Expression of Candidate Tumor Markers in Ovarian Carcinoma and Benign Ovary: Evidence for a Link Between Epithelial Phenotype and Neoplasia

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EpCAM, epithelial membrane antigen (EMA)–mucin 1 (MUC1), mesothelin, and CD9 have been reported to be overexpressed at the RNA level in ovarian carcinomas. By using immunohistochemistry, we profiled the protein expression of these gene products in ovarian carcinoma tissues and compared them with benign ovarian surface epithelium (OSE) and cortical inclusion cysts (CICs). Immunoreactivity for EMA and calretinin were used to define epithelial and mesothelial differentiation in nontumor tissues, respectively. Papillary serous (n = 16) and endometrioid (n = 10) tumors were immunopositive for EMA/MUC1 (100%), mesothelin (75% and 30%, respectively), CD9 (88% and 90%, respectively), and EpCAM (100%). All ovarian carcinomas and carcinoma cell lines tested were negative for calretinin. In nonneoplastic ovary, both OSE and CICs ranged from flat-to-cuboidal to stratified and ciliated in appearance. OSE with a cuboidal morphology had a similar immunoreactivity as omental peritoneum, expressing calretinin, mesothelin, and CD9. In contrast, CICs with stratified and ciliated epithelium show expression patterns similar to those in fallopian tubes. They frequently expressed EMA, EpCAM, mesothelin, and CD9. This immunophenotype is preserved in ovarian carcinomas, suggesting that Müllerian metaplasia signals the acquisition of these markers and that their expression is maintained in ovarian carcinomas that originate from this epithelium. HUM PATHOL 35:1014-1021. © 2004 Elsevier Inc. All rights reserved.

Key words: **EMA/MUC1, EpCAM, mesothelin, CD9, calretinin, EMA, ovary, carcinoma.**

Abbreviations: **EMA, epithelial membrane antigen; OSE, ovarian surface epithelium; IHC, immunohistochemistry; CICs, cortical inclusion cysts; hOSE, human ovarian surface epithelial [cell lines].**

The ovarian surface epithelium (OSE) covers the entire ovarian surface and varies morphologically from simple flat mesothelium to cuboidal to low pseudostratified columnar ciliated epithelium.[1,2] Although during early reproductive life the OSE is generally simple in appearance, with aging, the ovarian surface becomes more complex, with the development of cortical inclusion cysts (CICs) in most ovaries. CICs are largely epithelial in appearance, and the source of this epithelium is unclear. Potential sources include transfer of endometrial or salpingeal epithelium to the ovarian surface by exfoliation, direct contact with salpingeal epithelium by adhesions, and epithelial metaplasia of the OSE.[3] In the latter scenario, the incorporation of the epithelium into the ovarian cortex may occur by repair of ovulation sites, adhesions, and surface invaginations that develop during the process of ovarian atrophy (reviewed in Vanderhyden et al[4] and in Drapkin and Hecht[5]). The incidence of CICs increases with advancing age, and they are common in postmenopausal women. Although generally benign in nature, these epithelial rearrangements are widely thought to be the potential origin of most ovarian epithelial cancers. The more frequent appearance of epithelial invaginations and inclusion cysts in women with a hereditary predisposition for ovarian cancer supports this hypothesis (reviewed in Vanderhyden et al,[4] Drapkin and Hecht,[5] and Wong and Auersperg[6]).

In addition, the most common subtypes of ovarian epithelial tumors are histologically similar to other tumors arising in the female genital tract. For instance, serous carcinomas resemble tumors arising in the fallopian tubes, whereas ovarian endometrioid and mucinous carcinomas resemble endometrial and endocervical carcinomas, respectively. This similarity is consistent with the fact that although continuous with the mesothelial lining of the peritoneal cavity, the OSE shares a common embryologic origin with epithelia of Müllerian duct–derived tissues; they both arise from the coelomic epithelium in the area of the gonadal ridge. This common origin is evident in normal adult ovaries as the commonly observed histological transformation of the OSE to a more columnar and ciliated cell type, a process referred to as Müllerian metaplasia. In fact, OSE cells in culture can be driven to both epithelial and mesothelial differentiation.[1,2] Moreover, results from several observational studies support the hypothesis that dysplasia or hyperplasia arising within these epithelial inclusion cysts may represent a histological precursor to ovarian carcinoma.[7-10]

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Together, these morphological observations suggest that the OSE normally undergoes a physical relocation from the surface into the ovarian cortex through processes related to ovulation and aging. Once entrapped in the cortex, a conversion from the flat cuboidal mesothelial cells to stratified Müllerian epithelial cells can occur, perhaps under the influence of stromal hormones.[11] Malignant transformation of these CIC epithelial cells can then result in carcinomas with the morphological variations and characteristics of ovarian carcinomas. The particular molecular and genetic events associated with neoplastic transformation of the ovarian epithelium on the surface or in CICs are still largely unknown. However, recent large-scale genomewide studies of ovarian cancers have revealed that certain genes are overexpressed in ovarian cancer cell lines and primary tumors.[12-15] It is currently unclear where in the process of neoplastic transformation of the OSE these genes are involved, but their gene products may serve as useful tools in the early detection of ovarian cancer and perhaps in the development of novel therapeutics.

