博士論文

Comprehensive histopathological and immunohistochemical analysis of ovarian mucinous tumors

（卵巣粘液性腫瘍の発生と進展に関する病理組織学的検討）

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Abbreviations

MA	Mucinous cystadenoma
MBT	Mucinous borderline tumors
IMBT	Intestinal- type mucinous borderline tumors
EMBT	Endocervical- type mucinous borderline tumors
SBT	Serous borderline tumor
MCa	Mucinous adenocarcinoma
IMCa	Intestinal type mucinous adenocarcinoma
EMCa	Endometrioid adenocarcinoma
CCCa	Clear cell adenocarcinoma
HGSCa	High grade serous carcinoma
LGSCa	Low grade serous carcinoma
CRC	Colorectal carcinoma
BT	Brenner tumor
IC	Inclusion cyst
EC	Endometrial cyst
MCT	Mature cystic teratoma
SCA	Serous cystadenoma
TMA	Tissue micro array

Abstract

Ovarian mucinous tumors are generally classified into mucinous cystadenoma (MA), mucinous borderline tumors (MBTs) and mucinous adenocarcinoma (MCa). MBTs are subclassified into intestinal-type (IMBTs) and endocervical-like (EMBTs) tumors. Almost all MCas are considered to be of intestinal-type (IMCa). In this study we attempted to clarify the phenotypes, and direction of differentiation of mucinous epithelium that constitutes MA, MBTs and MCas by immunohistochemistry. A panel of antibodies that included gastric markers (claudin-18 [CLDN18], MUC5AC, and MUC6), intestinal markers (MUC2 and CDX2), Müllerian markers (ER, PgR, CA125, and vimentin), and cytokeratins (CK7 and CK20) was applied. Special attention was paid to the expression of CLDN18, which is a recently established gastric marker.

The results of this study showed that intestinal-type, and endocervical-like ovarian mucinous tumors are two distinct entities with completely different immunophenotype. Frequent and diffuse expression of gastric markers, along with less frequent and usually focal expression of intestinal markers in IMBTs and IMCas, suggested that these lesions, which have conventionally been regarded as “intestinal-type” are essentially of “gastrointestinal-type”. In addition, we showed that CLDN18 can serve as a useful diagnostic marker for IMBTs and IMCas, because EMBTs, ovarian non-mucinous adenocarcinomas and metastatic colorectal carcinomas involving the ovaries were CLDN18-negative. Lastly, we

found that most MAs are also of gastrointestinal-type. Coexistence of mature cystic teratoma, Brenner tumor and estrogen-receptor-positive Müllerian-type epithelium such as that of endometrial cyst were observed in a subset of MAs with gastrointestinal-type phenotype. Therefore, these coexistent lesions are candidate for the origin of gastrointestinal-type mucinous tumors.

General Introduction:

The author came to Japan from Afghanistan 5 years ago with a mission to study surgical pathology in Japan, and become the first practicing surgical pathologist in Afghanistan. While, being trained for diagnostic surgical pathology at the Department of Pathology of the University of Tokyo Hospital, the author began to have strong interest in gynecological pathology, especially in pathology of ovarian tumors. This thesis encompasses results of the comprehensive histological and immunohistochemical study performed on ovarian mucinous tumors. Every single case of ovarian mucinous tumor diagnosed at the University of Tokyo Hospital in the past 26 years is included in this study. As a consequence, this is one of the largest pathological studies performed to date regarding ovarian mucinous tumors.

Mucinous tumor is one of the major histological subtypes of ovarian epithelial neoplasms along with other subtypes (serous, clear cell and endometrioid tumors). In recent 10 years, significant advances were made in the

research field of non-mucinous ovarian tumors. For example, as for serous adenocarcinomas, their origin was shown to be fallopian tube epithelium. Further, almost all of them were proved to harbor *TP53* mutations. (1, 2) In clear cell and endometrioid adenocarcinomas, their association with endometriosis and involvement of *ARID1A* mutations were revealed (3). With regards to ovarian mucinous tumors, however, much about their origin and pathogenesis remain unclear. In this study, we tried to investigate the pathobiology of ovarian mucinous tumors from histological and immunohistochemical points of view.

According to the current WHO classification, mucinous tumors have been classified into mucinous cystadenoma (MA), mucinous borderline tumors (MBT), and mucinous adenocarcinoma (MCa). Around 80% of these tumors are benign or cystadenomas. The remainders are mucinous borderline tumor (MBT), noninvasive (intraepithelial or intraglandular) mucinous adenocarcinomas, and invasive mucinous adenocarcinoma. MBTs are subclassified into intestinal-type (IMBTs), and endocervical-like (EMBTs) tumors. In intestinal-type tumors, the epithelium is tall columnar in appearance and have small basal nuclei with intracytoplasmic mucin. Their epithelium is most often similar to gastrointestinal-tract epithelium such as gastric foveolar and pyloric-type epithelium, or intestinal-type epithelium with scattered goblet cells. In endocervical-like tumors, the epithelium is usually composed of tall columnar cells with intracytoplasmic mucin which show ciliated change on the surface. No goblet cells are found. They show some resemblance to normal uterine cervical

glandular epithelium. Almost all MCas are considered to be of intestinal-type (IMCa), and they are supposed to develop in a stepwise manner, from MA to IMBT and to IMCa. Endocervical-like mucinous adenocarcinomas are extremely rare. From diagnostic stand points, distinction between IMCas and metastatic colorectal carcinomas is often difficult. But it is critical in patient's management. As for MAs, subclassification such as intestinal-type and endocervical-type has not been clearly defined at this point, since the characteristics of their epithelium have not been evaluated in detail.

In this study, we performed comprehensive histopathological and immunohistochemical analysis of ovarian mucinous tumors. We analyzed consecutive ovarian mucinous tumors that were surgically removed at the University of Tokyo Hospital, including MAs, MBTs, and MCas, by immunohistochemistry. In addition a variety of benign and metaplastic ovarian mucinous lesions, non-mucinous and Müllerian duct derivatives were also analyzed.

This thesis comprise of three independent studies:

In the first part, we analyzed the phenotypes and directions of differentiation of the mucinous epithelium in mucinous borderline tumor by immunohistochemistry. The main purpose of this study was to elucidate the difference between intestinal-type, and endocervical-like mucinous tumors. Since, histological distinctions between the two subtypes are established more clearly in

borderline tumors than in carcinomas/adenomas, we focused specially in borderline tumors to start the first part of this thesis study. A panel of antibodies that included gastric markers (claudin-18 [CLDN18], MUC5AC, and MUC6), intestinal markers (MUC2 and CDX2), Müllerian markers (ER, PgR, CA125, and vimentin), and cytokeratins (CK7 and CK20) has been applied. In this study, special attention was paid on the expression of CLDN18, one of the claudins that constitutes a family of 27 proteins essential for the formation of tight junctions, and the maintenance of polarity in epithelial, and endothelial cells.

In the second parts, we attempted to investigate the expression of CLDN18 in various subtypes of ovarian adenocarcinomas. Since, intestinal-type mucinous adenocarcinoma was suspected to have gastric phenotype from morphological evaluation, we tried to confirm that by applying CLDN18 immunohistochemistry. We then, assessed the utility of CLDN18 immunohistochemistry in differentiating intestinal-type mucinous adenocarcinoma (IMCas) and other subtypes of non-mucinous ovarian adenocarcinomas, as well as metastatic colorectal adenocarcinomas (CRCs) involving the ovaries.

Lastly, we analyzed a series of mucinous cystadenomas immunohistochemically, using gastric, intestinal, and Müllerian markers. Our aim was to elucidate the direction of the differentiation of mucinous epithelium that arises in the ovary and to seek for a possible histogenetic linkage between

gastrointestinal and Müllerian-type epithelium in the ovary. CLDN18 immunohistochemistry was also performed in variety of benign and metaplastic mucinous lesions and Müllerian duct derivatives to determine, candidate lesions those can give rise to benign gastric-type mucinous epithelium in the ovary, which we have shown to be CLDN18 positive.

Claudin-18 overexpression in intestinal-type mutinous borderline tumor of the ovary

Background

According to the current classification of the World Health Organization (WHO), ovarian mucinous borderline tumors (MBTs) are further classified into two types: intestinal-type mucinous borderline tumors (IMBTs) and endocervical-like mucinous borderline tumors (EMBTs)(4).

These tumors have been given a variety of names in the past literature, and there is certain confusion with regard to their nomenclature. Some authors refer to IMBTs as “gastrointestinal”-type mucinous borderline tumors (5-8). Terms such as “seromucinous,” “Müllerian-type,” and “non-gastrointestinal type” are frequently used to refer to EMBTs (5, 6, 8-12). The inconsistency in nomenclature is primarily due to the subjective interpretation of the morphological features of IMBTs and EMBTs by each author. Paucity of the objective data regarding the phenotypes and direction of differentiation of the mucinous epithelium of IMBTs and EMBTs is another reason.

Distinction between IMBTs and EMBTs is important because their clinicopathological features differ significantly (5, 6, 10, 12-14). IMBTs comprise approximately 85% of MBTs. They are usually unilateral (over 90%) (13). Most IMBTs are large multicystic masses, and their epithelial component has been described as a mixture of intestinal-type, gastric-type, and endocervical-type mucinous epithelium that grows predominantly in glandular or cystic structures admixed with papillary and villous structures (10, 12, 13). Although

intestinal differentiation, which is represented by the presence of goblet cells and CDX2 immunoreactivity, has been regarded as a key feature of IMBTs (5, 10, 12, 13, 15) mucinous epithelium that resembles gastric foveolar-type epithelium is often the predominant component of IMBTs in our observations and in observations by others (5, 16). In fact, Ji *et al*. reported frequent expression of MUC5AC, a gastric foveolar epithelial marker, in IMBTs (17). The tumor cells of IMBTs show a variable degree (mild to moderate) of atypia, and coexistence of a benign-looking mucinous cystadenoma component is often observed. It is generally accepted that stepwise malignant transformation occurs from MA to IMBT and to usual type (non-endocervical type) intestinal-type mucinous adenocarcinoma (IMCa). However, the precise origin of these tumors remains unclear.

Compared with IMBTs, EMBTs are much less common and smaller, and they tend to occur in younger females (18-20). EMBTs are more frequently bilateral, and they show a paucilocular gross appearance with intracystic papillary projection (14, 20). Histologically, EMBTs are characterized by finely branching papillae with fibrovascular cores, and their architecture closely resembles that of serous borderline tumors (SBTs). The lining epithelium is composed of columnar mucin-containing cells and indifferent polygonal cells with eosinophilic cytoplasm (12, 14, 21). The former resemble endocervical cells. However, the glandular epithelium of EMBTs does not necessarily resemble that of typical endocervical glands because the above two types of cells are usually

admixed with each other, and cellular tufting and budding are prominent. Goblet cells are rarely found in EMBTs (12, 19, 20). EMBTs are frequently associated with endometriosis, (18, 19, 22) and they share common immunohistochemical features with SBTs and low-grade endometrioid carcinomas, such as positivity for estrogen receptor [ER] and progesterone receptor [PgR], suggesting the Müllerian nature of the neoplasm (5, 6, 8, 11).

Study aims

We herein attempted to clarify the phenotypes and directions of differentiation of the mucinous epithelium that constitutes IMBTs and EMBTs by immunohistochemical analysis. A panel of antibodies that included gastric markers (claudin-18 [CLDN18], MUC5AC, and MUC6), intestinal markers (MUC2 and CDX2), Müllerian markers (ER, PgR, CA125, and vimentin), and cytokeratins (CK7 and CK20) was applied. The expression of these markers was also assessed in SBTs to reveal the typical phenotype of Müllerian-type tumors. In this study, special attention was paid to the expression of CLDN18, which is a recently established gastric marker. CLDN18 is one of the claudins that constitutes a family of 27 proteins essential for the formation of tight junctions and the maintenance of polarity in epithelial and endothelial cells (23, 24). Positive immunoreactivity for CLDN18 has been shown in all types of gastric epithelium (foveolar-type, pyloric-type, and fundic-type) (25, 26). Thus, we

believe that CLDN18 is one of the best pan-gastric immunohistochemical markers available at this time.

Materials and methods

Tissue samples

A total of 79 ovarian MBTs (54 IMBTs and 25 EMBTs) from 75 patients were retrieved from the archives of the Department of Pathology of the University of Tokyo Hospital. These included 54 cases of unilateral IMBT and 17 cases of unilateral EMBT and four cases of bilateral EMBTs. Two of the IMBTs were in coexistence with mature cystic teratoma (MCT).

The discrepancy between the number of IMBTs and EMBTs is due to the relative rarity of EMBTs. We included all EMBTs that were resected between 1989 and 2011. Since IMBTs during this period far outnumbered EMBTs, we randomly selected 54 cases, which is a substantial number for comparative analysis. We also added 22 cases of SBT to the series. Hematoxylin and eosin (H&E)-stained slides of all cases were reviewed. Histological diagnosis was based on the most recent criteria of the WHO.

Preparation of tissue samples and immunohistochemistry

All tissue samples were fixed in formalin and embedded in paraffin. For immunohistochemistry, we arranged all MBTs (54 IMBTs and 25 EMBTs) and all 22 SBTs in tissue microarrays (TMAs) with duplicate 2-mm cores obtained

from each tumor. For those EMBT cases with bilateral involvement, tumors in the right and left ovaries were submitted separately. TMAs were cut into 4-μm thickness.

To perform immunohistochemistry deparaffinization, antigen retrieval with Ventana CC1 buffer has done. Immunohistochemistry has carried out using the Benchmark XT Automated immunohistochemistry system (Ventana Medical Systems, Tucson, AZ, USA). Primary antibody staining and detection curried out using Ventana/EIEW DAB Universal Kit with validated reagents. After staining on the instrument, slides were dehydrated through graded alcohols to xylene and a coverslips were applied.

For immunohistochemistry, MUC1 (clone:MA695), MUC2 (clone:Ccp58), MUC5AC (clone:CLH2), MUC6(clone:CLH5), Cytokeratin 20 (clone: Ks 20.8), and CA125 (clone: Ov 185:1) were obtained from Novocastra. CK7 (clone:OV-TL12/30) and Vimentin (clone: V9) obtained from DakoCytomation. CLDN18 (clone:poly) obtained from Zymed Laboratories. CDX2 (clone:CDX2-88) obtained from CellMarque, Estrogen receptor (clone:ER1D5) and Progesterone receptor (clone:A9621A) obtained from Ventana. Immunohistochemistry for CLDN18, MUC1, MUC2, MUC5AC, MUC6, CK7, CK20, CDX2, CA125, ER, PgR, and vimentin were performed in all ovarian borderline tumors. Antibodies used in this study are detailed in Table 1.

Interpretation of immunohistochemistry

Immunoreactivity was interpreted based on the presence of cytoplasmic staining for CK7, CK20, and vimentin; nuclear staining for CDX2, ER, and PgR; membranous staining (with or without cytoplasmic staining) for CA125; and luminal/apical or combined luminal and cytoplasmic staining for MUCs. CLDN18 expression was evaluated based on the existence of basolateral membrane staining. Evaluation of immunohistochemistry was performed by two authors (SAH and DM), who specialize in gynecological pathology. Immunohistochemical reactions were scored based on the percentage of positive cells and graded as 0 (totally negative), 1+ (1%–4%), 2+ (5%–14%), 3+ (15%–49%), and 4+ (≥50%). The average tumor cell positivity in two TMA cores was calculated. Each core was scored individually then the mean of the two readings was calculated. Appropriate positive and negative controls were included.

Hierarchical clustering of ovarian borderline tumors according to their immunophenotype

Unsupervised two-way hierarchical clustering was performed based on Euclid distances and average linkage clustering algorithms in sample directions and antibody directions using the Cluster software version 3.0 (Stanford University, http://bonsai.ims.u-tokyo.ac.jp/~mdehoon/software/cluster/software.htm#ctv).

All IMBTs, EMBTs, and SBTs were included in the analysis. For the expression level of each protein, data on the percentage of positive cells detected by immunohistochemistry were used. A heat map was drawn using the Java TreeView software (Alok, http://jtreeview.sourceforge.net/).

Statistical analysis

Statistical analysis was performed using Fisher's exact test. Statistical analyses were performed using the StatView software version 5.0 (SAS Institute, Cary, NC), and a value of $P < 0.05$ was considered to indicate statistical significance.

Results

Immunohistochemical comparison of IMBT and EMBT

To reveal the characteristics of mucinous epithelium that comprise IMBT and EMBT, we initially analyzed the expression of markers that are known to represent either gastric or intestinal differentiation. The results are shown in Table 2. Positive immunoreactivity for CLDN18, a pan-gastric marker, was observed in nearly all cases (98%) of IMBTs, whereas EMBTs were usually CLDN18-negative (Figure 1). CLDN18 stained more than 50% of the tumor cells (4+) in the majority of IMBTs (48 of 56 cases). Diffuse basolateral staining was detected, especially in IMBTs that comprised stratified columnar mucinous

epithelium that resembled gastric foveolar-type epithelium. However, we also found CLDN18-positivity in the epithelium of IMBTs that contained scattered goblet cells (Figure 1). Almost all EMBTs were completely negative for CLDN18 with the exception of one case that revealed focal positivity.

