for EMA, 100% (4/4) for HBME-1, 50% (2/4) for WT1 and 25% (1/4) for CA125. The positive staining rates for the epithelial markers were 25% (1/4) for MOC31, 75% (3/4) for TAG-72, 100% (4/4) for Ber-EP4, and 75% (3/4) for E-cadherin (Table III).

Inclusion cyst. The positive staining rates for mesothelial markers in the total inclusion cysts were 3% (3/92) for calretinin, 1% (1/92) for podoplanin, 3% (3/92) for D2-40 and 1% (1/92) for thrombomodulin. The positive staining rates for both markers in the inclusion cysts were 44% (41/92) for vimentin, 39% (36/92) for EMA, 51% (47/92) for HBME-1, 4% (4/92) for WT1 and 0% (0/92) for CA125. The positive staining rates for the epithelial markers in the inclusion cysts were 2% (2/92) for MOC31, 65% (60/92) for TAG-72, and 88% (81/92) for Ber-EP4 and 7% (7/92) for E-cadherin (Fig. 2).

Considering the histological subtypes of inclusion cysts, no significant differences in the positive rate are detected between mesothelial markers and epithelial markers. As for staining with both markers, the positive staining rates for vimentin, EMA and HBME-1 were lower in the cuboidal/flat type than that in the columnar/ciliated type cells (Table III).

The OSE has the characteristics of both mesencyhmal and epithelium cells and the tubal epithelium has epithelial characteristics. In contrast, inclusion cysts gain epithelial characteristics but lose mesencyhmal characteristics. MET was observed during inclusion cyst formation (Fig. 3).

Discussion

The normal OSE is a stationary mesothelium that retains the capacity to alter its state of differentiation to stromal or epithelial phenotypes in response to environmental cues. Human OSE cells typically form a cobblestone epithelial monolayer in culture and assume flattened epithelial or atypical, fibroblast-like forms with time and passages (14-16). Resta *et al* reported that a high frequency of hyperplastic and
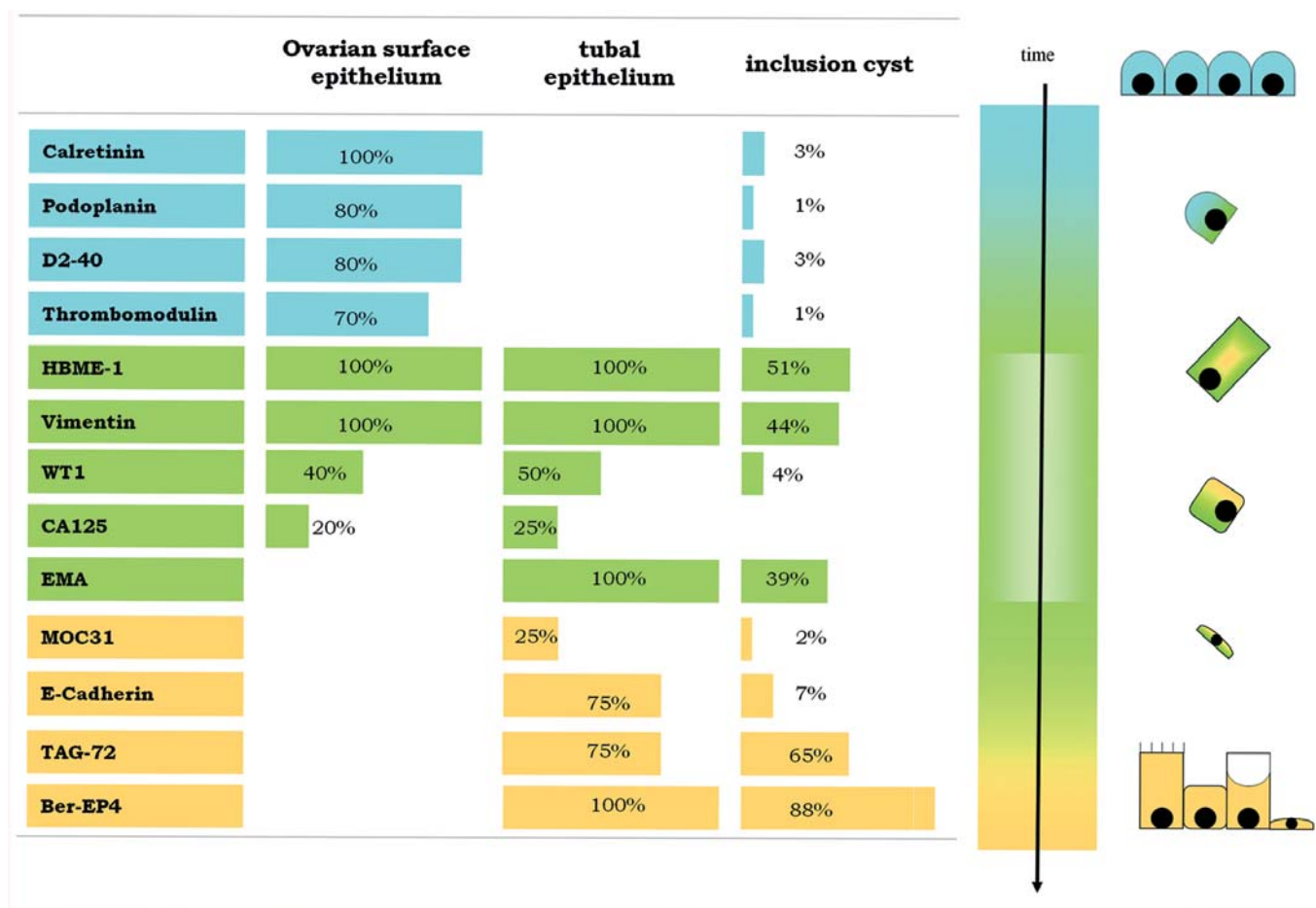
Figure 3. Immunohistochemical analysis of the OSE, tubal epithelium and inclusion cysts suggests that MET occurred during inclusion cyst formation. Calretinin, podoplanin, D2-40 and thrombomodulin were used as mesothelial markers. MOC31, E-cadherin, TAG-72, and Ber-EP4 were used as epithelial markers. HBME-1, vimentin, WT1, CA125 and EMA were used as both mesothelial and epithelial markers. OSE had the characteristics of both mesenchymal and epithelium cells and the tubal epithelium had epithelial characteristics. In contrast, inclusion cysts gained epithelial characteristics but lost mesenchymal characteristics.