As a first step in determining the potential roles of these genes in the diagnosis, detection, or treatment of ovarian cancer, we asked whether we could validate their expression in human ovarian tumor samples by immunohistochemistry (IHC). A review of the literature identified a large number of genes that are overexpressed in ovarian carcinomas relative to cultured ovarian surface cells. However, when we applied a limited set of restrictions, the list of genes was greatly reduced. We selected 5 genes for further study. Our data indicate that all 5 genes are overexpressed at the protein level in the most common ovarian carcinomas. Moreover, the results from our analysis on OSE and CICs provide a link between metaplasia of the surface epithelium, CICs, and ovarian carcinogenesis.

MATERIALS AND METHODS

Genomewide experimental approaches have been applied to the study of ovarian cancer. Attempts to identify genes involved in ovarian tumorigenesis have used primary human ovarian tumors and cell lines in a wide array of experimental methodologies, including differential display, serial analysis of gene expression, global gene expression profiling using cDNA arrays, comparative genomic hybridization, 2-dimensional gel electrophoresis of cellular proteins, and proteomic analysis of serum proteins. Published studies were identified in MEDLINE and reviewed. Genes conforming to the following parameters were selected: (1) genes up-regulated in ovarian cancers relative to normal ovarian surface epithelium and (2) genes appearing in at least 2 independent published studies. Among the genes fulfilling these criteria were *HE4, mesothelin, EpCAM, mucin 1,* and *CD9.* Relevant references are cited in Table 1. Those with commercial antibodies were chosen and included mucin 1 (also called MUC1, epithelial membrane antigen [EMA]), EpCAM (also called ESA, EGP40, 323/A3), mesothelin, and CD9. Studies on HE4 will be presented elsewhere. EMA and calretinin served as positive controls for epithelial and mesothelial differentiation, respectively.

TABLE 1. Genes Commonly Overexpressed in Ovarian Carcinomas*

Gene	Function	Reference No.
HE4	Epididymis-specific protease inhibitor	13, 34, 35, 12, 36, 37
Mucin1	Glycoprotein	13, 36, 37, 12
EpCAM	Glycoprotein	38, 39, 12, 34
Mesothelin	Glycoprotein	39, 12, 34
CD9	Transmembrane protein	36, 34

*This list includes genes that were identified in at least 2 independent studies.

Immunohistochemistry

Immunohistochemical localization of target proteins was performed on 4-micrometer sections from paraffin-embedded tissue. In addition to the aforementioned commercially available antibodies used in this study, we also employed primary antibodies for the estrogen receptor (ER) and the mesothelial marker calretinin. Antigen retrieval was performed as described in Table 2. Primary antibodies were detected with the Envision+ system that employs horseradish peroxidase–labeled polymer conjugated to goat anti-mouse immunoglobulin antibodies. Immune complexes were iden-

TABLE 2. Antibodies Used for Immunoperoxidase Staining

Gene	Source and Location (Mono/Poly)	Clone	Dilution	Retrieval Time in Min (method)
EpCAM	Dako, Carpinteria, CA	BerEp4	1:100	10 (protease)
Mesothelin	Novocastra, UK	5B2	1:50	30 (microwave)
CD9	Neomarkers, Fremont, CA	9CO1	Prediluted	30 (microwave)
EMA/Muc1	Dako	E29	1:200	30 (microwave)
Calretinin	Zymed, San Francisco, CA	Polyclonal	1:300	30 (microwave)
ER	Dako	1D5	1:200	30 (microwave)

NOTE. Incubation times and buffer in each case was 40 minutes, TBS.
Abbreviations: TBS, Tris-buffered saline; EMA, epithelial membrane antigen; ER, estrogen receptor.