In addition to CLDN18 expression, significant differences between IMBTs and EMBTs were found with regard to the expression of MUCs and CDX2 (Figure 2). MUC5AC, a gastric foveolar epithelial marker, was more frequently expressed in IMBTs (93%) than in EMBTs (72%) (P = 0.0307). Further, most IMBTs showed 3+ and 4+ immunoreactivity for MUC5AC. In contrast, MUC5AC expression in EMBTs was usually focal (1+ and 2+) or negative. Markers of intestinal differentiation, such as MUC2 and CDX2, were expressed in less than half of IMBTs (33% and 48%, respectively). Expression of MUC2 and CDX2 in IMBT was usually focal and patchy (1+ and 2+), and diffuse (4+) immunoreactivity for these markers was found only in 3% and 5% of the cases. EMBTs were almost always negative for MUC2 and CDX2. MUC6, a marker for gastric pyloric gland-type epithelium, was negative in most IMBTs and EMBTs. MUC1 expression was seen more frequently in EMBTs (100%) compared with IMBTs (44%).

Expression of conventional markers, including CK7, CK20, ER, PgR, CA-125, and vimentin, was also evaluated in IMBTs and EMBTs. The results are shown in Table 3. In our series, all IMBTs and EMBTs expressed CK7. The remaining markers were differentially expressed in IMBTs and EMBTs (P <

0.0001). IMBTs are roughly characterized by a CK20+/ER-/vimentin- immunophenotype, whereas most EMBTs display a CK20-/ER+/vimentin+ pattern (Figure 3). CK20 expression was observed in 80% of the IMBTs. The extent of CK20-positivity was variable among the IMBT cases. EMBTs were almost always negative for CK20. Markers that were positive in all EMBTs included ER, vimentin, and CA125. The expression of ER, vimentin, and CA125 in IMBTs was less frequent (4%, 2%, and 35%, respectively). Finally, PgR can be listed as another positive marker for EMBTs. However, the expression of PgR in EMBTs was slightly less frequent (80%) compared with that of ER, and ER staining tended to be more diffuse.

Immunophenotype of SBTs and hierarchical clustering of ovarian borderline tumors

We performed immunohistochemistry for all markers listed above in 22 cases of SBT. The results are shown in Table 4. Our investigation revealed that all SBTs were negative for CLDN18. Markers commonly expressed in SBTs included MUC1, CK7, ER, CA125, and vimentin. The dendrogram depicted in Figure 4 is the result of hierarchical clustering of IMBTs, EMBTs, and SBTs according to their immunoprofile. This dendrogram shows the degree of relatedness between the protein expression patterns detected by the 12 antibodies across the 101 cases of ovarian borderline tumors, with short branches indicating a high degree of similarity in the staining pattern. In the dendrogram, IMBTs

comprised a distinct group that was separate from the EMBT/SBT group. Based on the analyses of these 12 markers, EMBTs and SBTs were not clearly separated. Rather, similarities in the immunophenotypes of EMBTs and SBTs were highlighted in the dendrogram.

Discussion

Evidence of altered claudin expression in various human neoplasms has been accumulating rapidly. Expression of CLDN18 has been studied in various types of human cancers and normal tissues (26-34). Two alternatively spliced variants are present in mice: Variant 1 (claudin18a1) is expressed in the lung, whereas variant 2 (claudin18a2) is expressed in the stomach (30, 32). In normal human tissues, expression of claudin18a2 is confined to gastric epithelial cells (foveolar, endocrine, parietal, and chief cells) and duodenal Paneth cells, and is not expressed in other organs, such as the esophagus, colon, pancreas, and lung (26, 30, 32, 35). CLDN18 is now considered to be a highly selective immunohistochemical marker of gastric lineage, and its expression is considered to determine the gastric phenotype in neoplastic conditions (27-29, 34).

Sanada *et al*. (32) used immunohistochemistry to reveal that CLDN18 is highly expressed in normal gastric cells and that its expression is retained in approximately half of gastric cancers. Interestingly, they further showed that CLDN18 is downregulated in gastric epithelium with intestinal metaplasia and gastric cancers with an intestinal phenotype. Our group recently showed that a

subset of intrahepatic cholangiocarcinomas and pancreatic ductal carcinomas show a CLDN18-positive gastric phenotype (27, 28). It is of note that upregulation of CLDN18 occurs in the early stage of cholangiocellular and pancreatic carcinogenesis, as shown by CLDN18-positivity in precancerous lesions such as pancreatic intraepithelial neoplasias and biliary intraepithelial neoplasias.

The current study is the first to investigate CLDN18 expression in ovarian borderline tumors. We demonstrated that the CLDN18-positive immunophenotype is specifically observed in IMBTs and not in EMBTs or SBTs. Another gastric marker, MUC5AC, which is expressed in normal gastric foveolar epithelium, was also frequently expressed in IMBTs, giving further support to the gastric differentiation of the IMBT epithelium. Since, previous reports have shown that normal endocervical glands frequently express MUC5AC (36, 37) we believe that focal positivity observed in EMBTs are most likely due to the MUC5AC antibody reacting to Müllerian-type mucinous epithelium that does not necessarily have gastric foveolar-type characteristics. In the past, the presence of goblet cells has been emphasized as a characteristic of IMBTs that can be observed in almost all cases (10, 12, 13) and a number of studies have focused on the expression of intestinal markers such as CDX2 and MUC2 as key immunophenotypes of IMBT (5, 15, 38). However, similar to some of the previous reports (38, 39) CDX2 and MUC2 expression in IMBTs was observed

in less than half of the cases, and their immunoreactivity was often focal in this study. Therefore, we conclude that in general, IMBTs are essentially composed of gastrointestinal-type mucinous epithelium, the predominant component of which is gastric-type rather than intestinal-type epithelium. This notion coincides with the morphological assessment of IMBTs by us and other researchers who consider that most mucinous epithelium in IMBTs resembles foveolar-type gastric epithelium (5, 17). Therefore, we propose abandoning the nomenclature "intestinal-type mucinous borderline tumor" and replacing it with "gastrointestinal-type mucinous borderline tumor" to avoid further confusion.

Our immunohistochemical panel highlighted the differences between EMBTs and IMBTs. Similarities between EMBTs and SBTs have repeatedly been described from the morphological and immunohistochemical points of view (5, 6, 19). Recent studies have reported that EMBTs share features with low-grade endometrioid tumors (borderline tumors and carcinomas), such as frequent association with endometriosis and frequent loss of *ARID1A* expression (11).

Currently, it is not clear whether EMBTs are closer to SBTs or low-grade endometrioid tumors. We recognize EMBT as a distinct Müllerian-type tumor that shows a variable degree of mucin production. In fact, our study revealed Müllerian immunophenotypes of EMBTs, such as positivity for ER, PgR, CA-125, and vimentin. Furthermore, hierarchical clustering of ovarian borderline tumors (IMBTs, EMBTs, and SBTs) according to their protein expression resulted in grouping EMBTs and SBTs together in a cluster that was completely

separate from the IMBT cluster. Although the number of antibodies applied in this study was limited and there was limitation in terms of assessing intratumoral heterogeneity due to the use of TMAs, the data clearly show that EMBT and IMBT are two distinct neoplasms and that the former is a part of the ovarian Müllerian-type tumor spectrum.

From a diagnostic standpoint, pathologists may occasionally encounter ovarian mucinous tumors that are difficult to classify as either IMBT or EMBT. In such instances, the best immunohistochemical panel we propose is a combination of CLDN18, CK20, ER, and vimentin. IMBTs most frequently show a CLDN18+/CK20+/ER-/vimentin- pattern, whereas EMBTs are almost always CLDN18-/CK20-/ER+/vimentin+.

In summary, we report overexpression of a gastric marker, CLDN18, in ovarian IMBTs. The distinct nature of IMBTs and EMBTs was elucidated through immunohistochemical analyses using a panel of antibodies including CLDN18. We also showed that CLDN18 can serve as a good diagnostic marker to distinguish IMBT from EMBT. Taking these results into consideration, we hope to emphasize that IMBTs are essentially "gastrointestinal-type mucinous borderline tumors" and that EMBTs are "Müllerian-type mucinous borderline tumors."

CLDN18 is specifically expressed in intestinal-type mucinous adenocarcinoma (IMCa) among ovarian cancers

Background

Ovarian epithelial carcinoma is generally classified into five major categories: Mucinous adenocarcinoma (MCa), Endometrioid carcinoma (EMCa), Clear cell carcinoma (CCCa), High grade serous carcinoma (HGSCa) and Low grade serous carcinoma (LGSCa). Each of these subtypes is a distinct disease (40) .

Mucinous adenocarcinoma is relatively uncommon. Unlike mucinous borderline tumors, they have not been clearly subclassified into intestinal and endocervical-types. However, histological features of most mucinous adenocarcinomas are of (gastro-) intestinal-type, and most gynecological pathologists regard them as intestinal-type mucinous adenocarcinoma (IMCa). In fact, many studies revealed that nearly all cases of IMCas have coexisting areas of IMBTs, which accounts for 5 to 70% of the whole tumor (41-43). IMCa is known for its poor prognosis and chemoresistance nature. About 80% of these tumors are confined to the ovary (stage I) at the time of diagnosis (41, 42). Tumors in advance stage have an extremely poor prognosis.

The age of patients with IMCa ranges from 14-87 years with a mean of 39 to 50 years (41-43) . Most IMCas are from 8 to 40cm (mean 16-19) in greatest dimension (41). IMCa is reported to be typically unilateral. Only about 5% or less is bilateral (41, 42). They are usually cystic, microcystic and most often they

appear multicystic (44). Solid areas and firm nodules are also common. In 4% of cases, the tumors are predominantly or entirely solid (44). IMCa usually shows two different patterns of stromal invasion: expansile and infiltrative. In expansile invasion the tumor glands are closely packed, in a back to back manner with little or no intervening ovarian stroma. This type of invasion is relatively common and usually difficult to distinguish from non-invasive carcinoma or from IMBTs. In contrast, infiltrative invasion is easily recognized by irregular glands, tubules, tumor nests, cords and cells that haphazardly infiltrate within reactive ovarian stroma (42). This type of invasion is usually called destructive stromal invasion (42). The existence of infiltrative invasion always raises concern for metastatic carcinoma from elsewhere in the body.

For successful specific treatment, it is very important to correctly distinguish IMCas from other non-mucinous ovarian adenocarcinomas, because the treatment option and response to therapy is different in each subtype. Although identification of intracytoplasmic mucin is highly diagnostic in IMCa, many tumors lack obvious mucin in large parts of tumor, and their morphology simulate those of endometrioid or other subtypes of non-mucinous ovarian cancers. In such instances, distinction with other subtypes of non-mucinous ovarian adenocarcinoma is very difficult, and the tumor can easily be misdiagnosed as endometrioid or other type of non-mucinous ovarian cancer. Unfortunately, there are only few reliable immunohistochemical markers

available for distinction between IMCa and other subtypes of primary ovarian adenocarcinomas.

Another important issue in the diagnosis of primary ovarian IMCa is their distinction from metastatic carcinomas involving the ovary. Since, the ovary is a common site for metastatic involvement, the possibility of metastatic carcinoma always needs to be considered when an ovarian mucinous tumor is examined. Always, a high index of suspicion is needed by pathologists as well as gynecologists to avoid misclassification of metastatic tumor as a primary ovarian adenocarcinoma. Metastatic tumors that usually resemble ovarian IMCa, are from the gastrointestinal-tract, especially from the lower gastrointestinal-tract (45), appendix (46, 47), pancreas (48), biliary-tract and stomach (49). Carcinomas from the lower gastrointestinal-tract (colorectum) are the most frequent tumors that metastasize to the ovaries. Low grade or benign looking areas are often found within the metastatic tumors, and they intensify diagnostic confusion. In some cases, the primary site tumor may not be apparent at the time of diagnosis, and metastatic ovarian tumor can be the first manifestation of undiagnosed non-ovarian primary (49-51). Clinical information such as preoperative evaluation of tumor markers, tumor size information, and whether the ovarian tumor is unilateral or bilateral are reported to be helpful in distinguishing primary ovarian and metastatic adenocarcinoma. Microscopical findings and immunohistochemistry may also be useful. However, due to overlapping of the features, diagnostic uncertainty usually remains high.

In general, histological features are helpful to differentiate primary from metastatic mucinous tumors. Primary IMCa is more likely to have an expansile pattern of invasion, complex papillary pattern, microscopic cysts, and necrotic luminal debris (52). The presence of a coexisting ovarian lesion (MA and or IMBT) can be supportive for diagnosis of a primary ovarian tumor, although it should be noted that these benign looking components may also exist even in tumors metastatic to the ovary from elsewhere. Diagnostic criteria which support metastatic nature include nodular growth pattern, ovarian surface involvement, infiltrative pattern of invasion, infiltrative single cell pattern, hilar involvement, and signet ring cells (9, 49).

Recently Seidman et al (50) proposed an algorithm using two factors include tumor size and tumor laterality. Based on the proposed algorithm unilateral tumors with size being or greater than 10 cm are primary, while bilateral tumors, and unilateral tumors with size less than 10cm are all metastatic. According to the proposal, this algorithm will accurately classify tumors in over 90% of the cases. In another study, performed by *Khunamornpong et al*, in a retrospective sample of patients with unilateral tumors greater than 10 cm, the tumor was diagnosed as a primary ovarian tumor in 50% of the cases with 10-15cm, and 69% of those were greater than 15cm (51).

Immunohistochemistry shall play an important role in distinguishing the primary ovarian from metastatic tumors. Cytokeratin (CK) and CDX2 staining

have been frequently used for this purpose (39, 53). A CK7 positive/CK20 negative immunophenotypes is known to suggest a primary ovarian lesion, while CK7 negative/CK20 positive immunophenotype supports metastatic involvement (39, 53). However, mucinous tumors of the appendix or upper gastrointestinal tract origin stain with CK7 occasionally. Results of various previous studies suggest that we cannot rely on the CK7/CK20 immunoprofile alone.

None of the above diagnostic tools are decisive alone. Uncertainty is always remaining high. Supportive and additional examination including immunohistochemistry is usually needed.

Study aims

In this study, we investigated the significance of CLDN18 expression in IMCas and variety of non-mucinous ovarian adenocarcinomas, as well as metastatic CRCs involving the ovary. Since intestinal-type mucinous adenocarcinoma was suspected to have gastric phenotype from morphological evaluation, we tried to confirm that by applying CLDN18 immunohistochemistry. We then, assessed the utility of CLDN18 immunohistochemistry in differentiating intestinal-type mucinous adenocarcinoma and other subtypes of non-mucinous ovarian adenocarcinomas, as well as metastatic colorectal carcinomas (CRCs) involving the ovaries. We also examined the expression of

other conventional markers (CK7, CK20, CDX2, MUC2, MUC5AC, and ER) in order to establish a panel of markers that can be useful for differentiating IMCas and metastatic CRCs involving the ovary. The usefulness of proposed algorithm that relies on size and laterality is also evaluated in this study.

Materials and method

Cases of mucinous adenocarcinomas including primary IMCas and metastatic CRCs involving the ovaries were included in this study. Tumor samples were collected from 35 patients. These included 19 primary IMCas and 16 metastatic CRCs involving the ovaries. Fifteen of the IMCas were retrieved from the archives of the Department of Pathology at the University of Tokyo Hospital. Four of the IMCas were collected from the Department of Pathology at University of Teikyo Hospital. The histological slides of each case were reviewed, and the diagnosis was made according to the most recent WHO classification. Distinction between primary and metastatic tumor was based on the morphological and clinical presentation and in some cases with an aid of immunohistochemistry. Tumors were diagnosed as primary IMCa when they exhibited typical morphological features as described for primary IMCa (49, 51, 52). Clinicopathological data of all patients were reviewed. The data regarding patients age, tumor size (maximal dimension), and laterality of all cases (primary and metastatic) were collected.

Following histological features were evaluated for all tumors: tumor growth pattern, types of invasion, surface involvement, and co-existing ovarian lesions.

To evaluate the usefulness of proposed algorithm (50) in our cases, the primary and metastatic mucinous adenocarcinomas were compared by two factors including tumor laterality (unilateral versus bilateral) and tumor size (10cm or over versus less than 10 cm).

Non-mucinous variants of primary ovarian adenocarcinoma

Various subtypes of ovarian non-mucinous adenocarcinomas (n=202), were collected from the archives of the Department of Pathology, The University of Tokyo Hospital. These included 95 cases of clear cell adenocarcinoma (CCCa), 38 cases of endometrioid adenocarcinoma (EMCa), 58 cases of high grad serous adenocarcinoma (HGSCa) and 11 cases of low grade serous adenocarcinoma (LGSCa).

Preparation of the sample and Immunohistochemistry

All tissue samples were fixed in formalin and embedded in paraffin. For immunohistochemistry, we arranged CCCa (n=95), EMCa (n=38), HGSCa (n=58), LGSCa (n=11) and IMCa (n=12) in tissue microarrays (TMAs). The

TMAs were constructed from formalin fixed paraffin embedded tissues. Immunohistochemical comparison between primary IMCas and other non-mucinous primary ovarian adenocarcinomas were performed using the sets of tumors included in these TMAs.

In order to perform comparison between primary IMCas and metastatic CRCs involving the ovaries, we used a representative whole tissue section, so that the distribution of the positive cells can be thoroughly evaluated. Additional cases of IMCas were evaluated in whole section. Both primary IMCas (n=19), metastatic CRCs involving the ovary (n=16) were stained for comparison.