metaplastic changes occur in the surface epithelium or inclusion cysts of ovaries with contralateral epithelial ovarian tumors, endometrial adenocarcinomas and polycystic ovary disease (17). The high incidence of hyperplastic and mullerian metaplasia on the surface epithelium or in the inclusion cysts of patients with epithelial tumors of the contralateral ovary stresses the potential role of these changes as a substrate for the development of ovarian cancer (5). In this study, we found that the OSE has both mesenchymal and epithelial characteristics and the tubal epithelium has epithelial characteristics. In contrast, we found that inclusion cysts gained epithelial characteristics but lost mesencyhmal characteristics indicating that MET occurs during inclusion cyst formation. Our immunohistochemical study supports the conclusions of previous studies.

In our study, we used 13 epithelial and mesenchymal marker antibodies to examine MET during inclusion cyst formation. Ordóñez reported that there are many immuno-histochemical markers available that are useful for distinguishing between epithelioid mesotheliomas and pulmonary adenocarcinomas (18). Over the past two decades, an increasing number of antibodies that are useful for distinguishing between epithelioid mesotheliomas and pulmonary adenocarcinomas have been developed, many of

which have become commercially available. We selected 13 antibodies referring to related reports (19-21).

We used HBME-1, vimentin, EMA, WT1 and CA125 as both mesothelial and epithelial markers. The OSE that lines inclusion cysts is two to three times more metaplastic in women with epithelial ovarian tumors than in women without such cancers (5). Ovarian cancer markers including CA125 and CA19-9 are significantly more expressed in the epithelium of inclusion cysts than in the OSE, suggesting that early malignant changes occur more frequently in OSE lined inclusion cysts than in the ovarian surface itself (12). In our study, no expression of CA125 was detected in inclusion cysts, probably because we used inclusion cysts in ovaries from endometrial cancer patients, not from ovarian cancer patients. We examined the number of inclusion cysts in ovarian cancer cases; nevertheless, we only found a small number of stage I ovarian cancer cases and no cases beyond stage II. Therefore, we checked the number of inclusion cysts in ovaries from endometrial cancer cases and found a lot of inclusion cysts in ovaries from endometrial cancer cases, especially postmenopausal cases. The expression of HBME-1, vimentin and EMA was different among different histological inclusion cyst types. The columnar/ciliated type showed characteristics similar to the tubal epithelium, while the

cuboidal/flat type characteristics were similar to mesothelial cells. These different phenotypes among inclusion cysts may display different expression patterns.

E-cadherin is the 'master gene' of epithelial phenotype induction and mesenchymal to epithelial transition (MET) has been shown to occur in E-cadherin cDNA transfected fibroblast cell lines and tumor cell lines (22,23). Our results showed clearly different expression among OSE, tube, and inclusion cysts, suggesting that MET occurs during inclusion cyst formation.

The literature on MET or EMT in ovarian cancer is limited compared to that of other human cancers with most based on the evaluation of EMT-associated changes in response to external stimuli in cultured ovarian cancer cell lines. Few studies have evaluated cancer markers using tissue arrays or have implicated known EMT markers with increasing tumor stage. However, immunohistochemical studies and those using cell lines provide evidence that epithelial ovarian tumors undergo EMT during disease progression. One can argue that this transition is not complete and tumors may retain both the epithelial and mesenchymal phenotypes or they may undergo MET as described above, and shown for other cancers (24,25).

However, epithelial-mesenchymal transition (EMT) dedifferentiation of the tumor cells, which is a redifferentiation towards an epithelial phenotype that resembles a mesenchymal to epithelial transition, is detectable in other carcinoma, especially colorectal carcinomas (26). This indicates that malignant progression is based on dynamic processes that cannot be explained solely by irreversible genetic alterations, but must be additionally regulated by the tumor environment (27).

In summary, we found that the OSE had the characteristics of both mesenchymal and epithelium cells and that the tubal epithelium had epithelial characteristics. In contrast, inclusion cysts gained epithelial characteristics, but lost mesenchymal characteristics. MET was observed during inclusion cyst formation. To our knowledge, this is the first study on MET during inclusion cyst formation detected by immunohistological analysis.

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