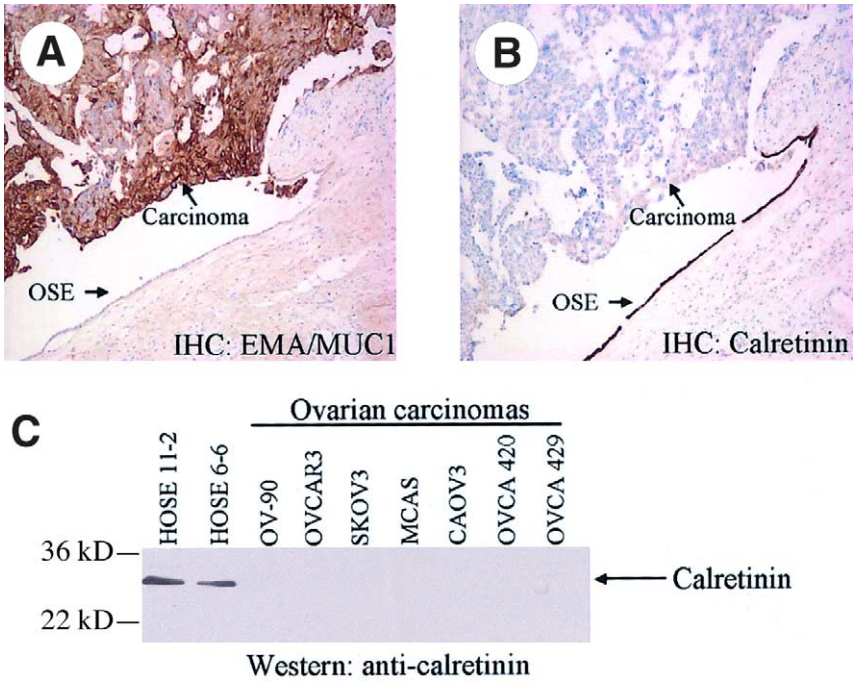

FIGURE 1. Epithelial and mesothelial controls (10 × objective). All cases of carcinoma were reactive for epithelial membrane antigen (EMA; A) and negative for calretinin (B). Calretinin highlighted the residual normal ovarian surface epithelium (OSE), whereas EMA was negative. (C) Ovarian cancer cell lines show restricted calretinin protein expression. Human OSE (hOSE) cell lines expressed calretinin, whereas ovarian cancer cell lines do not.

tified by using a peroxidase reaction with DAB+ as chromogen (Envision+ detection system, K4006, Dako Corp, Carpinteria, CA). Positive controls were established for each stain, including peripheral nerve (EMA); mesothelioma (mesothelin); tonsil (CD9); and normal skin, appendix, or muscle (EMA). Slides were counterstained with methyl green.

Cell Lines, Lysate Preparation, and Western Blot Analysis

Human ovarian surface epithelial (hOSE) cell lines were immortalized by infection with a replication-defective retrovirus encoding the human papillomavirus oncoproteins E6 and E7 as described elsewhere[16] and were nontumorigenic in nude mice. All the ovarian cell lines, except MCAS, were derived from the ascitic fluid of women with advanced carcinoma. OV-90, OVCA-420, and OVCA-429 were generated from women with serous tumors; OVCAR-3, SKOV-3, and CAOV3 were derived from women with poorly differentiated ovarian adenocarcinomas; MCAS is a line derived from the solid component of a mucinous cystadenocarcinoma.[17] All lines were cultivated in M199 media (Sigma, St. Louis, MO) supplemented with MCDB105 (Sigma) containing 10% fetal bovine serum at 37°C in a 10% CO_2-containing atmosphere. Cells were harvested at 80% confluence and lysed in ice-cold NETN buffer (20 mM Tris-HCl [pH 8], 150 mM NaCl, 1 mM EDTA, 0.5% NP-40) for 45 minutes. Insoluble material was removed by centrifugation.

Soluble cell lysates were resolved on 4% to 12% gradient Tris-Glycine gels (Invitrogen Corporation, Carlsbad, CA) and transferred onto nitrocellulose membranes (Invitrogen) with a semidry blotter. Membranes were incubated with anti-calretinin polyclonal rabbit antibodies (Zymed, South San Francisco, CA) at a dilution of 1:1000. After subsequent washes, a Horseradish peroxidase linked anti-rabbit Ig secondary antibody (Amersham Biosciences, Piscataway, NJ) was added. The blots were developed with a chemiluminescence kit from Amersham Biosciences.

Case Selection

After institutional review board approval, the computer records of the Departments of Pathology of Brigham and Women's Hospital and Beth Israel-Deaconess Medical Center were queried for the diagnosis of papillary serous or endometrioid type (grade 1-2) ovarian carcinoma. Slides of each case were reviewed, and only tissue sections of tumor with adjacent residual ovary were selected. In addition, we identified cases in which benign ovaries were removed incidentally for another procedure (7 cases) or as prophylaxis for familiar cancer (8 cases). In all, 26 cases of cancer (16 papillary serous and 10 endometrioid type) and 11 normal ovaries were examined.

Histological Evaluation of Staining

Tumors were considered positive if >50% of the tumor cells in the tissue section were reactive. Staining patterns in OSE and CIC are described in the Results section.

