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Claudin-18 overexpression in intestinal-type mucinous borderline tumour of the ovary

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Aims: Mucinous borderline tumours of the ovary are subclassified as intestinal-type (IMBT) and endocervical-like (EMBT), which differ in their clinicopathological features. In this study, we attempted to elucidate characteristics of the mucinous epithelium in each subtype.

Methods and results: The expression of claudin-18, a marker of gastric differentiation, MUCs, CDX2, CK7, CK20, oestrogen receptor (ER), progesterone receptor (PgR), CA-125 and vimentin in IMBTs ($n = 54$), EMBTs ($n = 25$) and serous borderline tumours (SBTs) ($n = 22$) were compared by immunohistochemistry. Claudin-18 positivity was identified in 98% of the IMBTs, whereas only 4% of the EMBTs were claudin-18-positive. Expression of intestinal markers such as CDX2 and MUC2 was relatively infrequent in IMBTs (48% and 33%, respectively). Müllerian-lineage markers such as ER, PgR and vimentin were expressed rarely in IMBTs, while most EMBTs and SBTs were positive for these markers. Hierarchial clustering revealed a close association between EMBTs and SBTs, while IMBTs were clearly separate.

Conclusions: Claudin-18 positivity is a specific phenotype that is characteristic of IMBTs. Frequent and diffuse expression of gastric markers, along with less frequent and usually focal expression of intestinal markers, suggests that IMBTs are essentially composed of gastrointestinal-type mucinous epithelium (gastric-type epithelium with a variable degree of intestinal differentiation).

Keywords: claudin-18, mucinous borderline tumour, ovary

Introduction

According to the current classification of the World Health Organization (WHO), ovarian mucinous borderline tumours (MBTs) are classified further into two types: intestinal-type mucinous borderline tumours (IMBTs) and endocervical-like mucinous borderline tumours (EMBTs).[1] These tumours have been given a variety of names in the literature, and there is a degree of confusion regarding their nomenclature. Some authors refer to IMBTs as 'gastrointestinal'-type mucinous borderline tumours.[2–5] Terms such as 'seromucinous', 'Müllerian type' and 'non-gastrointestinal type' are used frequently to refer to EMBTs.[2,3,5–9] The inconsistency in nomenclature is due primarily to the subjective interpretation of the morphological features of IMBTs and EMBTs by each author. The

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paucity of 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.[2,3,7,9–11] IMBTs comprise approximately 85% of MBTs. They are usually unilateral (more than 90%).[10] Most IMBTs are large multicystic masses, and their epithelial component has been described as a mixture of intestinal-, gastric- and endocervical-type mucinous epithelium that grows predominantly in glandular or cystic structures admixed with papillary and villous structures.[7,9,10] Although intestinal differentiation, which is represented by the presence of goblet cells and CDX2 immunoreactivity, has been regarded as a key feature of IMBTs,[2,7,9,10,12] mucinous epithelium that resembles gastric foveolar-type epithelium is often the predominant component of IMBTs in observations by ourselves and others.[2,13] In fact, Ji *et al.*[14] have reported frequent expression of MUC5AC, a gastric foveolar epithelial marker, in IMBTs. The tumour 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 mucinous cystadenoma to IMBT to usual type (non-endocervical type) mucinous adenocarcinoma. However, the precise origin of these tumours remains unclear.

Compared with IMBTs, EMBTs are much less common and smaller, and tend to occur in younger females.[15–17] EMBTs are more frequently bilateral, and show a paucilocular gross appearance with intracystic papillary projection.[11,17] Histologically, EMBTs are characterized by finely branching papillae with fibrovascular cores, and their architecture resembles closely that of serous borderline tumours (SBTs). The lining epithelium is composed of columnar mucin-containing cells (which resemble endocervical cells), and polygonal cells with eosinophilic cytoplasm.[9,11,18] 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 found rarely in EMBTs.[9,16,17] EMBTs are associated frequently with endometriosis,[15,16,19] and share common immunohistochemical features with SBTs and low-grade endometrioid carcinomas, such as positivity for oestrogen receptor (ER) and progesterone receptor (PgR), suggesting the Müllerian nature of the neoplasm.[2,3,5,8]