To perform immunohistochemistry, deparaffinization and antigen retrieval with Ventana CC1 buffer was done. Immunohistochemistry was carried out using the Benchmark XT Automated immunohistochemistry system (Ventana Medical Systems, Tucson, AZ, USA). Primary antibody staining and detection were carried out using Ventana/EIEW DAB Universal Kit with validated reagents. After staining on the instrument, slides were dehydrated through graded alcohols to xylene and a coverslips were applied.

Immunohistochemistry for CLDN18 was performed in all cases of IMCas, non-mucinous variants of ovarian adenocarcinomas and all metastatic CRCs involving the ovary. To compare the immunophenotype of IMCas and metastatic CRCs involving the ovary, additional markers such as MUCs (MUC2 and

MUC5AC), cytokeratins (CK7 and CK20), CDX2 and ER were also stained. Appropriate positive and negative control was used for all antibodies.

Interpretation of immunohistochemistry

Immunoreactivity was interpreted based on the presence of cytoplasmic staining for CK7, CK20, nuclear staining for CDX2 and ER, luminal/apical or combined luminal and cytoplasmic staining for MUCs. CLDN18 expression was evaluated based on the existence of basolateral membrane staining. Evaluation of immunohistochemistry was performed by two authors (SAH and DM), who specialize in gynecological pathology. Immunohistochemical reactions were scored based on the percentage of positive cells and graded as 0 (totally negative), 1+ (1%–4%), 2+ (5%–14%), 3+ (15%–49%), and 4+ (≥50%). For TMA sections, the average tumor cell positivity in two cores was calculated. Each core was scored individually, and then the mean of the two readings was calculated.

Statistical analysis

Statistical analysis was performed using $\chi 2$ test for comparison between IMCas and non-mucinous subtypes of ovarian adenocarcinomas, and Fisher's exact test for comparison between IMCas and metastatic CRCs involving the ovary. Statistical analyses were performed using the StatView software version

5.0 (SAS Institute, Cary, NC), and a value of $P < 0.05$ was considered to indicate statistical significance.

Results

In this study, none of the patients with primary IMCas had any history of previously diagnosed adenocarcinoma in other organs. In one patient, a synchronous advanced adenocarcinoma existed in the ascending colon. In this specific patient, the tumors in the ovary and ascending colon were resected simultaneously.

The patients' age with primary IMCas varied from 24–72. In 2 (10%) patients, the tumor involved bilateral ovaries. In 17 (90%) patients, the tumors were unilateral, and left ovarian involvement was relatively more frequent. Tumors sizes varied from 7cm to 30 cm. In 17 (90%) patients, the tumors were more than 10cm in greatest dimension. In 2 patients (10%), the tumors were less than 10cm. Histologically, the majority of tumors in this study showed expansile pattern of invasion. Only 3 cases, showed destructive or infiltrative stromal invasion. One of the tumors had a mature cystic teratoma in the background.

All metastatic ovarian tumors in this study had typical morphological features of metastatic CRCs involving the ovaries (52). Tumors in this study originated from different primary sites including cecum (n=2), ascending colon (n=4), transverse colon (n=2), sigmoid colon (n=5), and rectum (n=3). Nodularity,

surface involvement, and stromal reaction were present in almost all cases. The patients' age varied from 28 to 74. Bilateral ovarian involvement was found in 7 (44%) patients. In 9 (56%) patients the tumors were unilateral and right and left ovaries were equally involved. The tumors were more than 10cm in 11 (69%) patients, while less than 10cm in 4 (25%). In one patient, the size information was not available. Among metastatic bilateral tumors, 4 of them were less than 10cm, while 3 of them were greater than 10cm. Unilateral metastatic tumors (n=8) were all greater than 10cm in size. There is no unilateral metastatic tumor with size less than 10cm in this study. The summery of tumor size and laterality in both primary IMCas and metastatic CRCs involving the ovary is shown in Table 5. In 11 patients, both tumors (primary and metastatic) were resected simultaneously. In the remaining cases primary and metastatic tumors were resected separately. The time interval between metastatic tumor detection and primary tumor surgery varied from 1 to 3 years.

Results of immunohistochemistry:

CLDN18 is specifically expressed in IMCa among ovarian cancers

The results of immunohistochemistry (TMA analysis) are summarized in Table 6.

CLDN18 expression was analyzed among verity of primary ovarian adenocarcinomas. Our immunohistochemical analyses revealed that CLDN18 is

exclusively expressed in IMCas. Diffuse membranous staining was found in nearly all cases of IMCas (11/12). There was only one case that was exceptionally CLDN18-negative. In that patient, coexistence of mature cystic teratoma was observed in the same ovary. In contrast to IMCas, nearly all other subtypes of non-mucinous ovarian adenocarcinomas (CCCa, EMCa, HGSCa, and LGSCa) were CLDN18-negative. Focal expression was found in three cases (6%) of endometrioid adenocarcinoma due to mucinous metaplasia which is commonly found in EMCas. The representative histology and CLDN18 expression of each subtype is shown in Figure 5.

Differential expression of CLDN18 and MUC5AC among IMCas and metastatic CRCs involving the ovary:

In this study we also focused on the immunohistochemical comparison between IMCas and metastatic CRCs, since CRCs are the most frequent origin of metastatic ovarian cancers which cause diagnostic confusion. The results of immunohistochemistry are summarized in Table 7.

As a result, positive immunoreactivity for CLDN18 was observed in majority (85%) of IMCas, whereas metastatic CRCs involving the ovary were usually CLDN18-negative (Figure 6). CLDN18 positivity was observed in more than 50% of the tumor cells (4+) in nearly half of the IMCas. In 3 cases, CLDN18 was exceptionally negative. Those cases included one that had mature

cystic teratoma in the background. In that case the coexisting low grade (IMBT and MA) components were also CLDN18-negative. Almost all metastatic CRCs involving the ovary were completely negative for CLDN18 (Figure 6) with the exception of two cases that revealed very focal positivity.

In addition to CLDN18 expression, significant differences between primary IMCas and metastatic CRCs were found with regard to the expression of MUC5AC and CK7 (Figure 7). MUC5AC, was more frequently expressed in IMCas (84%) than in metastatic CRCs (19%) (P=0.0001). MUC5AC expression in IMCas was usually moderate-to-diffuse (2+, 3+ and 4+). In contrast, only three cases of metastatic CRCs expressed very focal (1+) MUC5AC positivity. CK7 was exclusively expressed in IMCas (100%), whereas it was usually negative in metastatic CRCs involving the ovaries (P<0.0001). MUC2 was more frequently expressed in metastatic CRCs (87%) than in primary IMCas (37%) (P=0.0022). CK20 and CDX2 were almost always expressed by metastatic CRCs involving the ovaries (100%), whereas their positivity was slightly lower in primary IMCas (74%). CK20 and CDX2 expression was usually diffuse in metastatic CRCs involving the ovary, while it was usually focal and patchy in primary IMCas (Figure 8).

In summary, IMCas usually demonstrate CK7+/MUC5AC+/CLDN18+ immunophenotype. Metastatic CRCs in the ovaries are usually CK7-/CK20+/CDX2+/MUC5AC-/CLDN18-.

Discussion

The expression pattern of various CLDNs in normal tissues, benign and malignant tumors is complex and mostly appears organ dependent (31). The abnormal expressions of various CLDNs have recently fascinated researchers (54). In general, the association between expression pattern of CLDNs and cancer is not fully studied. However, recently studies on cancer showed that the over or underexpression of at least one of the CLDNs is seen in various types of human cancers, suggesting that they probably have a role in cancer initiation or cancer progression. For example, underexpression of CLDN1 and CLDN7 occur in breast, colon, and head and neck cancer, whereas overexpression of CLDN3, and CLDN4 occur in ovarian, prostate, uterine and breast cancers (55-59). Some authors also reported the overexpression of CLDN18 in intrahepatic cholangiocarcinomas and pancreatic ductal carcinomas (27, 28).

In the previous chapter, we have shown CLDN18 overexpression in intestinal-type mucinous borderline tumor. This has led to the assumption that CLDN18 overexpression occurs in IMCas, too.

This is the first study to evaluate CLDN18 in ovarian IMCas. Our results showed that CLDN18 is overexpressed in nearly all cases of IMCas with diffuse pattern of expression. Interestingly, all other non-mucinous variants of ovarian adenocarcinomas were negative, except for few exceptional EMCas. Since, CLDN18 expression represents gastric phenotype in neoplastic conditions (26-29,

34), its expression in ovarian IMCas suggests that they have a gastric phenotype as well. Combined with the data in the previous chapter, we now conclude that IMBTs and IMCas are the same lineage of tumor characterized by CLDN18-positive, gastric phenotype. At this point, it is not clear whether CLDN18 expression has any prognostic value or not. To clarify this issue, further investigation in a larger case series is needed.

Irrespective of their role or contribution in cancer progression the CLDNs recently hold a great hope as a target for the future therapeutic intervention. Their unusual expression pattern in various types of human cancers suggests the utility for detection, diagnosis and treatment of drug resistant human cancer (58, 65-67). IMCa is known for its chemoresistant nature to conventional therapeutic agent. CLDN18 overexpression in ovarian IMCa, as shown in this study suggests that it may be a potential target for future cancer therapy.

The differential expression pattern of CLDN18 in IMCas and other subtypes of non-mucinous ovarian carcinomas has a significant diagnostic value. To date there is no specific marker available distinguishing IMCa from other subtypes of ovarian non-mucinous adenocarcinomas, especially endometrioid carcinoma, CCCa and HGSCa. Therefore, in problematic cases when histological distinction is difficult, CLDN18 can serve as a useful marker for differential diagnosis.

One of the biggest challenges in diagnosis of ovarian mucinous tumor is its distinction from metastatic tumors. In fact, the ovary is a common site for metastatic involvement. Tumors of the gastrointestinal-tract, breast and uterine cervix often metastasize to the ovaries. In every patient with mucinous adenocarcinoma, it is necessary to rule out the possibility of metastatic involvement. Among all tumors, those with colorectal origin most frequently metastasize to the ovaries and cause diagnostic confusion. During the last decades, there has been a dramatic change in diagnostic criteria for differentiation between primary ovarian and metastasis from elsewhere (42, 49). According to the recently published reports, the frequency of primary mucinous adenocarcinoma in the ovary is much less common compared to what have been previously reported (15, 50). Metastatic mucinous adenocarcinomas were reported to be much more frequent than primary mucinous adenocarcinoma. Some reports described that most of the cases that were previously diagnosed as primary, were actually metastatic from non-ovarian primaries. A ratio of metastatic to primary tumor, which is 3.2:1, has previously reported (50). Histological feature and immunohistochemistry is useful for differentiation. However, many features may be overlapping between primary and metastatic tumors.

According to the proposed algorithm (50), if ovarian mucinous tumor is unilateral, and over 10cm in size, it is most likely primary IMCa. Bilateral tumors and unilateral tumors with size less than 10cm are metastatic. This algorithm is

useful for tumor classification. But, according to our observation, there was some difference in the distribution of metastatic mucinous adenocarcinoma in our study compared to what have been previously proposed. In our study, we focused only on metastatic ovarian lesions from colorectal primaries. Most of the metastatic CRCs are tended to be larger than those reported by Seidman et al. In general, our data showed that primary IMCa with bilateral ovarian involvement and metastatic CRCs with size over than 10cm is not uncommon. 90% of IMCas in this study were unilateral, while the remaining 10% displayed bilateral ovarian involvement. In 90% of IMCas, the tumors were over than 10cm in greatest dimension, which is consistent with Sideman's algorithm. However, we have got different data with regards to metastatic tumors. In our study, 56% of metastatic tumors were unilateral, while 44% were bilateral. Most of the metastatic tumors in our study were larger than 10 cm. 69% of metastatic tumors in our study was more than 10cm in greatest dimension. In addition the frequency of bilaterality was lower (7/16, 44%), compared to 94% reported by Seidman et al.

According to our results the algorithm of size and laterality cannot be highly predictive and reliable for classifying tumor as primary or metastatic. Therefore, in addition to clinical and histological information other auxiliary methods for differential diagnosis always should consider, especially immunohistochemistry.

Immunohistochemistry is also widely used for distinction between primary and metastatic tumor, although the number of immunohistochemical markers available right now is not sufficient. In recent years, immunohistochemistry, especially differential expression of cytokeratins (CK7, CK20) and CDX2 staining, has been widely used as an aid for distinction between primary IMCas and metastatic CRCs involving the ovary. Since, CK7 is usually expressed in primary ovarian IMCas while, metastatic CRCs usually express CK20 and CDX2 (39, 53).

Our immunohistochemical comparison revealed that “CLDN18” is a novel marker which is useful for distinguishing, primary IMCas from metastatic CRCs involving the ovary. Our study demonstrated diffuse CLDN18 expression in 85% of ovarian IMCas with the exception of three cases, including the one that arose in association with mature cystic teratoma. In contrast, metastatic CRCs were nearly always CLDN18-negative with exception of two cases, which revealed very focal positivity. MUC5AC is another marker that differentially expressed in primary IMCas and metastatic CRCs. Therefore, it can be another useful marker for differential diagnosis between these two lesions.

Expression of CK20 was found in all cases of metastatic CRCs. However, CK20 expression was variable in primary IMCas. They were frequently positive in IMCas, but not in a diffuse manner. In contrast, most cases of metastatic CRCs were diffusely positive for CK20. Looking at the results, we believe that a panel

including (CLDN18, CK7, CK20, MUC5AC, and CDX2) is the best combination of markers for differential diagnosis between primary IMCas and metastatic CRCs involving the ovary. It is also important to know that CLDN18 usefulness is limited to IMCa vs. metastatic CRCs in the ovary, since it can't be used for distinction between IMCa and metastatic pancreatic and gastric carcinomas which are reported to be CLDN18-positive formerly (26-28, 32).

The results of our study and previous reports showed a significant role for CLDNs expression in various types of human neoplasms. Its clinical application can be significant in terms of tumor detection, tumor diagnosis and tumor treatment. According to our findings, CLDN18 immunohistochemistry has a significant role in diagnosis of intestinal-type ovarian mucinous tumors. We can easily distinguish intestinal-type and endocervical subtypes of ovarian mucinous tumors by using CLDN18 immunohistochemistry. In terms of distinction between mucinous and non-mucinous subtypes of ovarian tumors, and distinction between primary IMCas and metastatic CRCs involving the ovary, CLDN18 immmunohistochemistry plays a significant role. All these finding suggest a significance clinical role for Claudin18 immunohistochemistry which is important for the patients' management.

Additionally, since a large numbers of human cancers overexpress various CLDN family members, along with the cell-surface localization of CLDNs, it makes these proteins (CLDNs) attractive molecular targets for cancer treatment.

However, the clinical application of CLDNs-targeted therapy may face several obstacles. The agents designed to disrupt tight junctions, such as CPE (clostridium perfringens enterotoxin) increases tight-junction's permeability which may be beneficial in providing enhanced uptake of chemotherapeutic agents. Since, CPE has been the most frequently studied CLDN-targeted therapeutic and its ability to rapidly lyse tumour cells of several cancer types has been demonstrated. Therefore, CPE might be best suited to local administration such as in the intraductal treatment of breast carcinoma in situ (88) or intraperitoneal treatment of ovarian cancer (89).

Majority of mucinous cystadenomas (MAs) are of gastrointestinal-type and their small subset originate from Müllerian-type epithelium

Background

Mucinous cystadenoma (MA) is the most common mucinous tumor in the ovary. They are always benign and comprise 80% of all ovarian mucinous tumors (4, 40). These tumors are usually large, and unilateral in about 95% of cases. About 5% of these tumors are bilateral (4, 40). They have a smooth external surface and typically composed of multiple smooth walled cystic lesions with various sizes. Histologically, a non-stratified mucinous epithelium resembling gastric foveolar-type epithelium is usually observed in mucinous cystadenoma (40). Intestinal-type mucinous epithelium including various numbers of goblet cells is also occasionally observed. The epithelium in MA is tall columnar with intracellular mucin having a small basal nuclei (40). Stratification and tufting are generally absent. MA generally lacks atypia or they may have a very mild or focal atypia (4). Rarely these tumors appear multilocular cystic with papillary architecture.

The immunophenotype of these tumors are not fully understood, and not much has been described in the literature. These tumors are usually diagnosed simply as “mucinous cystadenoma” without subclassification such as intestinal-type or endocervical-type as we do in mucinous borderline tumors.

They are parts of ovarian epithelial neoplasm and currently accepted as a precursor for IMBTs and IMCas. It is generally believed that stepwise malignant transformation occurs from MAs to IMBTs and to IMCas. According to some of

the previous studies nearly all cases of IMCas have areas of MA as well as IMBT (41-43). Thus, it can be speculated that most MAs are of gastrointestinal-type lineage.

The histogenesis of these tumors is not clearly understood. There are several hypotheses regarding the histogenesis of these tumors. Some investigators believe that most of these tumors are derived from ovarian surface epithelium that undergoes a metaplastic process (68-71). At the same time, a teratomatous origin is suggested. Mucinous tumors are present in 11% of ovarian mature cystic teratomas (MCT), and conversely around 5% of ovarian mucinous tumors contain a teratoma (68, 72-75). Since, mature cystic teratomas (MCT) have gastrointestinal-type mucinous epithelium, it is certainly possible that mucinous tumors associated with MCTs arise from those gastrointestinal elements in MCT and have a germ cell or teratomatous origin, rather than ovarian surface epithelium (75).