RESULTS

Epithelial Versus Mesothelial Antigens

To establish internal controls for these studies, we examined the expression patterns of calretinin and EMA (MUC1). Calretinin is a calcium-binding protein that is expressed in mesothelial cells and mesotheliomas but not in adenocarcinomas of various sites.[18,19] Recent studies have shown that calretinin is expressed robustly in OSE and its invaginations and in CICs lined by flat-to-cuboidal epithelium.[20,21] In contrast, EMA is a high molecular weight transmembrane glycoprotein expressed in many carcinoma and nonneoplastic epithelia, but is not expressed in OSE or peritoneum.[22,23]

All our cases of ovarian carcinoma were reactive

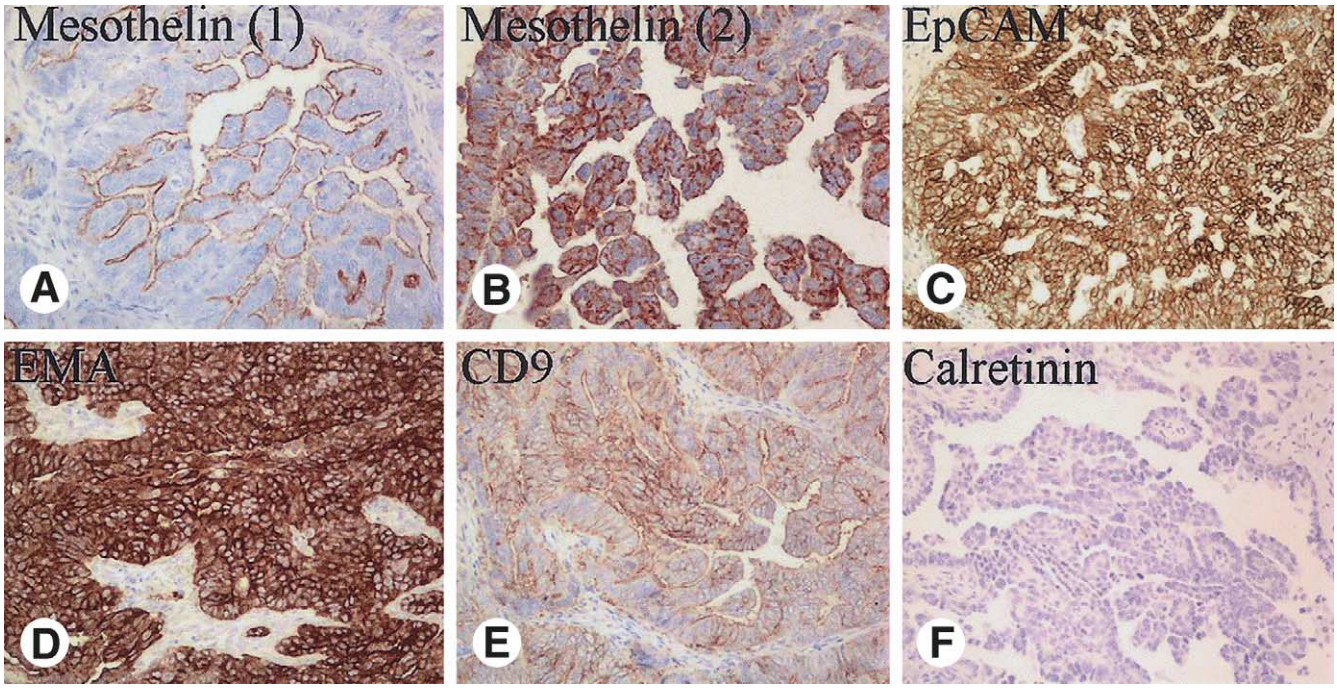

FIGURE 2. Papillary serous ovarian carcinoma (20 × objective). Immunohistochemistry using antibodies against specified proteins. Mesothelin generally had an apical staining pattern (A) but occasionally stained more extensively (B). EpCAM (C) and EMA/MUC1 (D) had strong diffuse membranous and cytoplasmic staining. CD9 (E) had a membranous pattern with apical accentuation. Calretinin stains (F) were all negative.

for EMA and negative for calretinin (Fig 1A and B). In addition, calretinin highlighted the residual normal OSE (Fig 1B). We confirmed this observation by using normal hOSE and a battery of ovarian cancer cell lines in a Western blot analysis. When compared with hOSE cell lines, which expressed calretinin, none of the ovarian cancer cell lines were positive, consistent with our IHC data (Fig 1C). Equal protein loading was confirmed by Western blotting for β-tubulin (data not shown).