In this study, we have 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 tumours. 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, a family of 27 proteins essential for the formation of tight junctions and the maintenance of polarity in epithelial and endothelial cells.[20,21] Positive immunoreactivity for CLDN18 has been shown in all types of gastric epithelium (foveolar-type, pyloric-type and fundic-type).[22,23] 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, 17 cases of unilateral EMBT, and four cases of bilateral EMBTs. Two of the IMBTs coexisted 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. Because IMBTs during this period far outnumbered EMBTs we selected 54 cases randomly, which is a substantial number for comparative analysis. We also added 22 cases of SBT to the series. Haematoxylin and eosin (H&E)-stained slides of all cases were reviewed. Histological diagnosis was based on the most recent criteria of the WHO.

IMMUNOHISTOCHEMISTRY

All tissue samples were fixed in formalin and embedded in paraffin. For immunohistochemistry, we arranged all the MBTs and SBTs in tissue microarrays (TMAs) with duplicate 2-mm cores obtained from each tumour. For those EMBT cases with bilateral involvement, tumours in the right and left ovaries

were submitted separately. TMA sections were cut at 4-μm thickness.

Immunohistochemistry for CLDN18, MUC1, MUC2, MUC5AC, MUC6, CK7, CK20, CDX2, CA125, ER, PgR and vimentin were performed in all ovarian borderline tumours. Antibodies used in this study are detailed in Table 1. For all antibodies, immunostaining was performed according to standard techniques using an autostainer (BenchMark XT; Ventana Medical Systems, Inc., Tucson, AZ, USA). 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 (S.A.H. and D.M.), who specialize in gynaecological 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 tumour cell positivity in two TMA cores was calculated, and then a grade was given. Appropriate positive and negative controls were included.

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, oestrogen receptor; PgR, progesterone receptor.

HIERARCHICAL CLUSTERING OF OVARIAN BORDERLINE TUMOURS 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 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 StatView software version 5.0 (SAS Institute, Cary, NC, USA), and a value of $P < 0.05$ was considered statistically significant.

Results

IMMUNOHISTOCHEMICAL COMPARISON OF IMBT AND EMBT

To reveal the characteristics of the mucinous epithelia that comprise IMBT and EMBT, we initially analysed 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 tumour 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. 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 expressed more frequently in IMBTs (93%) than

Table 2. Claudin-18, MUCs, and CDX2 expression in intestinal-type and endocervical-like mucinous borderline tumours

	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	

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 fewer 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 in only 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%).

The 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 expressed differentially in IMBTs and EMBTs ($P < 0.0001$). IMBTs are characterized roughly 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, and was of variable extent; 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 was another positive marker for EMBTs, but expression 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 TUMOURS

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 immunoprofiles. This dendrogram shows the degree of relatedness between the protein expression patterns detected by the 12 antibodies across the 101 cases of ovarian borderline tumours, 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