The association of mucinous tumor with Brenner tumor (BT) is also reported in the past. Metaplastic mucinous epithelium is occasionally observed in the center of transitional cell nests of the Brenner tumor (BT). It is generally believed that those mucinous tumors associated with BT arise from areas of metaplastic mucinous epithelium existing in the BT nests (73, 75, 76). Molecular change including activating KRAS mutation is also reported more frequently in mucinous ovarian tumors. The frequency is higher compare to other histological

subtypes. According to the previous studies, mutation of KRAS is more common in endocervical-type mucinous tumors than those of gastrointestinal-type (85-87).

The association between gastrointestinal-type mucinous epithelium of the mucinous cystadenomas and Müllerian-type epithelium such as those of endometrial cyst (EC), and other ER-positive epithelium has not been clearly illustrated in the past. According to our observation, mucinous cystadenoma is occasionally associated with Müllerian-type lesions such as endometrial cyst (EC). However, still it is largely unknown whether Müllerian-type epithelium or epithelium of Müllerian duct derivative has a potential to bear gastrointestinal-type mucinous epithelium or not.

Study aims:

In this study, we attempted to elucidate the direction of differentiation of mucinous epithelium that constitutes mucinous cystadenomas. Special attention was paid to the existence of gastrointestinal-type mucinous epithelium, and its association with Müllerian-type epithelium.

To determine the histogenesis of gastrointestinal-type mucinous epithelium, we have also attempted to clarify the distribution of CLDN18-positive gastric-type mucinous epithelium in variety of ovarian lesions, including benign teratomatous and metaplastic mucinous epithelium, and non-neoplastic Müllerian duct derivatives.

Materials and methods

Tissue sample

In this study, the following tissue samples were immunohistochemically analyzed: Mucinous cystadenomas, mucinous epithelium in mature cystic teratomas, metaplastic mucinous epithelium in ovarian lesions such as Brenner tumor and endometrial cyst, non-mucinous ovarian lesions and non-neoplastic Müllerian duct derivatives.

Ovarian Mucinous Cystadenoma

A large series of MAs including unilateral tumors from 139 patients were retrieved from archives of Department of Pathology at The University of Tokyo Hospital. In all of these cases, the histological slides have been reviewed, and tumor diagnosis was made according to the most recent WHO classification. The histological features of all tumors including types of epithelium lining the cystic cavities, and tumor growth pattern were evaluated. Coexistence of other lesions in the ovary especially, endometriosis, endometrial cyst, mature cystic teratoma, and Brenner tumor were also recorded.

Teratomatous and metaplastic mucinous epithelium in the ovary

Gastric and intestinal-type mucinous epithelium in MCT, and variety of metaplastic mucinous epithelium in the ovary were also analyzed in this study. We analyzed 13 cases of MCTs that contained gastric-type and/or colonic-type

mucinous epithelium, five endometrial cysts (ECs) with focal mucinous metaplasia, and three cases of Brenner tumors (BTs) with focal mucinous metaplasia.

Benign non-mucinous ovarian lesions and non-neoplastic Müllerian duct derivatives.

Benign non-mucinous ovarian lesions, including eight serous cystadenomas (SCAs), 10 endometrial cysts (ECs) without mucinous metaplasia, and five surface epithelial inclusions, were added to the series. Further, the following cases of non-neoplastic Müllerian duct derivatives were also included in this study: Fallopian tube epithelium of six patients, endometrial epithelium of 41 patients (11 in the proliferative phase, 12 in the secretory phase, six in the menstrual phase, and 12 in the gestational phase), endocervical epithelium of six patients, and endometriosis of six patients.

Preparation of tissue sample and immunohistochemistry

All tissue samples were fixed in formalin and embedded in paraffin. The tumors were initially evaluated, on morphological basis, for the presence or absence of gastrointestinal-type mucinous epithelium, and Müllerian-type epithelium. As previously described, gastrointestinal-type mucinous epithelium is

tall columnar in appearance, and the cells have small basal nuclei and intracytoplasmic mucin. They look similar to gastric foveolar and pyloric-type epithelium, or epithelium of intestine which have goblet cells. Müllerian-like epithelium is also tall columnar or cuboidal in appearance, show ciliation and focal papillary change on the surface with no goblet cells. Morphologically they are similar to the uterine cervical glandular epithelium or epithelium of EMBT and SBTs.

A representative slide was chosen for morphologically purely gastrointestinal-like MAs and purely Müllerian-like MAs. For those cases in which transition from Müllerian-like epithelium to gastrointestinal-like epithelium was observed, or suspected, slides that contained such areas were selected for immunohistochemistry.

A representative whole tissue section was also selected for the remaining benign teratomatous mucinous lesions, metaplastic mucinous lesions, non-mucinous and non-neoplastic Müllerian duct derivatives. We arranged benign endometrial epithelium in another TMA with a single 2-mm core obtained from each case. Both whole sections slides and TMAs were cut into 4μm thickness for immunohistochemical analysis. Immunohistochemistry was performed in a method which is previously described (refer to page 16).

To clarify the characteristics of mucinous epithelium in Mas, we performed immunohistochemistry. Although they usually consisted of gastric

foveolar-type epithelium, in some cases the characteristics of epithelium was vague and difficult to specify by histological examination only. For conclusive evaluation of the epithelial differentiation, we performed immunohistochemistry for CLDN18 (as a gastric marker), CDX2 (as an intestinal marker) and ER (as a Müllerian marker) in all cases of MAs. To detect CLDN18-positive gastric-type mucinous epithelium in benign ovarian lesions and Müllerian duct derivatives, all teratomatous mucinous lesions, metaplastic mucinous lesions, non-mucinous ovarian lesions and non-neoplastic Müllerian duct derivatives were stained with CLDN18 only.

Interpretation of immunohistochemistry

Immunoreactivity was interpreted based on the presence of nuclear stating for, ER and CDX2. CLDN18 expression was evaluated based on the existence of basolateral membrane staining. Immunohistochemical reactions were scored based on the percentage of positive cells and graded as 0 (totally negative), 1+ (1%–4%), 2+ (5%–14%), 3+ (15%–49%), and 4+ (≥50%).

Finally, we defined the epithelium showing CLDN18+/CDX2±/ER- immunophenotype as pure gastrointestinal-type epithelium, and the epithelium showing CLDN18-/CDX2-/ER+ immunophenotype, designated as pure Müllerian-type epithelium. The epithelium showing CLDN18+/CDX2±/ER+ immunophenotype, designated as "mixed gastrointestinal/Müllerian-type epithelium".

Statistical analysis

Statistical analysis was performed using Fisher's exact test. Statistical analyses were performed using the StatView software version 5.0 (SAS Institute, Cary, NC), and a value of $P < 0.05$ was considered to indicate statistical significance.

Results:

Morphologically, 14 cases of MAs coexisted with mature cystic teratoma (MCT), 6 cases were in transition or in coexistence with endometrial cyst (EC), 2 cases coexisted with endometriosis and one case coexisted with Brenner tumor.

In majority of mucinous cystadenomas the lining epithelium consists of gastric foveolar-type mucinous epithelium with flat apex that show no to mild stratification. The nuclei are uniformly located at the base of the cells. In addition, over 30 cases of MAs demonstrated varying numbers of goblet cells, suggesting their intestinal-type differentiation.

In a small number of cases, the lining epithelium represented non–gastrointestinal. The existence of ciliated change on the surface with focal papillary formation, and nuclear feature different from gastrointestinal-type epithelium were detected in these cases. Unlike, gastrointestinal-like MAs their nuclei were located in the mid part of cytoplasm, and had round shaped. These

features suggest Müllerian-type differentiation. There were also some cases which showed histological transition from morphologically Müllerian-type epithelium to morphologically gastrointestinal-type mucinous epithelium. In these cases, gastrointestinal-type mucinous epithelium usually predominated. In 7 cases of mucinous cystadenomas, the cyst wall showed endometrial cyst-like changes such as fibrosis, hyalinization and hemosiderin deposition.

Results of immunohistochemistry

Mucinous cystadenomas

The results of immunohistochemistry in mucinous cystadenomas are summarized in Table 8 and 9.

Our immunohistochemistry results, revealed gastrointestinal phenotype as defined by CLDN18 and/or CDX2 positivity in vast majority of mucinous cystadenomas (93%, 129/139). CLDN18 was positive in 91% (127/139) of the cases, and almost all the cases showed diffuse expression pattern, since more than 50% of tumor cells were CLDN18 positive. CDX2 (intestinal marker) was expressed in 40/139 (29%) of MAs. The expression was focal with exception of three cases, which revealed diffuse positivity (Figure 9 and Figure 10). Based on these results, 71% (99/139) of mucinous cystadenoma in our study can be

categorized as a purely gastrointestinal-type MAs, which characterized by (CLDN18+/CDX2±/ER-) immunophenotype (Figure 9 (A-D) and Figure 10).

In 12 cases of MAs, we found purely gastrointestinal-type mucinous epithelium (CLDN18+/CDX2±/ER-) in transition from Müllerian-type (CLDN18-/CDX2-/ER+) epithelium (Figure 11). In most of these cases, predominant component was gastrointestinal-type. In 18 cases of MAs, the tumors contained mixed gastrointestinal and Müllerian-type epithelium characterized by CLDN18+/CDX2±/ER+ immunophenotype. The positivity of ER in these cases varied from weak or focal to diffuse and strong. We regard this finding as evidence, which suggest transformation of Müllerian-type epithelium to gastrointestinal-type epithelium. The representative areas are shown in Figure 12.

In 8 (6%) cases, the tumors showed only ER positivity, and we regarded as “pure Müllerian-type”. In these cases immunoreactivity for other markers (CLDN18 and CDX2) was completely negative. This immunophenotype is purely Müllerian (CLDN18-/CDX2-/ER+). The representative histology of purely Müllerian-type MAs along with their ER, CLDN18 and CDX2 expression is shown in Figure 13. Around 1% of mucinous cystadenoma in this study represented non-specific histological and immunohistochemical feature. All three markers were negative in these cases. They were considered as mucinous

cystadenoma NOS (Not otherwise specified). The summary of each subtype with their specific immunophenotype is shown in Table 9.

In our study, the frequency of coexistence of MCT was relatively high, since 14/139 (10%) of the cases were in association with MCT. In contrast, the frequency of coexistence of BTs was less than MCT. Only one of the tumors in our study was in coexistence with BT. Our results showed a slight difference between those cases of MAs in association with MCT, and those with no association. The CLDN18 positivity was found in nearly all cases of MAs associated with MCT with one exceptional case. In contrast, CDX2 expression in MAs which coexisted with MCT was higher than those cases with no association. CDX2 positivity was found in 9/14 (65%) of MAs in association with MCT, while CDX2 positivity in MAs with no association with MCT was 31/125 (26%) (P=0.0109). This is consistent with the previous studies (15), suggesting that, MA associated with MCT exhibits immunohistochemical features similar to lower gastrointestinal type mucinous tumor.

CLDN18 expression in benign ovarian lesions and Müllerian duct derivatives.

CLDN18 expression in benign ovarian lesions and Müllerian duct derivatives are listed in Table 10. Among variety of benign-looking mucinous epithelium of the ovary, CLDN18 expression was demonstrated exclusively in

gastric-type mucinous epithelium in mature cystic teratomas, and metaplastic mucinous epithelium in (benign/borderline) Brenner tumors. In mature cystic teratomas, diffuse CLDN18 expression was observed in gastric foveolar epithelium, fundic glands, and pyloric glands, while intestinal (colonic) type mucinous epithelium containing goblet cells was always negative. Focal CLDN18 expression was seen in the metaplastic mucinous epithelium that lines the inner lumen of the transitional cell epithelium in two of three (2/3, 67%) benign/borderline Brenner tumors. The CLDN18 expression in benign teratomatous and metaplastic mucinous epithelium in BTs is shown in Figure 14.

The epithelium that lined the ECs was CLDN18-negative in all cases (0/10). We evaluated CLDN18 expression in focal metaplastic mucinous epithelium in the cyst wall of ECs, which was also negative (0/5). Other ovarian lesions, such as surface epithelial inclusions (n=5) and serous cystadenomas (n=8), Müllerian duct derivatives that included fallopian tube epithelium (n=6), endometrial glands in different stages of the menstrual period (n=41), endocervical epithelium (n=6), and epithelium of endometriotic lesions (n=6), were all negative for CLDN18.

Discussion

Mucinous cystadenomas are currently classified under ovarian epithelial tumors and account for 17% of all ovarian neoplasms. Controversy has surrounded the histogenesis of ovarian mucinous tumors. Several theories to describe the histogenesis of ovarian mucinous tumors have been previously suggested. Some researchers believe that, most ovarian mucinous tumors arise from surface epithelium. Some other investigators suggested that ovarian mucinous tumors are actually of teratomatous origin, since coexisting teratoma was found in 11% of ovarian mucinous tumors (15), while around 5% of teratomas were associated with mucinous cystadenoma or adenocarcinomas in the same ovary (16, 77). It is now generally accepted that some mucinous cystadenoma arise from gastrointestinal-type elements that exist in mature cystic teratoma (75). It has also been reported that an ovarian mucinous tumor can contain both components of surface epithelium, and teratomatous epithelium (68, 69, 76).

Another ovarian lesion that is currently believed to contribute to ovarian mucinous tumorigenesis is Brenner tumor. In a study by Waxman et al, 66 of 460 Brenner tumors were associated with mucinous cystadenoma or rarely with adenocarcinoma (79). It is of note that epithelium of Brenner tumors have tendency to undergo mucinous metaplasia. In general, those mucinous tumors which are associated with Brenner tumors are thought to originate from areas of

mucinous metaplasia within transitional epithelium of Brenner tumors nests (80, 81). Silverberg et al. showed a transition between the epithelium of mucinous cystadenoma and epithelium of the Brenner tumor (80). In addition to teratoma and Brenner tumor, mucinous tumors in association with sertoli-leydig cell tumors and granulosa cell tumor has also been previously reported (82-84). According to all these reports, the heterogeneous origin of ovarian mucinous tumor has gradually been accepted. However, the histogenetic relationship of gastrointestinal-type mucinous epithelium and Müllerian-type epithelium such as those of endometrial cysts in the ovary has not been clearly described in the past.

In this study, our immunohistochemistry revealed that most of the MAs contained gastrointestinal-type mucinous epithelium which is characterized by CLDN18 and CDX2 expression. CLDN18-positivity in nearly all cases of MAs suggests that these tumors are part of the ovarian gastrointestinal-type tumor lineage. Since, they have similar immunophenotype with IMBTs and IMCas, we consider them as a benign counterpart or precursor lesion for ovarian IMBTs and IMCas. In addition the existence of CLDN18-positive mucinous epithelium in mature cystic teratomas and Brenner tumors, along with CLDN18/and or CDX2 expression in nearly all MAs in association with mature cystic teratomas and Brenner tumor support the hypothesis that the origin of gastrointestinal-type mucinous neoplasms of the ovary maybe these lesions.

In this study, we succeeded in demonstrating the potential of Müllerian duct derivatives to bear gastrointestinal-type mucinous epithelium. In this study, in 12 cases (9%) of mucinous cystadenomas, we found an area of transition from Müllerian-type epithelium (CLDN18-/CDX2-/ER+) to gastrointestinal-type mucinous epithelium (CLDN18+/CDX2±/ER-). Interestingly in three of them, the tumors were in association with endometrial cyst. Transition from Müllerian-type epithelium (CLDN18-/CDX2-/ER+) of ECs to gastric foveolar-type epithelium (CLDN18+/CDX2±/ER-) of MAs was observed (Figure 9 E, F).

Furthermore, cases that showed mixed gastrointestinal/Müllerian phenotype were seen in 18 (14%) of MAs. Based on all these findings, we believe that a subset of gastrointestinal-type MA is derived from Müllerian duct derivatives, such as endometriosis. Surface epithelial inclusions and Müllerian-type lesion such as serous cystadenomas assessed in our series were all negative for CLDN18. Müllerian duct derivatives such as endocervical epithelium, endometrium, and tubal epithelium were all negative for CLDN18. We believe that, gastrointestinal-type epithelium arise from Müllerian-type epithelium through metaplastic/neoplastic process.

Another important observation was the existence of CLDN18-/CDX2-/ER+ MAs. This has not been clearly defined in the past. The morphology of the mucinous epithelium of the cyst was closest to the Müllerian-type or endocervical-type mucinous epithelium seen in other lesions such as EMBTs.

The tumors showed no cytological atypia and minimal papillary growth. Therefore, we postulate that “Müllerian-type mucinous cystadenoma” would be the most appropriate diagnosis for those cases (Figure 13). Until now, there was no established subclassification of MAs. However, according to our findings, there are two different kinds of MAs, gastrointestinal and Müllerian (endocervical).

In summary, our results showed that mucinous cystadenomas can be subclassified into two major subtypes, gastrointestinal-type and Müllerian-type. Most of the mucinous cystadenomas show differentiation toward gastrointestinal-type mucinous epithelium which characterized by CLDN18 and/or CDX2 expression and negative immunoreactivity for ER. These tumors are considered as a benign counterpart of ovarian gastrointestinal-type mucinous tumor lineage. They have similar immunophenotype with IMBTs and IMCas and considered to be a precursor lesion for these tumors. Since the transition from Müllerian-type epithelium to gastrointestinal-type mucinous epithelium is seen in some of our cases, we conclude that gastrointestinal-type epithelium in the ovary can arise not only from teratomatous lesions or Brenner tumors but also from Müllerian duct derivatives (Figure 15).