Localization of Antigens in Tumors

We then proceeded to ask whether the candidate ovarian tumor markers we identified (Table 1) are overexpressed at the protein level in our set of ovarian serous and endometrioid carcinomas and how these patterns compare to normal ovarian tissues. We found that the majority of tumors stained with EMA, mesothelin, CD9, and EpCAM (Fig 2). Conversely, all the tumors were negative for calretinin (Fig 2; Table 3). Interestingly, mesothelin expression was more prominent in the serous-type tumors, whereas it was only weakly reactive in endometrioid histology. EMA, EpCAM, and CD9 localized to the cell membrane, whereas mesothelin showed an apical or luminal pattern. CD9 staining accentuated a luminal distribution and did not stain every adjacent cell in an epithelial sheet.

Ovarian Surface Epithelium

Staining of normal tissues was more complex and correlated with the local histology. The ovarian surface epithelium ranged from flat to cuboidal to stratified and ciliated in appearance. Transitions between these epithelial architectures were typically abrupt and discontinuous. Cuboidal and flat-surface epithelium tended to stain with calretinin but not with EMA (Fig 3), consistent with our controls (Fig 1). EpCAM reactivity paralleled that of EMA in normal ovarian tissues. Mesothelin reactivity was confined to areas with stratification or plump cuboidal cells. The apical mesothelin staining pattern seen in tumors was also present in the normal surface cells. However, mesothelin staining was generally very weak and focal at the ovarian surface, compared with the carcinomas and underlying cortical inclusion cysts (see below). CD9 staining was variable but was generally confined to cuboidal surface epithelium and, focally, in stratified areas of the surface. In general, OSE staining with these markers paralleled the staining seen in reactive mesothelium or peritoneum (omentum; Fig 5).

Cortical Inclusion Cysts

Cortical inclusion cysts also had variable histology ranging from flat or cuboidal to stratified and ciliated. There were only occasional cysts showing transitions between histologies (see Figure 4). The predominant staining patterns are listed in Table 4. The majority of CICs with flat or cuboidal histology stained similar to the ovarian surface with reactivity for calretinin but not for EMA, EpCAM, or mesothelin (Table 4). However, CICs with stratified histology had reactivity with EMA, EpCAM, and mesothelin but were negative for calretinin (Fig 4; Table 4). CD9 was generally negative in CIC

TABLE 3. Staining Pattern in Tumors

Group (No. of Cases)	n (%)				
	Calretinin	EMA	Mesothelin	CD9	EpCAM
Papillary serous (16)	0 (0)	16 (100)	12 (75)	14 (88)	16 (100)
Endometrioid (10)	0 (0)	10 (100)	3 (30)	9 (90)	10 (100)

Abbreviation: EMA, epithelial membrane antigen.

(Table 4). The fallopian tube served as an epithelial control with reactivity for EMA, EpCAM, and mesothelin, but not for calretinin (Fig 5; Table 4). The CD9 staining pattern of the fallopian tube showed patchy staining of basally located cells (Fig 5). Interestingly, normal proliferative endometrial glands stained with EMA, CD9, and EpCAM but not for mesothelin, except in areas of ciliated metaplasia.

As mentioned above, cysts with a transition between flat or cuboidal and stratified/ciliated histologies were rare but when present did adopt a mixed antigen reactivity (Fig 4, bottom panel). In these cases, flat or cuboidal cell morphology was associated with calretinin positivity, whereas stratified or ciliated epithelium was EMA/MUC1 positive. These findings suggest that CICs house a potential transition in morphology that is accompanied by a change in tumor antigen expression. The stroma around the CIC with stratified or ciliated histology was not histologically distinct. However, occasionally, both the stromal cells and adjacent epithelial lining were ER positive (data not shown).

DISCUSSION

In human ovarian carcinomas, overexpression of EMA/MUC1, EpCAM, mesothelin, and CD9 has been shown in some studies to occur at the RNA level (see Table 1). In this article, we have verified that the corresponding proteins are highly expressed in both serous and endometrioid carcinomas. Calretinin, a marker of mesothelial cells and OSE, was not expressed in these tumors and served as a control in these experiments. Expression of EMA/MUC1, EpCAM, and CD9 was present in both endometrioid and serous subtypes, whereas mesothelin was more significantly associated with the serous phenotype. Similar findings for EpCAM recently have been reported.[24] Immunohistochemical distinctions between serous and well to moderately differentiated endometrioid histologies are not surprising because these histologies are known to be biologically and clinically distinct.[25] For example, serous cancers are more aggressive and more frequently associated with mutations in the p53 gene, and the overexpression of p53 is often used to separate serous from endometrioid subtypes.[26] Further studies will determine whether mesothelin can distinguish histological subtypes.