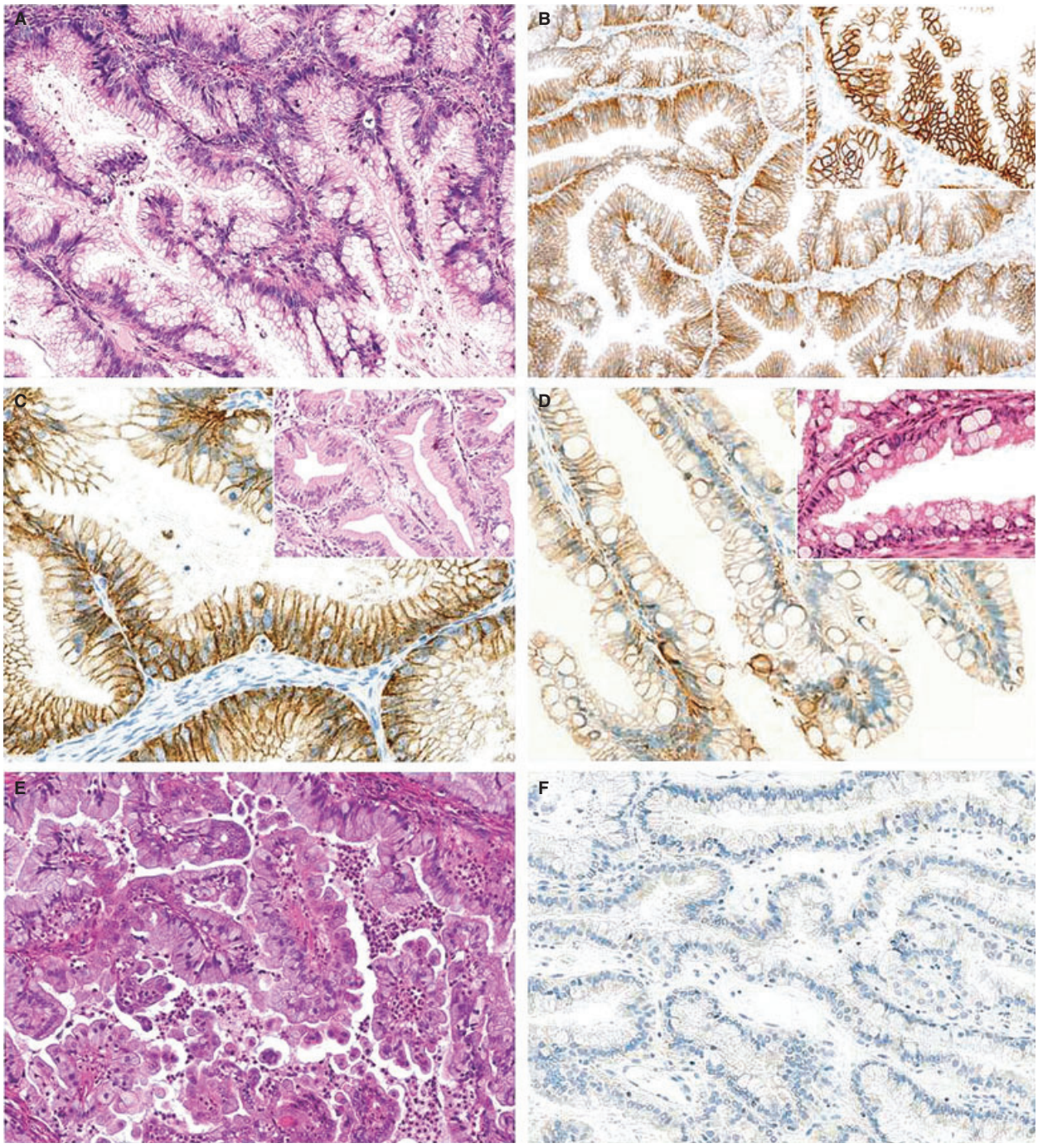

Figure 1. (**A**) Representative histology of intestinal-type mucinous borderline tumour (IMBT). (**B**) Diffuse membranous expression of CLDN18 in IMBT. (**C**) CLDN18 expression in gastric foveolar-type mucinous epithelium of IMBT. CLDN18 positivity is observed in the majority of the tumour cells.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

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.