Final conclusions:

The results of this study showed that:

1. Intestinal-type and endocervical-type ovarian mucinous tumors are two distinct entities with completely different immunophenotype.
2. We can accurately and easily distinguish intestinal-type and endocervical-type ovarian mucinous tumors by using CLDN18 immunohistochemistry.
3. Our results showed that, lesions which have been conventionally regarded as intestinal-type tumors are essentially of gastrointestinal-type. Since, the predominant components are usually gastric-type epithelium, rather than intestinal-type.
4. IMCa share similar immunophenotype with IMBTs and is considered to be a malignant subtype of ovarian gastrointestinal-type mucinous tumor category.
5. In this study, we showed the utility of CLDN18 immunohistochemistry in distinction between IMCas, and non-mucinous ovarian adenocarcinomas, and also between IMCas and metastatic CRCs involving the ovary, which is clinically significant.
6. We also showed that mucinous cystadenomas consist of two different subtypes. The majority of which is gastrointestinal-type characterized by CLDN18+/CDX2±/ER- immunophenotype. Müllerian-type MAs which characterized by CLDN18-/CDX2-/ER+ immunophenotype is rather rare.

7. Mucinous cystadenomas show similar immunophenotype with IMBTs and IMCas and are considered as a benign counterpart and precursor lesion for IMBTs and IMCas.
8. In addition to, MCT and BTs, we found some evidence that a subsets of gastrointestinal-type mucinous ovarian tumors originate form Müllerian duct derivatives such as endometriosis.

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Table 1.

Antibodies used for immunohistochemistry:

Antibody	Dilution	Clone	Manufacturer
CLDN18	1:1000	Poly	Zymed
MUC1	1:100	MA695	Novocastra
MUC2	1:20	Ccp58	Novocastra
MUC5AC	1:100	CLH2	Novocastra
MUC6	1:100	CLH5	Novocastra
CDX2	1:200	CDX2-88	Cell Marque
CK7	1:100	OV-TL12/30	DakoCytomation
CK20	1:100	Ks 20.8	Novocastra
ER	Prediluted	ER1D5	Ventana
PgR	Prediluted	A9621A	Ventana
CA125	1:200	Ov 185:1	Novocastra
Vimentin	1:1000	V9	DakoCytomation

CLDN18, claudin-18; CK7, cytokeratin 7; CK20, cytokeratin 20; ER, estrogen receptor; PgR, progesterone receptor.

Table 2.

Claudin-18, MUCs, and CDX2 expression in intestinal-type and endocervical-like mucinous borderline tumors:

	CLDN18		**MUC1**		**MUC2**		**MUC5AC**		**MUC6**		**CDX2**	
	IMBT	EMBT	IMBT	EMBT	IMBT	EMBT	IMBT	EMBT	IMBT	EMBT	IMBT	EMBT
-	1	24	30	0	36	24	4	7	42	19	28	25
1+	0	0	9	3	9	1	2	5	6	2	12	0
2+	1	1	7	4	6	0	5	9	5	4	7	0
3+	4	0	5	5	3	0	9	3	1	0	6	0
4+	48	0	3	13	0	0	34	1	0	0	1	0
Total	53/54 (98%)	1/25 (4%)	24/54 (44%)	25/25 (100%)	18/54 (33%)	1/25 (4%)	50/54 (93%)	18/25 (72%)	12/54 (22%)	6/25 (24%)	26/54 (48%)	0/25 (0%)
P	<0.0001		<0.0001		0.0042		0.0307		>0.9999		<0.0001	

IMBT (Intestinal-type mucinous borderline tumor)

EMBT (Endocervical-type mucinous borderline tumor)

Table 3.

Expression of cytokeratins and Müllerian markers in intestinal-type and endocervical-like mucinous borderline tumors.

	CK7		**CK20**		**ER**		**PgR**		**CA125**		**Vimentin**	
	IMBT	**EMBT**	**IMBT**	**EMBT**	**IMBT**	**EMBT**	**IMBT**	**EMBT**	**IMBT**	**EMBT**	**IMBT**	**EMBT**
-	0	0	11	24	52	0	53	5	35	0	53	0
1+	1	0	9	1	0	0	0	1	10	0	1	1
2+	2	0	10	0	2	0	1	6	4	0	0	2
3+	3	1	15	0	0	1	0	8	4	0	0	9
4+	48	24	9	0	0	24	0	5	1	25	0	13
Total	54/54 (100%)	25/25 (100%)	43/54 (80%)	1/25 (4%)	2/54 (4%)	25/25 (100%)	1/54 (2%)	20/25 (80%)	19/54 (35%)	25/25 (100%)	1/54 (2%)	25/25 (100%)
P	>0.0999		<0.0001		<0.0001		<0.0001		<0.0001		<0.0001	

IMBT (Intestinal-type mucinous borderline tumor)

EMBT (Endocervical-type mucinous borderline tumor)

Table 4.

Immunophenotype of serous borderline tumors:

	CLDN18	MUC1	MUC2	MUC5AC	MUC6	CDX2	CK7	CK20	ER	PgR	CA125	Vimentin
-	22	0	22	21	22	22	0	22	0	1	0	0
1+	0	0	0	1	0	0	0	0	0	1	0	2
2+	0	0	0	0	0	0	0	0	0	0	0	0
3+	0	0	0	0	0	0	1	0	0	3	0	3
4+	0	22	0	0	0	0	21	0	22	17	22	17
Total	0/22 (0%)	22/22 (100%)	0/22 (0%)	1/22 (5%)	0/22 (0%)	0/22 (0%)	22/22 (100%)	0/22 (0%)	22/22 (100%)	21/22 (95%)	22/22 (100%)	22/22 (100%)

Table 5.

Summary of primary IMCas, and metastatic CRCs involving the ovary according to the tumor size and laterality:

		Primary IMCa	Metastatic CRC
Laterality	Unilateral	17/19 (90%)	9/16 (56%)
	Bilateral	2/19 (10%)	7/16 (44%)
Tumor size	≥10cm	17/19 (90%)	11/16 (69%)
	<10cm	2/19 (10%)	4/16 (25%)

IMCas (Intestinal-type mucinous adenocarcinomas)

CRCs (colorectal adenocarcinoma).

Summary of metastatic colorectal carcinomas (CRCs) involving the ovary based on their laterality and size:

	Tumors <10cm	**Tumors ≥10cm**
Unilateral	0	3*
Bilateral	4	8

*The 3 cases (unilateral ≥10cm) which have been consistent with the algorithm.

Summary of primary Intestinal-type mucinous adenocarcinomas (IMCas) based on their laterality and size:

	Tumors <10cm	**Tumors ≥10cm**
Unilateral	2	15
Bilateral	0	2

Table 6.

CLDN18 immunohistochemistry among variety of ovarian mucinous, and non-mucinous adenocarcinomas.

Type of carcinomas	CLDN18 expression	P. value
Intestinal type mucinous carcinoma (IMCa)	11/12 (92%)	
Endometrioid adenocarcinoma (EMCa)	3/38 (6%)	
High grade serous carcinoma (HGSCa)	0/58 (0%)	<0.0001
Low grade serous carcinoma (LGSCa)	0/11 (0%)	
Clear cell adenocarcinoma (CCCa)	0/95 (0%)	

Table 7.

Immunohistochemical comparison between primary IMCas, and metastatic CRCs involving the ovary.

	CK7		CK20		CDX2		MUC2		MUC5AC		CLDN18		ER	
	IMCas	CRCs	IMCas	CRCs	IMCas	CRCs	IMCas	CRCs	IMCas	CRCs	IMCas	CRCs	IMCas	CRCs
0	0	14	5	0	5	0	12	2	3	13	3	14	18	16
1+	0	1	4	2	2	0	5	4	4	3	1	1	0	0
2+	1	0	3	2	5	0	1	8	5	0	3	1	0	0
3+	2	1	6	9	5	0	0	0	5	0	4	0	1	0
4+	16	0	1	3	2	16	1	2	2	0	8	0	0	0
Total	19/19 (100%)	2/16 (12%)	14/19 (74%)	16/16 (100%)	14/19 (74%)	16/16 (100%)	7/19 (37%)	14/16 (87%)	16/19 (84%)	3/16 (19%)	16/19 (85%)	2/16 (12%)	1/19 (5%)	0/16 (0%)
P	<0.0001		0.057		0.0473		0.0022		0.0001		<0.0001		0.9999	

IMCas (Intestinal-type mucinous adenocarcinomas)

CRCs (colorectal adenocarcinoma).

Table 8.

CLDN18, CDX2, and ER expression in ovarian mucinous cystadenoma:

	CLDN18	CDX2	ER
-	12	99	101
1+	3	16	11
2+	6	10	8
3+	29	11	6
4+	89	3	13
	127/139 (91%)	40/139 (29%)	38/139 (27%)

Table 9. IHC results in mucinous cystadenoma

Expression of CLDN18, CDX2 and ER in mucinous cystadenomas according to their specific phenotypes

Groups	Tumors specific categories	Immunoexpression	Total cases	IHC results on representative section			Coexisting lesions			
				CLDN18	CDX2	ER	MCT	Brenner	EM cyst	Endometriosis
A	Pure GI-type MAs	CLDN18+/CDX2+/ER-	99	97/97	28/97	0/97	13	1	2	1
		CLDN18-/CDX2+/ER-		0/2	2/2	0/2	0	0	0	0
	Pure GI-type MAs (CLDN18+/CDX2±/ER-) in transition from M-type epithelium (CLDN18-/CDX2-/ER+). (The predominant components in the tumors was GI-type epithelium)		12	12/12	5/12	12/12	0	0	3	0
B	MAs with Mixed phenotypes (CLDN18+/CDX2±/ER+)		18	18/18	5/18	18/18	1	0	0	1
C	Pure Müllerian-type MAs	CLDN18-/CDX2-/ER+	8	0/8	0/8	8/8	0	0	1	0
D	NOS (non-specific-type MAs)	CLDN18-/CDX2-/ER-	2	0/2	0/2	0/2	0	0	0	0

GI-type MAs: gastrointestinal-type mucinous cystadenomas. M-type: Müllerian-type

Table 10.

CLDN18 expression in benign ovarian mucinous lesions and non-neoplastic mullerian duct derivatives:

Type of lesions	CLDN18 positivity
Mucinous cystadenoma (MA)	
MA with no association with MCT or EC	107/116
MA in association with MCT	13/14
MA in transition with EC	3/3
MA in coexistence with EC	1/3
MA in association with endometriosis	2/2
MA in association with BT	1/1
Gastric-type mucinous epithelium in MCT	5/6
Colonic-type mucinous epithelium in MCT	0/7
ECs without mucinous metaplasia	0/10
Metaplastic mucinous epithelium in EC	0/5
Metaplastic mucinous epithelium in BT	2/3
Surface epithelial inclusion	0/5
Serous cystadenoma	0/8
Fallopian tube epithelium	0/6
Endometrial epithelium	0/41
Endocervical epithelium	0/6
Endometriosis	0/6

MCT (mature cystic teratoma), EC (endometrial cyst), BT (brenner tumor).

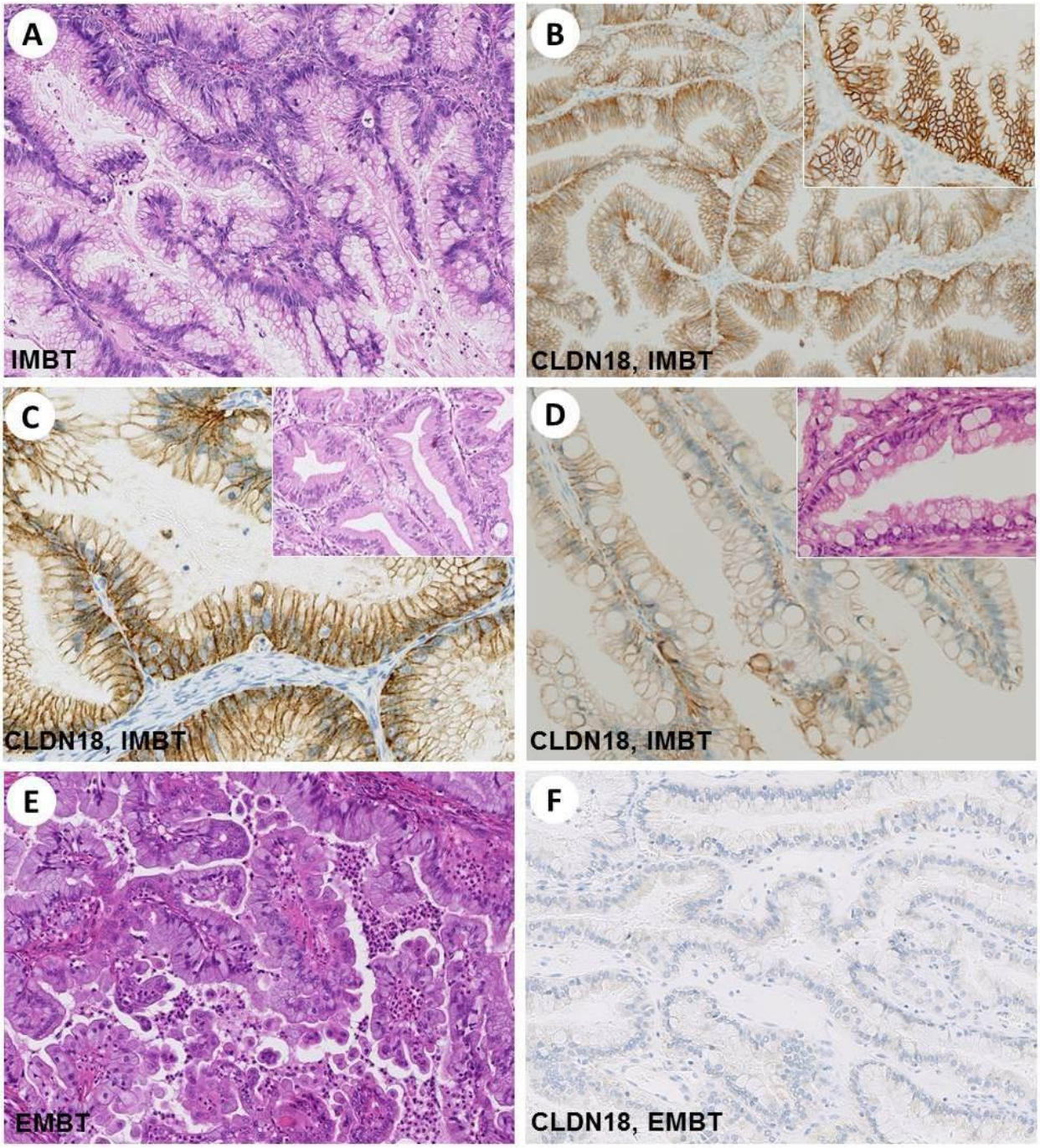


Figure 1. (A) Representative histology of intestinal-type mucinous borderline tumor. (B) Diffuse membranous expression of CLDN18 in intestinal-type mucinous borderline tumor. (C) CLDN18 expression in gastric foveolar-type mucinous epithelium of intestinal-type mucinous borderline tumor. Diffuse basolateral staining is observed in the Goblet cell-rich area of the intestinal-type mucinous borderline tumor. (D) CLDN18-positivity is observed in the majority of the tumor cells. (E) Representative histology of endocervical-like mucinous borderline tumor characterized by prominent papillary structures and stromal inflammation. (F) CLDN18 is completely negative in an endocervical-like mucinous borderline tumor.