We found that the epithelium in CICs is distinguished by absence of calretinin and presence of EMA/MUC1 immunoreactivity, a phenotype similar to that seen in the epithelial cells of the fallopian tube. Mesothelin is a glycoprotein that is abundantly expressed in peritoneal mesothelial cells and in peritoneal mesotheliomas.[27] CD9 is a broadly expressed, transmembrane protein of the tetraspanin superfamily with a putative role in endothelial cell function.[28] Whereas mesothelin is variably expressed in OSE, it is strong in CIC. In contrast, CD9 is abundantly expressed on the OSE and hilar vasculature but is lost in CICs.

The observation that benign CICs and ovarian carcinomas coexpress EMA/MUC1, EpCAM, and mesothelin, whereas the OSE is negative for these markers suggests that ovarian carcinomas do not emerge directly from the transformation of surface mesothelial cells but rather require an additional alteration. Our data suggest that the additional step involves a metaplastic transformation of the normal surface to a multilayered or ciliated Müllerian epithelium. Such metaplasia is most often observed in CICs. Moreover, the presence of these markers in the ensuing carcinomas suggests that once acquired by the CIC Müllerian epithelium, the expression of these markers is preserved in neoplasia.

Various models to explain ciliated epithelium in CIC have been postulated. One mechanism proposes that Müllerian-type epithelium arises from transfer of endometrial or tubal epithelium to the ovarian surface by exfoliation or adhesions.[3,4] Although we cannot exclude this transfer model, we found occasional CICs that show a morphological transition from flat to Müllerian-type epithelium, suggesting in situ metaplasia. In addition, surface invaginations of OSE with stratification maintained the peritoneal (calretinin-positive) staining pattern. The metaplasia model for ciliated cyst formation is further supported by the uncommitted phenotype of OSE. OSE has features of epithelial and mesenchymal origin that are characterized by expression of cytokeratin, laminin, and collagen IV as well as vimentin and collagen I and III. In addition, E-cadherin

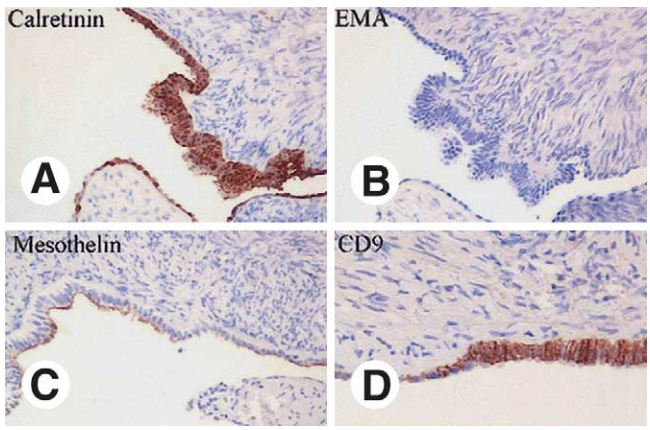

FIGURE 3. Ovarian surface epithelium (OSE) immunoreactivity (20× objective). Immunohistochemistry using antibodies against specified proteins. OSE is calretinin positive (A) and epithelial membrane antigen is negative (B), even in areas of multilayering. (C) Mesothelin is diffusely reactive but is enhanced and adopts an apical pattern in areas of multilayering. (D) CD9 was generally confined to areas of multilayering.