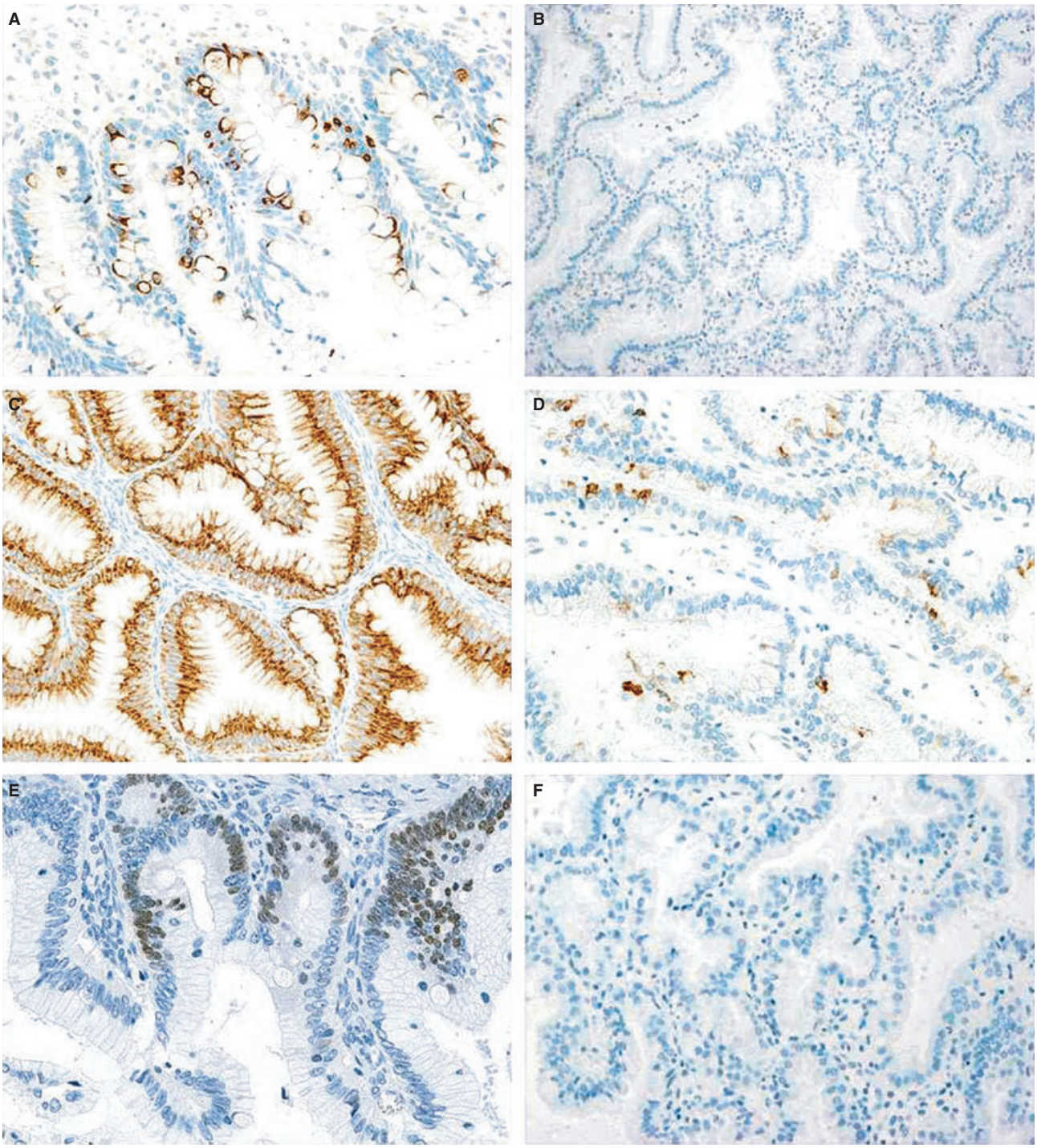

Figure 2. Expression of MUC2, MUC5AC and CDX2 in (**A**,**C**,**E**) intestinal-type mucinous borderline tumour (IMBT) and (**B**,**D**,**F**) endocervical-like mucinous borderline tumour (EMBT). (**A**) Focal MUC2 expression in a goblet cell-rich IMBT. (**B**) EMBT is negative for MUC2. (**C**) Diffuse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

Expression of CLDN18 has been studied in various types of human cancers and normal tissues.[23–31] 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.[27,29] In normal human tissues, expression of

Table 3. Expression of cytokeratins and Müllerian markers in intestinal-type and endocervical-like mucinous borderline tumours

	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	>0.9999		<0.0001		<0.0001		<0.0001		<0.0001		<0.0001	