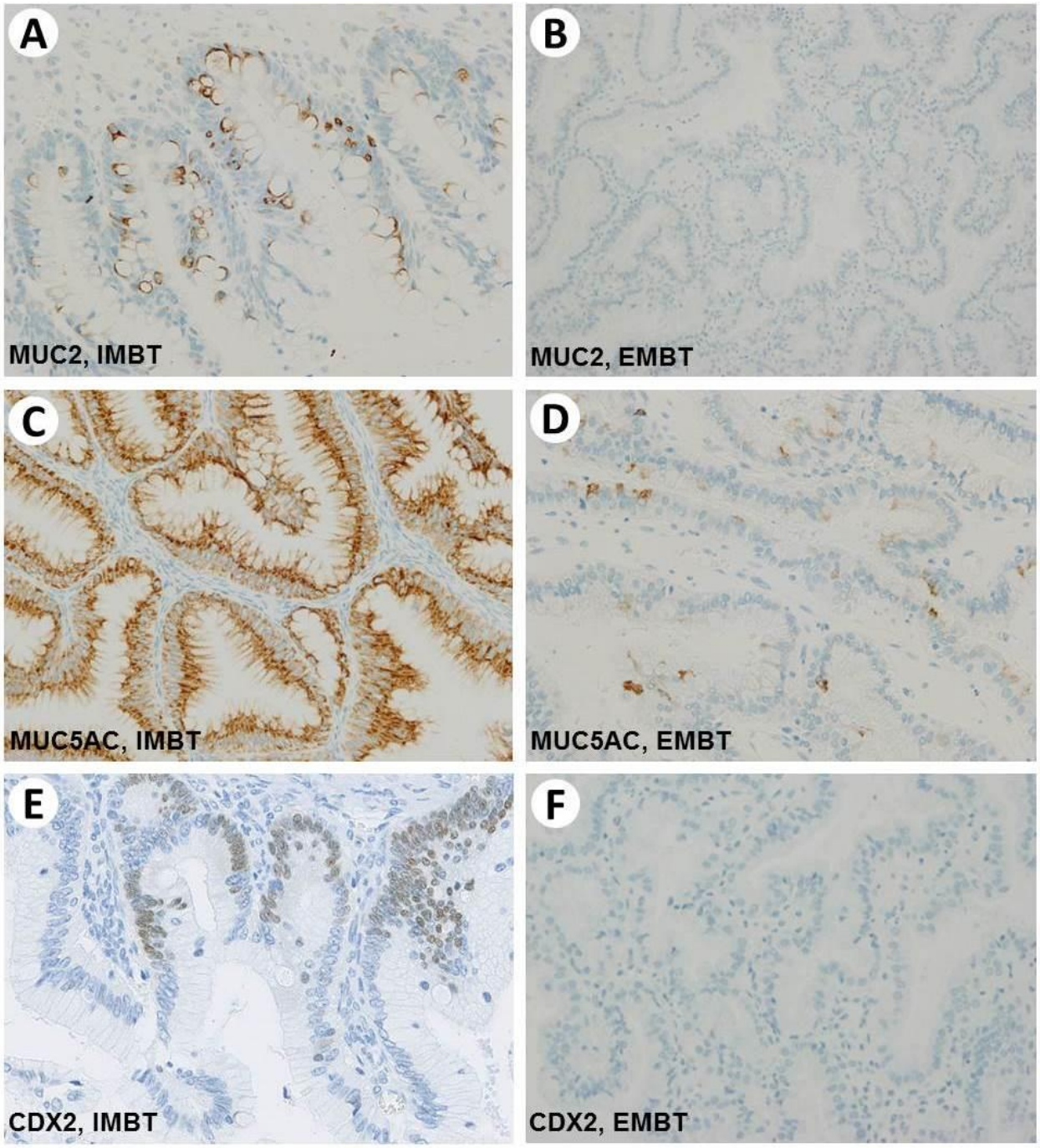


Figure 2. Expression of MUC2, MUC5AC, and CDX2 in (A, C, E) intestinal-type mucinous borderline tumor and (B, D, F) endocervical-like borderline tumor. (A) Focal MUC2 expression in a goblet cell-rich intestinal-type mucinous borderline tumor. (B) Endocervical-like borderline tumor negative for MUC2. (C) Diffuse MUC5AC expression in intestinal-type mucinous borderline tumor. (D) MUC5AC expression in endocervical-like mucinous borderline tumors is often focal. (E) Patchy and focal CDX2 positivity in intestinal-type mucinous borderline tumors. (F) CDX2 is always negative in endocervical-like mucinous borderline tumors.

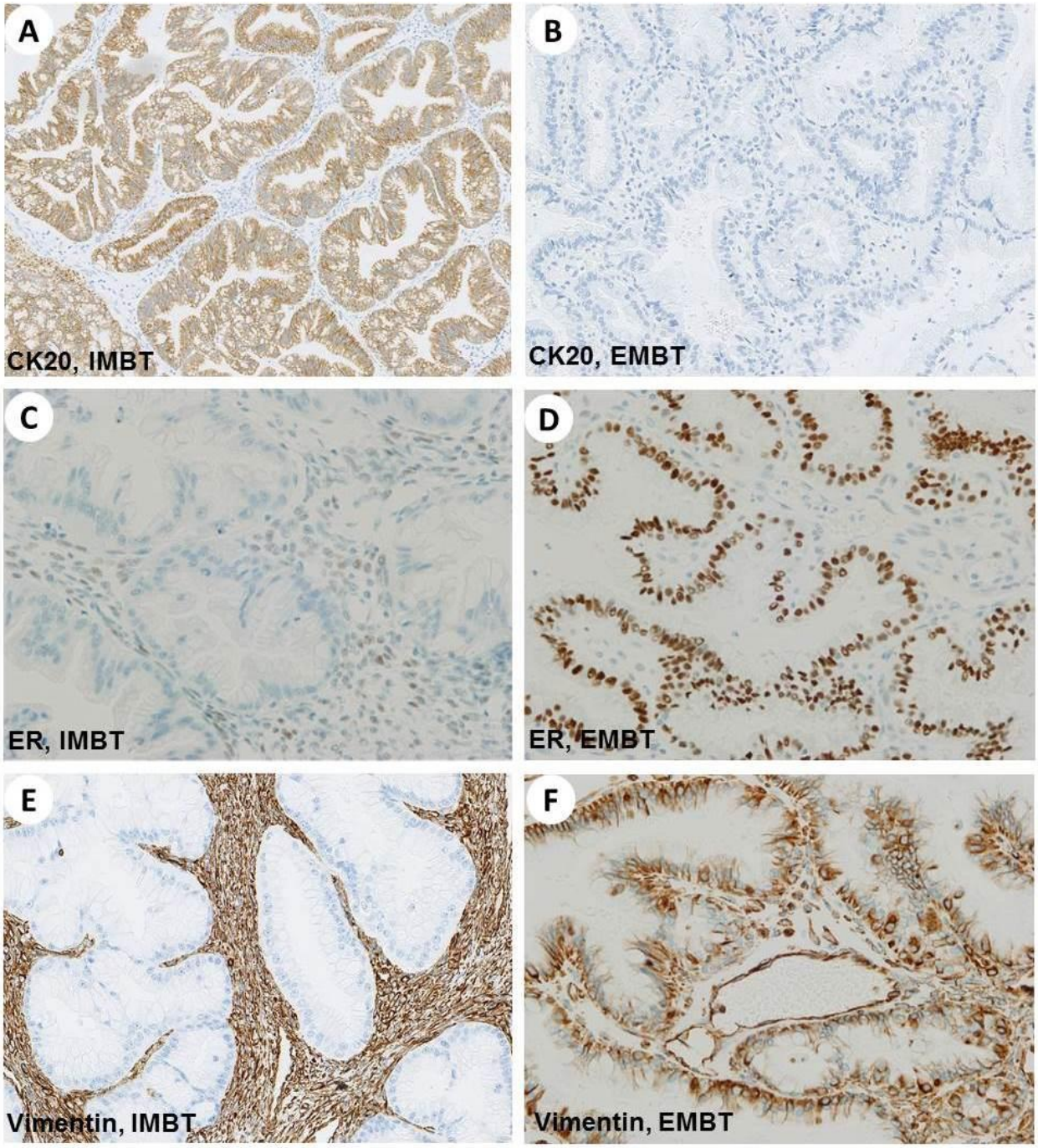


Figure 3. Expression of CK20, ER, and vimentin in (A, C, E) intestinal-type mucinous borderline tumors and (B, D, F) endocervical-like borderline tumors. (A) Intestinal-type mucinous borderline tumor showing strong CK20 expression. (B) CK20 is negative in endocervical-like mucinous borderline tumors. (C) ER is usually negative in intestinal-type mucinous borderline tumors, while (D) endocervical-like mucinous borderline tumors always shows diffuse and strong nuclear positivity. (E) Vimentin expression is seen only in the stroma of intestinal-type mucinous borderline tumors. The tumor cells are vimentin-negative. (F) Vimentin expression in an endocervical-like mucinous borderline tumor. Many of the tumor cells show positive immunoreactivity along with stromal cells.

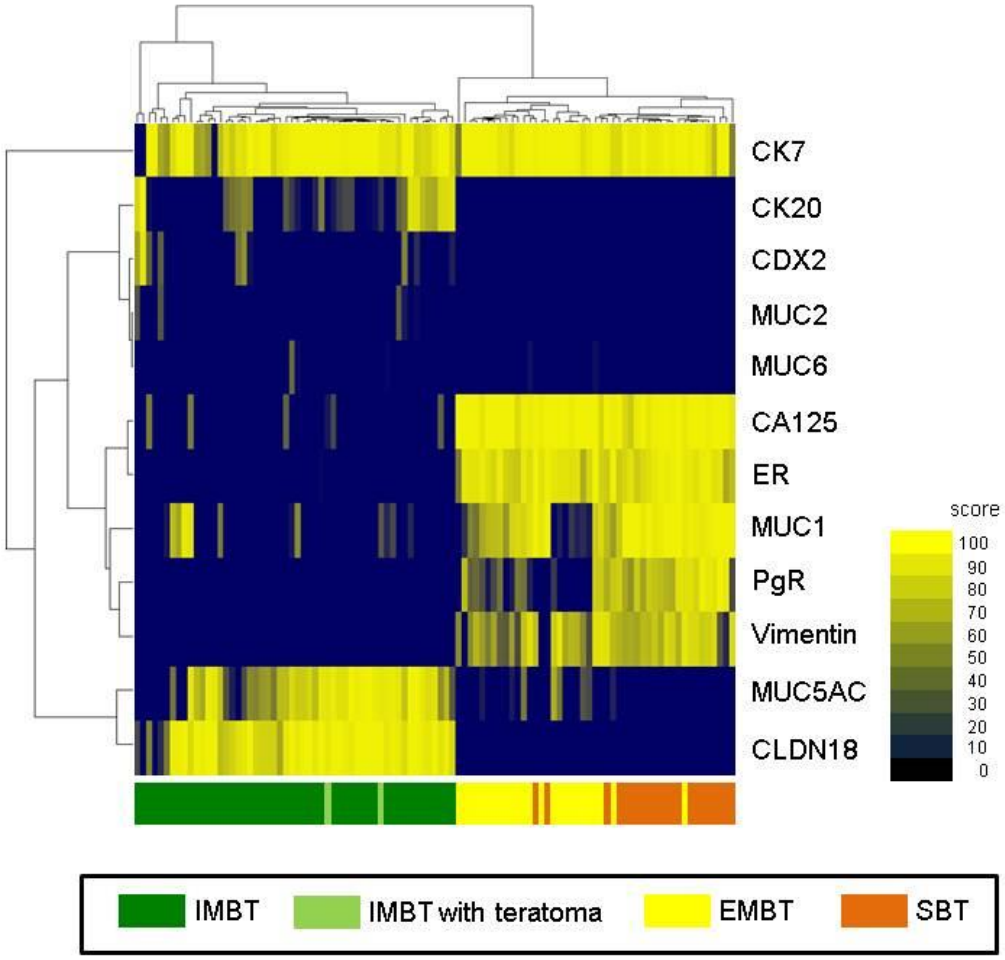


Figure 4. Unsupervised two-way hierarchical clustering based on the protein expression of ovarian borderline tumors. Intestinal-type mucinous borderline tumors were grouped separately from endocervical-like and serous borderline tumors. Similarities between the immunoprofiles of endocervical-like borderline tumors and serous borderline tumors are demonstrated.

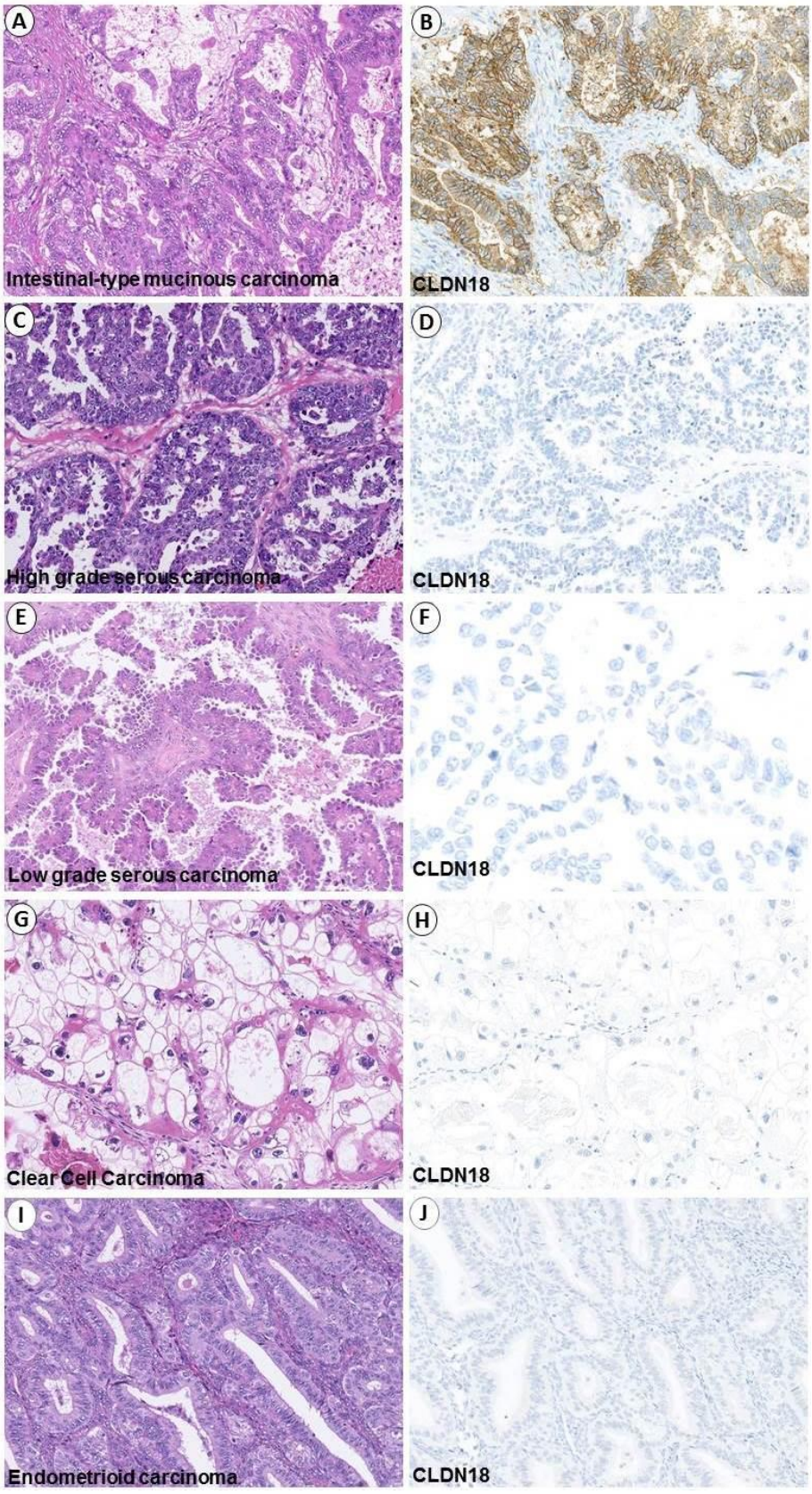


Figure 5. (A) Representative histology of IMCa. CLDN18 is diffusely positive in IMCas (B).

(C) Representative histology of HGSCa. CLDN18 is always negative in HGSCa (D).

(E) Representative histology of LGSCa. CLDN18 is always negative in LGSCa (F)

(G) Representative histology of CCCa HE. CLDN18 is always negative in CCCa (H)

(I) Representative histology of EMCa HE. CLDN18 is negative in nearly all EMCas (J).

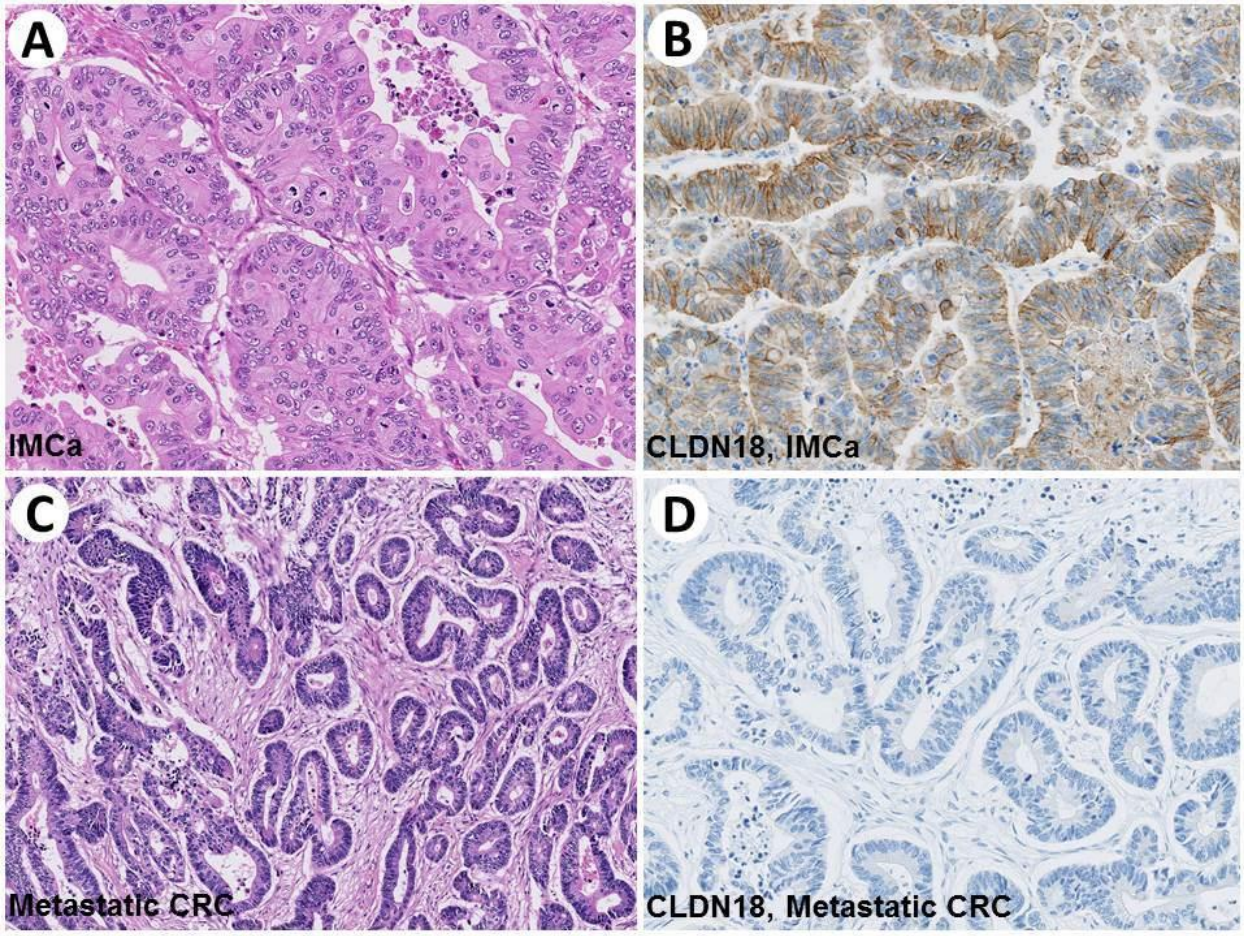


Figure 6. CLDN18 expression in primary IMCa and Metastatic CRC involving the ovary. (A) Representative histology of primary IMCa. (B) CLDN18 is usually positive in primary IMCa. (C) Metastatic CRC involving the ovary HE. (D) Metastatic CRC involving the ovary is usually CLDN18 negative.