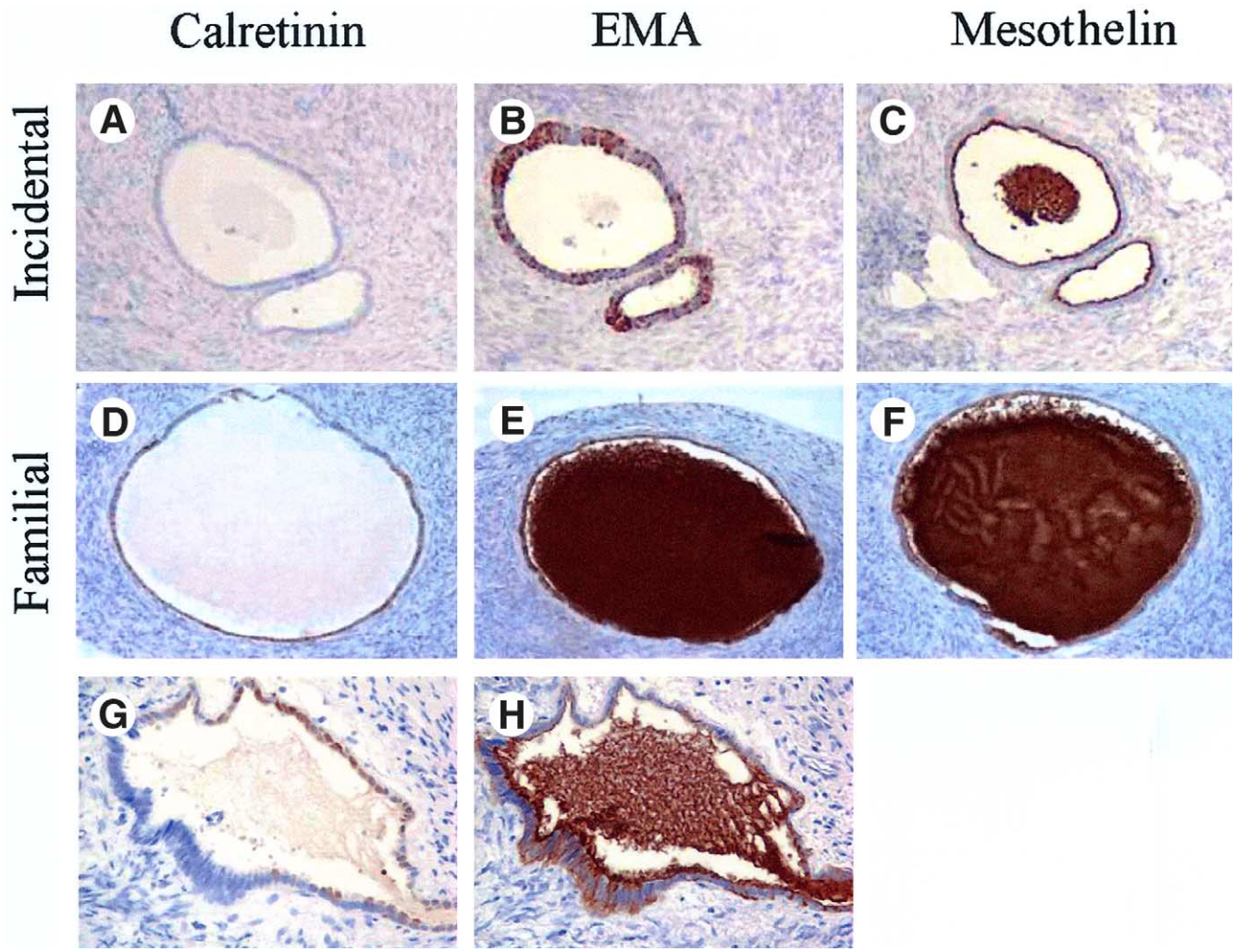

FIGURE 4. Cortical inclusion cyst (CIC) immunoreactivity (20 × objective). (A-C) Immunohistochemistry for calretinin, epithelial membrane antigen (EMA), and mesothelin. EMA and mesothelin are positive in cysts with stratified or ciliated epithelium, whereas calretinin is negative (D-F). The staining of CICs for calretinin, EMA, and mesothelin was similar in ovaries removed for cancer prophylaxis (familial) and those removed for other reasons (incidental). (G, H) In cysts with a histological transition from flat to multilayered or ciliated epithelium, EMA is positive only in the metaplastic or ciliated areas.

TABLE 4. Staining Pattern in Surface and CIC Epithelium

Area	Histology	Calretinin	EMA	Mesothelin	CD9	EpCAM	ER
Surface							
1	Flat	+	−	−	−	−	−
2	Cuboidal	+	−	+ (apical)	+	−	+
3	Stratified	+	−	+ (apical)	+	+/−*	+
CIC							
1	Flat/cuboidal	+	−	+ (apical)	−	−	−
2	Cuboidal	−	−/+*	+ (apical)	−	+	+*
3	Stratified	−	+	+	−	+	+
4	Stratified (ciliated)	−	+	±	+	+	+
Peritoneum (omentum)							
	Flat, papillary	+	−	+	+/−*	−	−
Fallopian tube							
		−	+	+ (apical)	+	+	+
Endometrium							
	Proliferative	NA	+	−†	+	+	+

Abbreviations: CIC, cortical inclusion cysts EMA, epithelial membrane antigen; ER, estrogen receptor; NA, not available.
*Scattered patches of cells are positive.
†Positive in areas of ciliated metaplasia.