claudin18a2 is confined to gastric epithelial cells (foveolar, endocrine, parietal and chief cells) and duodenal Paneth cells, and is not expressed in other organs, including the oesophagus, colon, pancreas and lung.[23,27,29,32] 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.[24–26,31] Sanada *et al.*[29] 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 showed further that CLDN18 is down-regulated in gastric epithelium with intestinal metaplasia and in gastric cancers with an intestinal phenotype. Our group showed recently that a subset of intrahepatic cholangiocarcinomas and pancreatic ductal carcinomas show a CLDN18-positive gastric phenotype.[24,25] It is of note that up-regulation 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 tumours. We demonstrated that the CLDN18-positive immunophenotype is observed specifically in IMBTs and not in EMBTs or SBTs. Another gastric marker, MUC5AC, which is expressed in normal gastric foveolar epithelium, was also expressed frequently in IMBTs, giving further support to the gastric differentiation of the IMBT epithelium. Because previous reports have shown that normal endocervical glands frequently express MUC5AC,[33,34] we believe that focal positivity observed in EMBTs are due most probably 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[7,9,10] and a number of studies have focused on the expression of intestinal markers such as CDX2 and MUC2 as key immunophenotypes of IMBT.[2,12,35] However, similar to some previous reports,[35,36] CDX2 and MUC2 expression in IMBTs was observed in fewer than half the cases, and their immunoreactivity was often focal in this study. Therefore, we conclude that in general IMBTs are composed essentially of gastrointestinal-type mucinous epithelium, the predominant component of which is gastric- rather than intestinal-type epithelium. This notion coincides with the morphological assessment of IMBTs by us and other researchers who consider that most