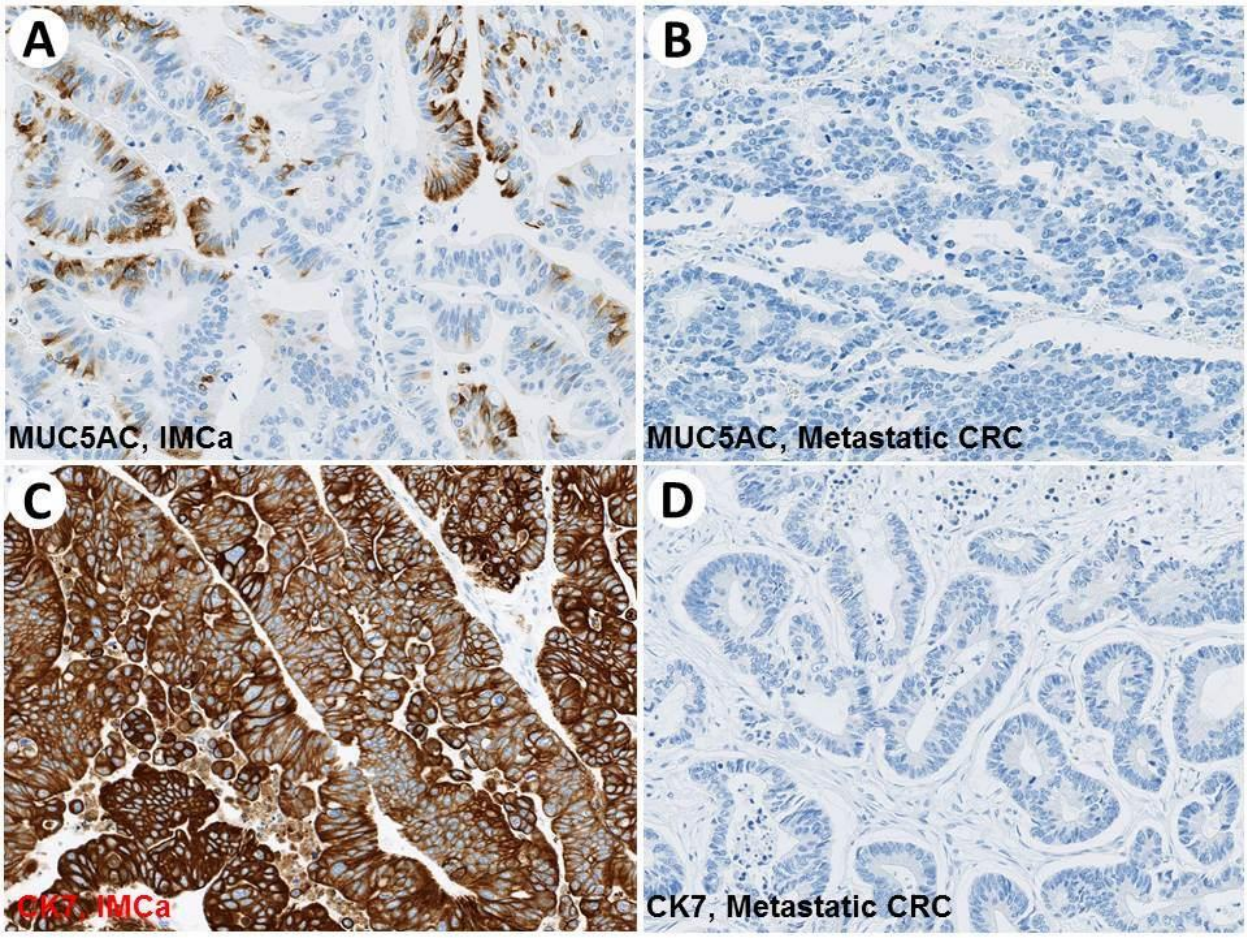


Figure 7. MUC5AC and CK7 expression in primary IMCa and metastatic CRC involving the ovary. (A) MUC5AC is focally positive in majority of primary IMCas. (B) Metastatic CRC involving the ovary is almost always CLDN18 negative. (C) Primary IMCa is diffusely positive for CK7. (D) CK7 is always negative in metastatic CRC involving the ovary.

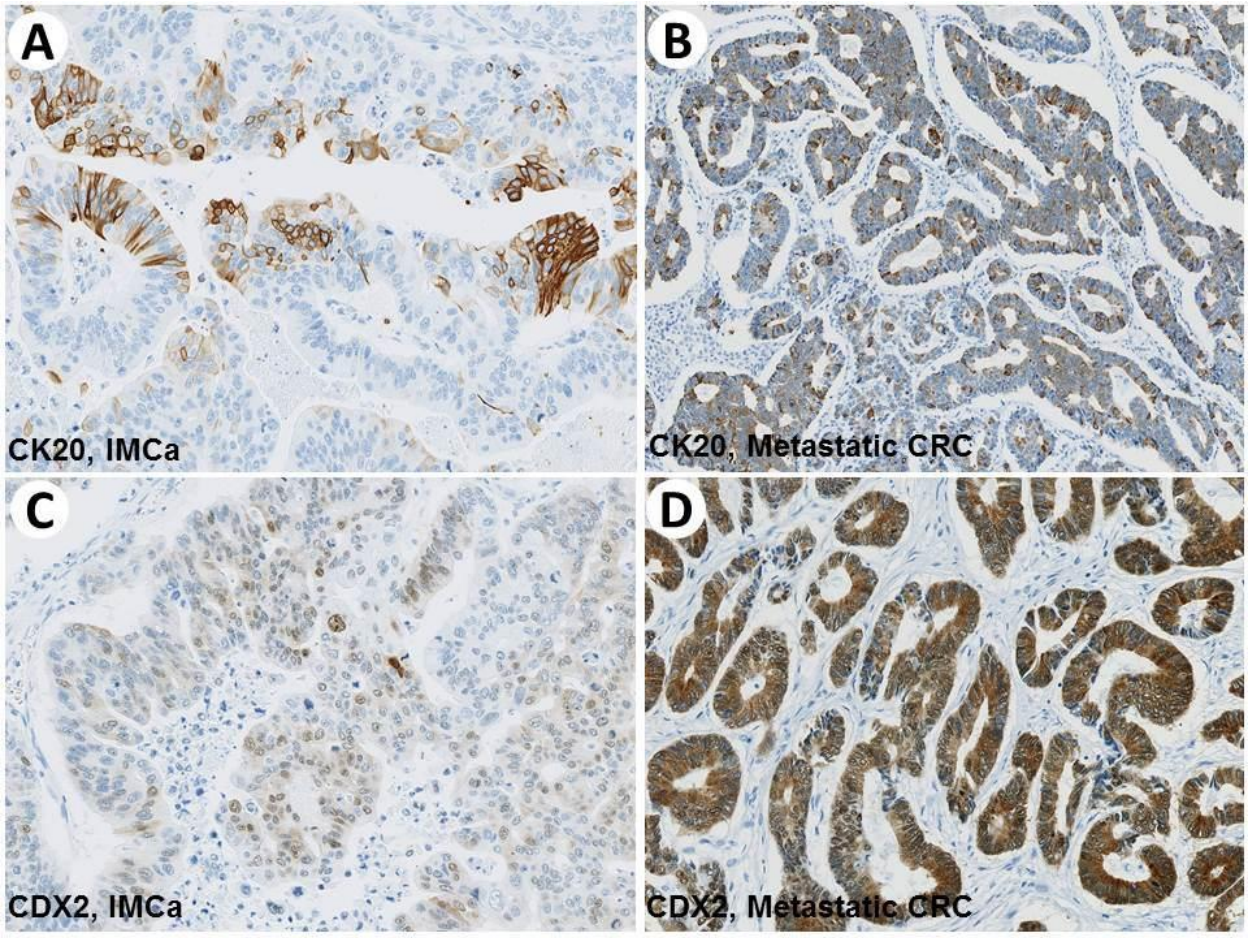


Figure8. Expression of CK20 and CDX2 in primary IMCa and metastatic CRC involving the ovary. (A) CK20 is focally expressed in primary IMCa, while it expression in metastatic CRC is usually diffuse (B). CDX2 expression in primary IMCa is focal and weak (C). In contrast to IMCas, CDX2 expression in metastatic CRC is usually diffused and strong (D).

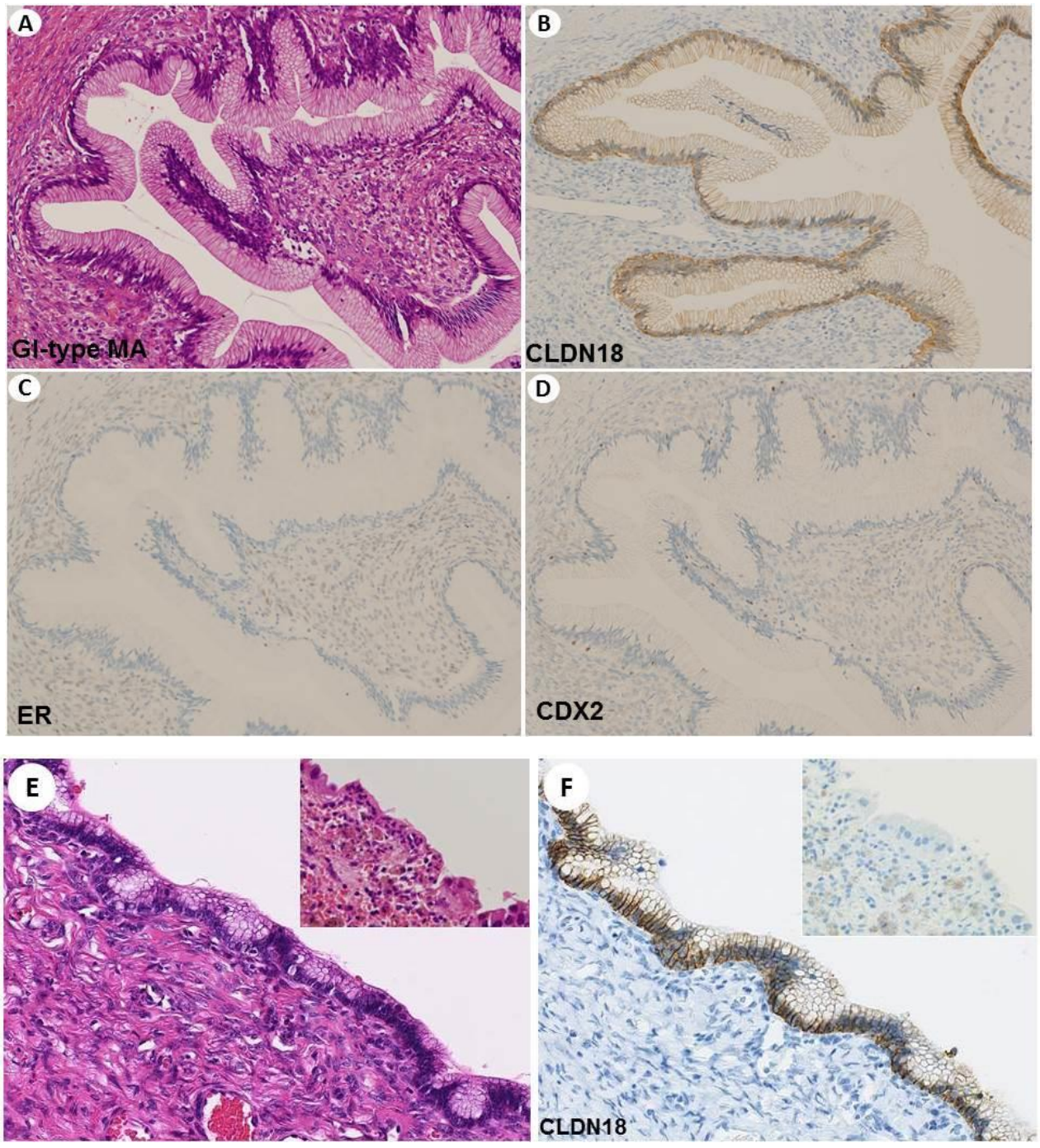


Figure 9. Gastrointestinal-type mucinous cystadenoma. (A) Representative histology of gastrointestinal-type mucinous cystadenoma composed of gastric foveolar-type epithelium. (B) Tumor shows diffused basolateral membranous staining for CLDN18. (C) ER is usually negative in gastrointestinal-type MAs. (D) CDX2 expression is often negative in MAs. (E) Gastrointestinal-type mucinous cystadenoma, that showed transition from endometrial cyst (inset), and (F) its CLDN18 expression. The mucinous cystadenoma expresses CLDN18 diffusely, whereas the epithelium of the background endometrial cyst is negative.

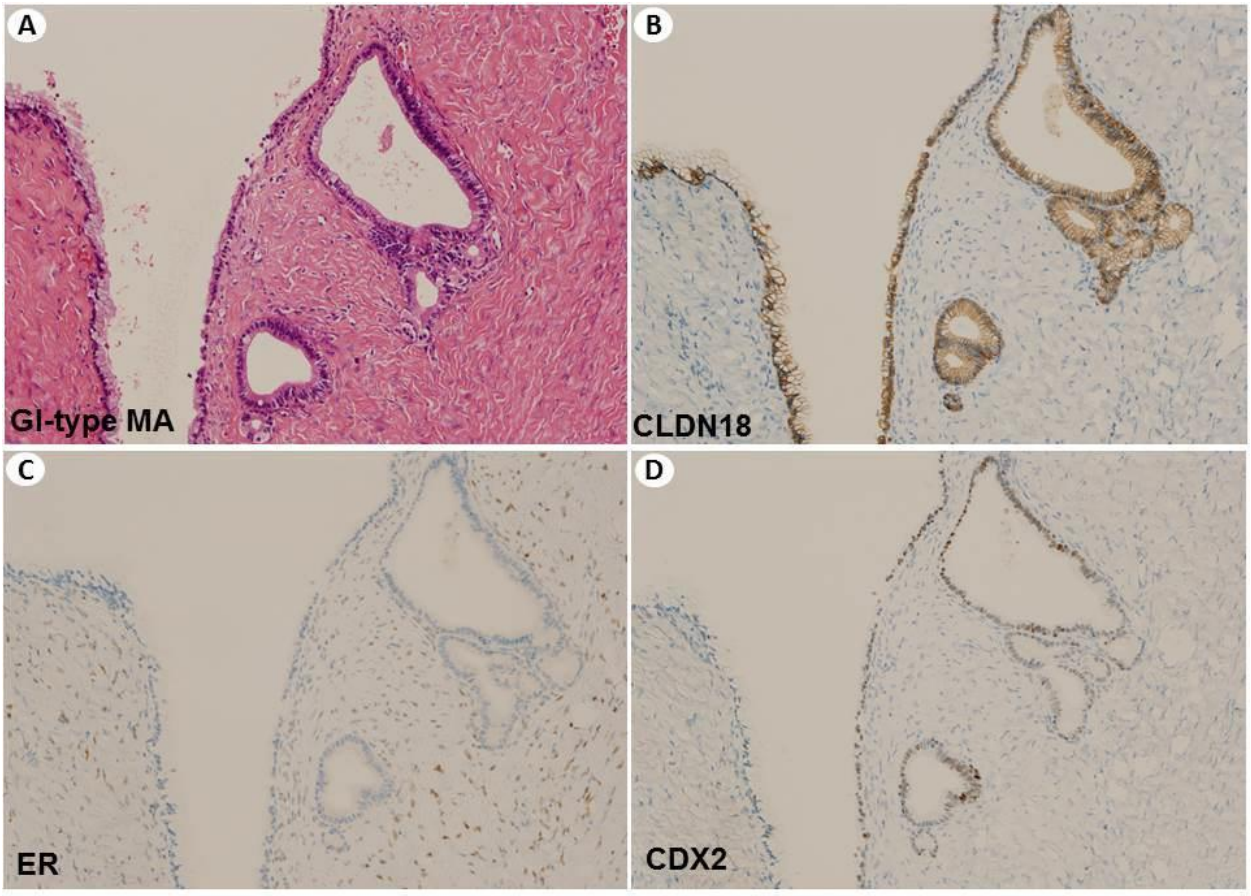


Figure 10. Gastrointestinal-type mucinous cystadenoma.(A) Representative histology of gastrointestinal-type mucinous cystadenoma composed of gastric foveolar-type epithelium. (B) Tumor shows diffused basolateral membranous staining for CLDN18. (C) ER is usually negative. (D) CDX2 expression is focally observed in some cases.