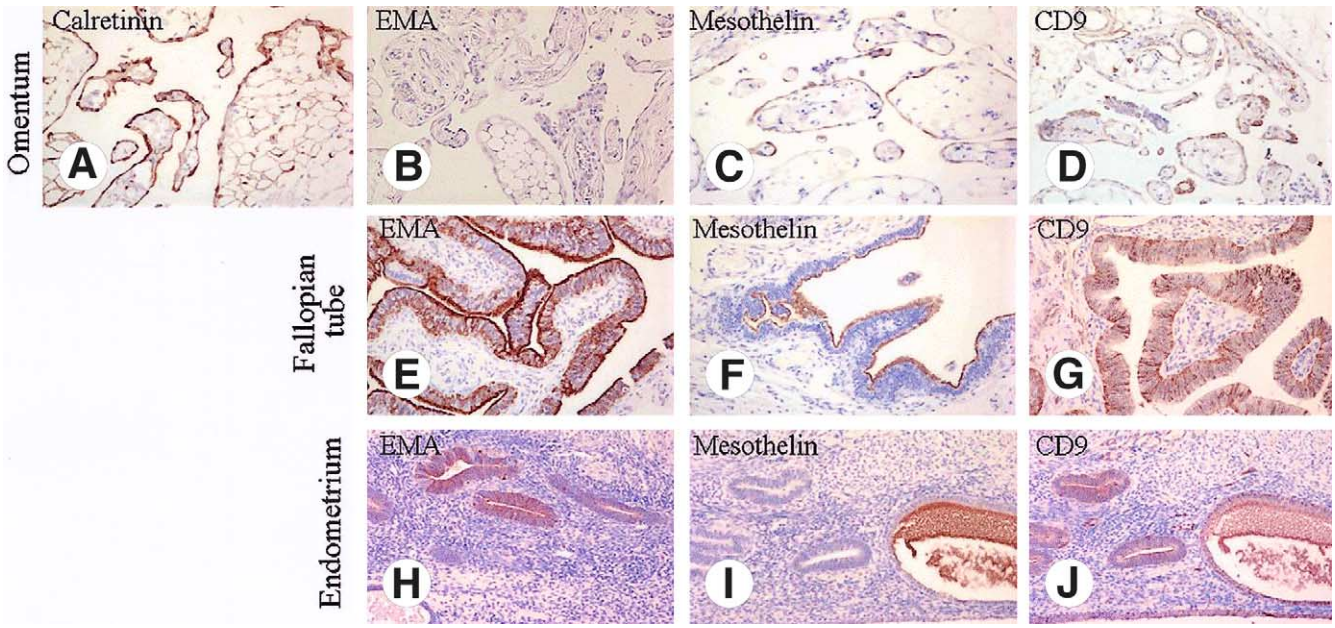

FIGURE 5. Immunoreactivity in fallopian tube, peritoneum, and endometrium. Immunohistochemistry using antibodies against specified proteins. Omentum is positive for calretinin (A) and mesothelin (C) but is negative for epithelial membrane antigen (EMA; B); staining for CD9 is patchy (D). Fallopian tube is positive for EMA (E), mesothelin (F), and CD9 (G). Endometrium is positive for EMA (H) and CD9 (J); mesothelin is positive only in glands with ciliated metaplasia (I), and mesothelin staining is apical, similar to the tumor. CD9 staining in endometrium (J) is strong, but patchy.

induces mesenchymal-to-epithelial transition in human OSE, which results in the expression of estrogen, progesterone, and androgen receptors.[29-31] To explore the transfer hypothesis for endometrioid carcinomas, we stained normal Müllerian tissues for the tumor markers (Fig 5). Mesothelin stains normal fallopian tube and areas of ciliated metaplasia in endometrium but not normal cycling endometrial glands. Mesothelin expression, therefore, is more closely related to histological differentiation than to the site of origin.

In conclusion, we used IHC to validate the expression of several putative tumor markers at the protein level on human ovarian cancer tissues. The studies used to define our list of candidate tumor markers were based on the assumption that OSE and ovarian carcinomas can be distinguished at the RNA level. Although this is a valid assumption, our findings suggest that additional steps are required beyond the simple transformation of the OSE. Specifically, we found that although these markers were indeed overexpressed in the tumors we tested, they were also present in Müllerian-type epithelium within benign CICs. As such, these results suggest that some of the markers identified as ovarian tumor markers are actually markers of Müllerian differentiation, not necessarily bona fide markers of neoplastic transformation. These observations support the hypothesis that the OSE is not the direct precursor of ovarian epithelial neoplasms but instead must first undergo metaplasia to become Müllerian-like.[8,9] A subsequent neoplastic event within this Müllerian epithelium may trigger oncogenic transformation. Support for this model comes from studies in sheep that showed that ovulation causes oxidative base damage to the cells in the vicinity of the rupture site and that such DNA damage is associated with up-regulation of the p53 tumor suppressor.[32] Because one mechanism of CIC formation is related to ovulation, DNA damage to OSE cells during this process may result in formation of CICs with cells harboring DNA damage. Perhaps in conjunction with these events, exposure to the high levels of the gonadotropic hormones (follicle-stimulating hormone and luteinizing hormone) seen during menopause may predispose the CIC epithelium to neoplastic transformation. Recent studies using murine models of ovarian epithelial tumors have supported the notion that the OSE is the cell of origin of ovarian carcinomas.[33]

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