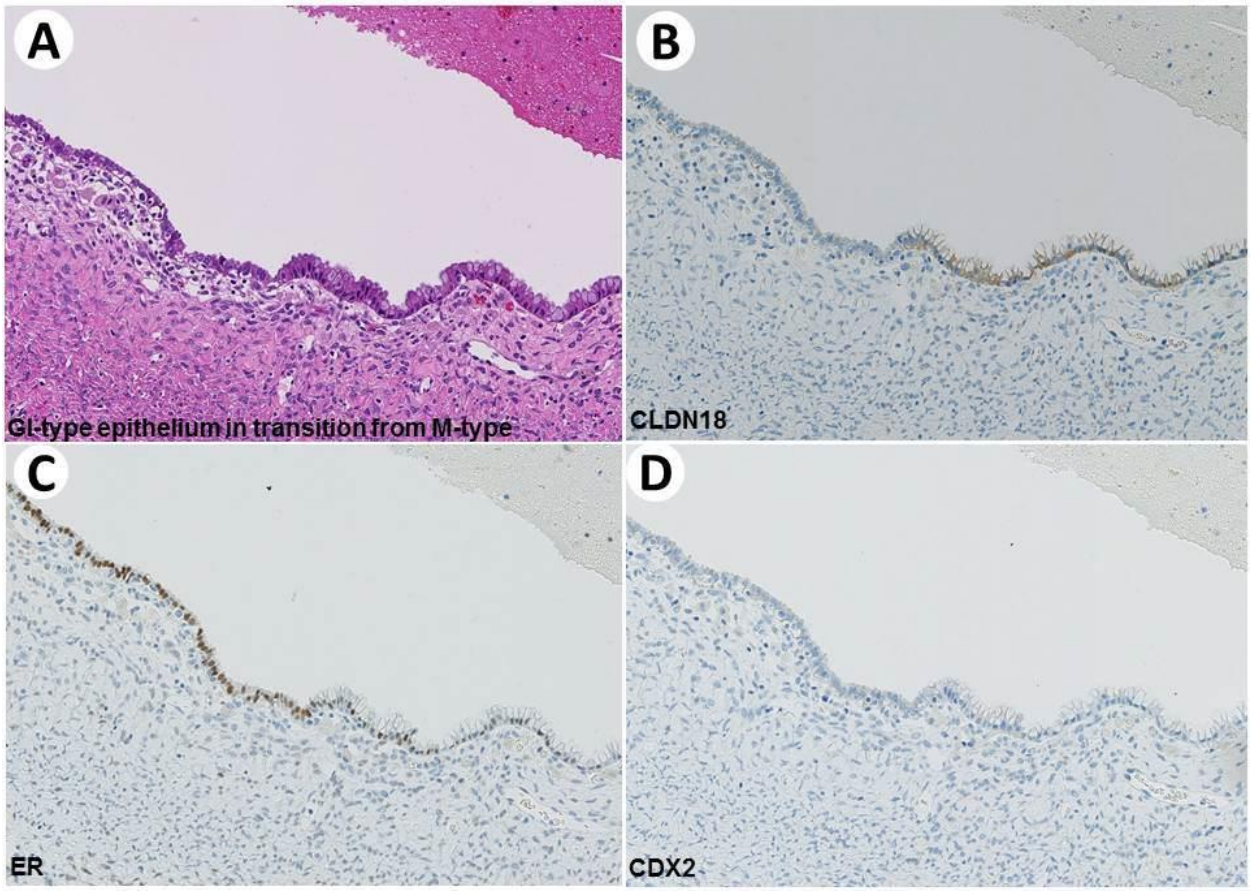


Figure 11. Gastrointestinal-type mucinous epithelium showing transition from Müllerian-type epithelium (A). CLDN18 expression is negative in areas with Müllerian-type epithelium, positive for areas with gastrointestinal-type epithelium (B). Diffused nuclear staining for ER is seen in areas with Müllerian-type epithelium, while ER expression is gradually losing in area with gastrointestinal-type epithelium (C). CDX2 expression is completely negative in both types of epithelium (D).

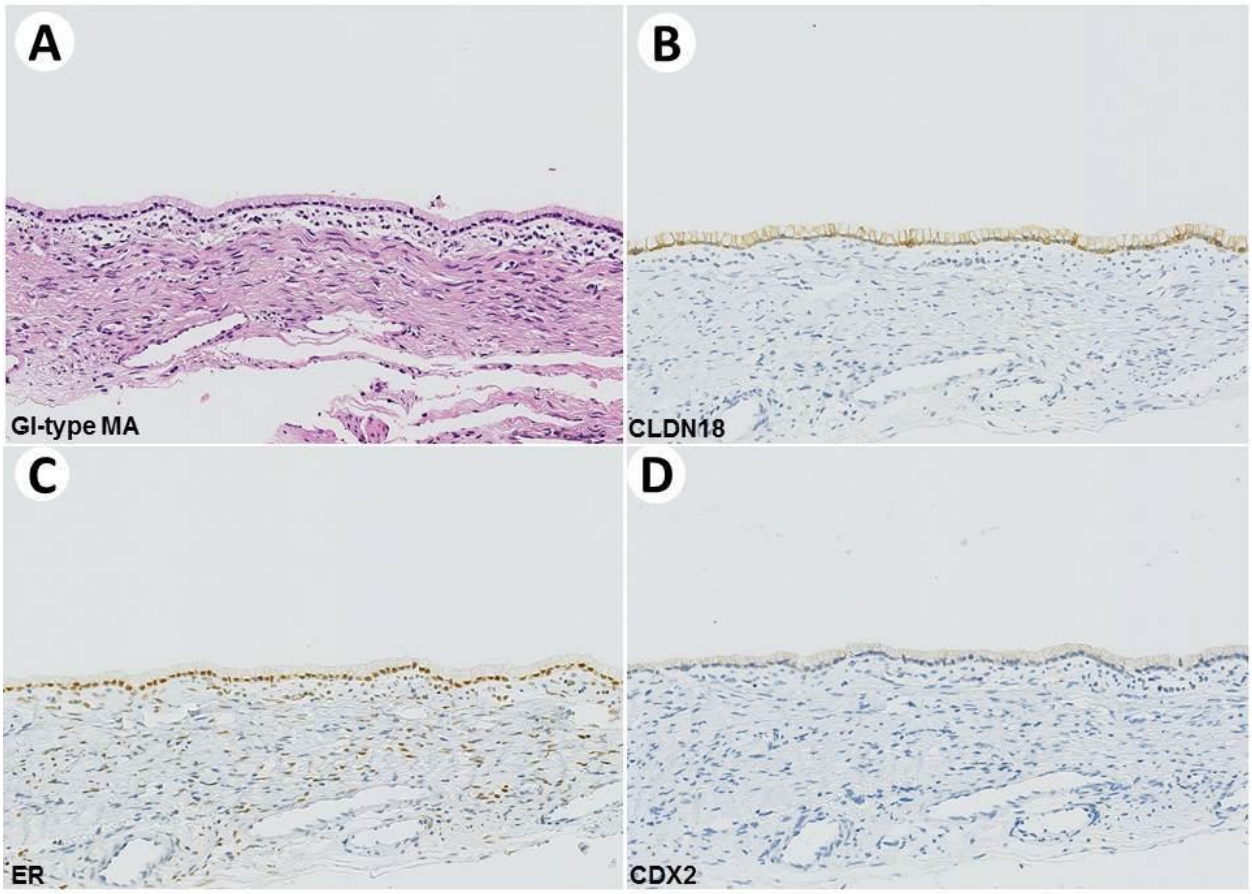


Figure12. Mucinous cystadenoma with mixed phenotypes (gastrointestinal and Müllerian). Representative histology (A). CLDN18 is diffusely positive in the tumor cells (B). Diffused nuclear staining for ER is also observed in morphologically gastrointestinal-type mucinous epithelium (C). CDX2 is completely negative (D).

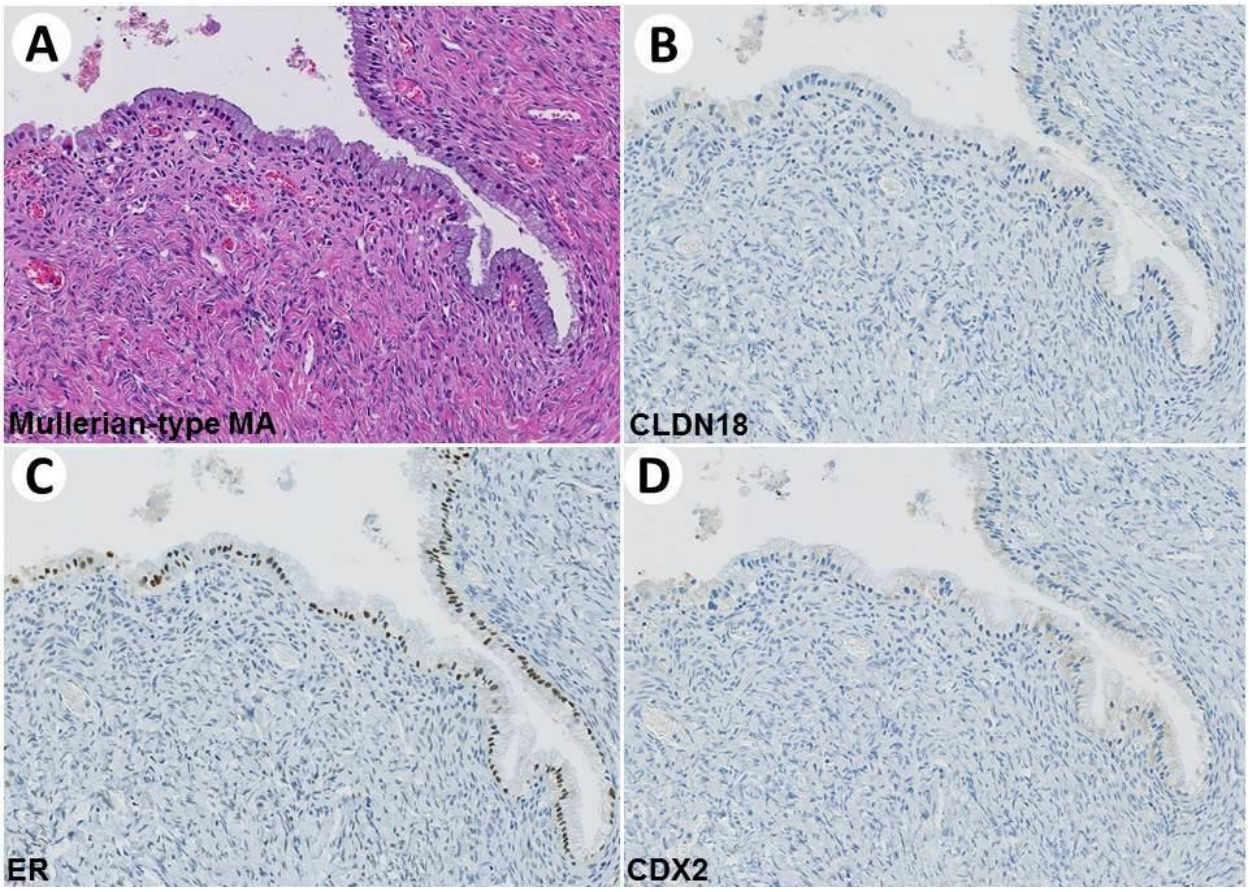
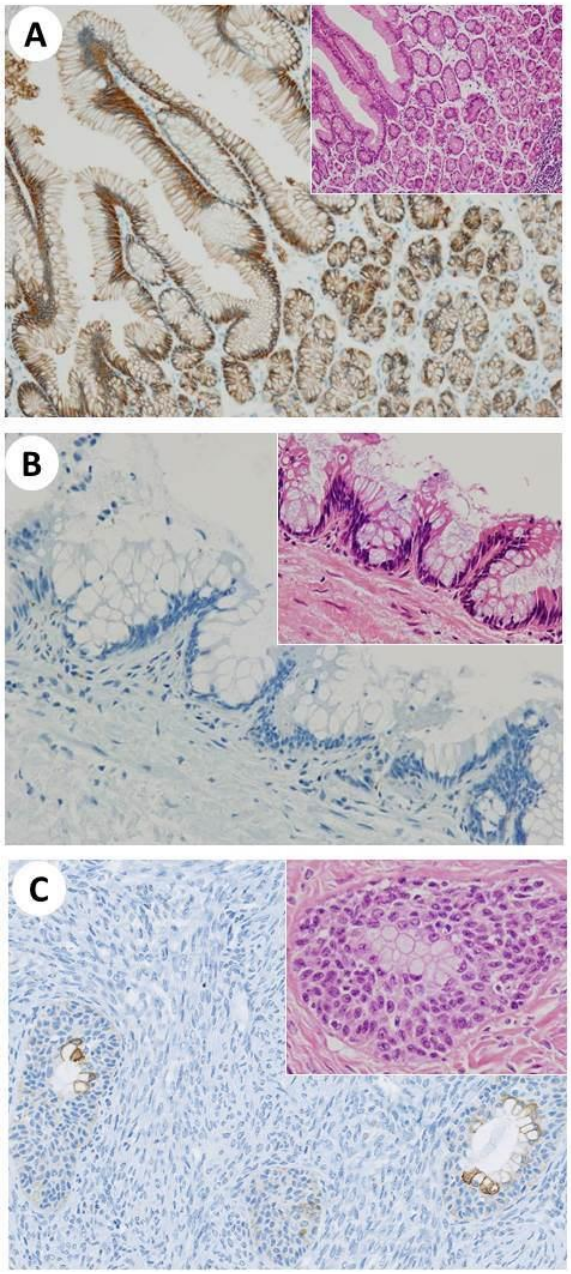


Figure 13. Müllerian-type mucinous cystadenoma representative histology (A). CLDN18 is always negative in Müllerian-type MAs (B). Diffused nuclear staining for ER is seen in Müllerian-type MA(C). CDX2 expression is completely negative (D).

Figure 14.



CLDN18 expression in mucinous epithelium of teratoma and Brenner tumor. (A) Diffuse CLDN18 expression in gastric-type mucinous epithelium in mature cystic teratoma. (B) Colonic-type epithelium in mature cystic teratoma is negative for CLDN18. (C) Metaplastic mucinous epithelium in a Brenner tumor showing focal CLDN18 expression.

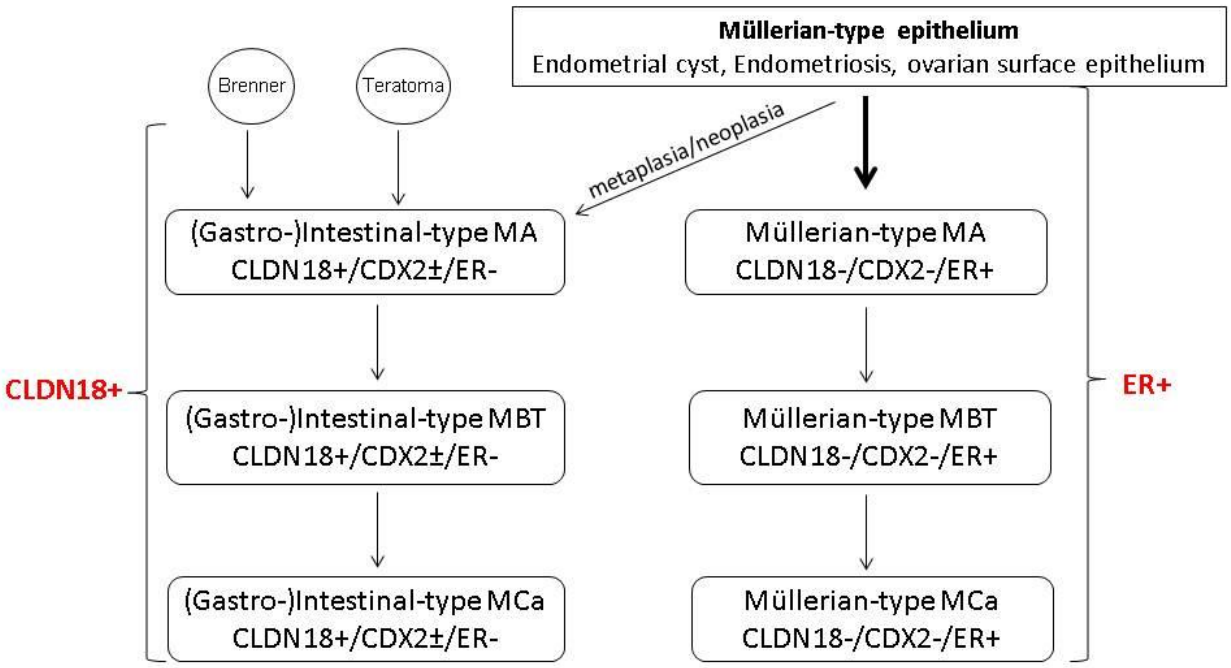


Figure 15.

Schematic demonstration of ovarian mucinous tumorigenesis

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