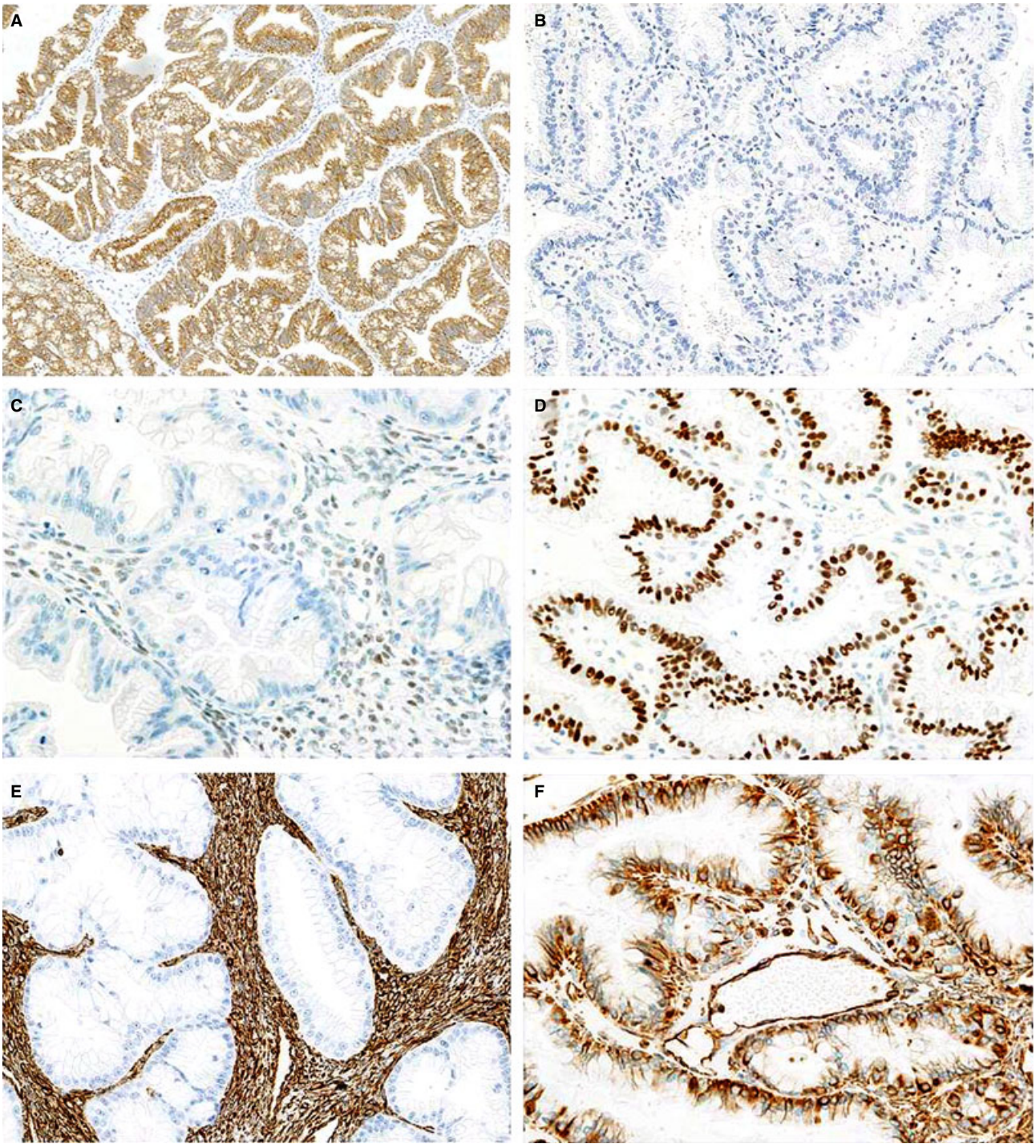

Figure 3. Expression of CK20, ER and vimentin in (**A**,**C**,**E**) intestinal-type mucinous borderline tumour (IMBT) (**B**,**D**,**F**) endocervical-like borderline tumour (EMBT). (**A**) IMBT showing strong CK20 expression. (**B**) CK20 is negative in EMBT. (**C**) ER is usually negative in IMBTs.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

mucinous epithelium in IMBTs resembles foveolar-type gastric epithelium.[2,14] Therefore, we propose abandoning the nomenclature 'intestinal-type mucinous borderline tumour' and replacing it with 'gastrointestinal-type mucinous borderline tumour' to avoid further confusion.

Table 4. Immunophenotype of serous borderline tumours

	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%)

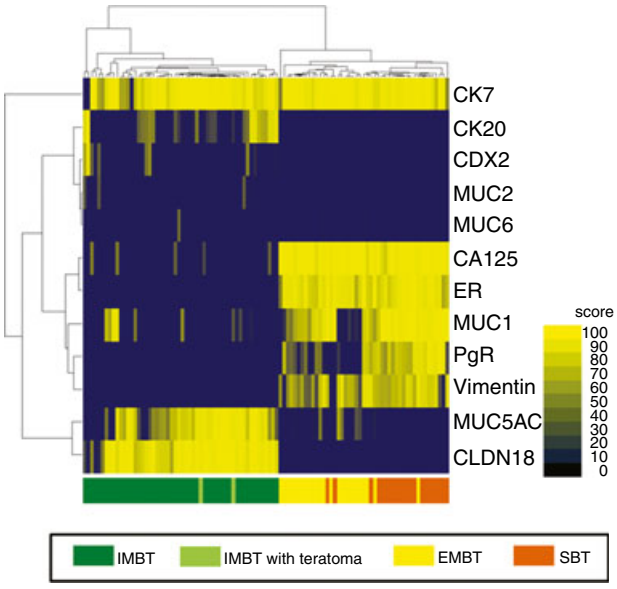

Figure 4. Unsupervised two-way hierarchical clustering based on the protein expression of ovarian borderline tumours. Intestinal-type mucinous borderline tumours (IMBT) were grouped separately from endocervical-like mucinous borderline tumours (EMBT) and serous borderline tumours (SBT). Similarities between the immunoprofiles of EMBTs and SBTs are demonstrated.*

Our immunohistochemical panel highlighted the differences between EMBTs and IMBTs. Similarities between EMBTs and SBTs have been described repeatedly from the morphological and immunohistochemical points of view.[2,3,16] Recent studies have reported that EMBTs share features with low-grade endometrioid tumours (borderline tumours and carcinomas), such as frequent association with endometriosis and frequent loss of ARID1A expression.[8] Currently, it is not clear whether EMBTs are closer to SBTs or low-grade endometrioid tumours. We recognize EMBT as a distinct Müllerian-type tumour that shows a variable degree of mucin production. In fact, our study revealed a Müllerian immunophenotype for EMBTs, including positivity for ER, PgR, CA-125 and vimentin. Furthermore, hierarchical clustering of ovarian borderline tumours (IMBTs, EMBTs and SBTs) according to their protein expression resulted in the grouping of 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 show clearly that EMBT and IMBT are two

*Figure 4 was corrected on 07/08/2013 after first online publication 06/05/2013. "CLDN18" was incorrectly spelled "CLND18".

distinct neoplasms and that the former is a part of the ovarian Müllerian-type tumour spectrum.

From a diagnostic standpoint, pathologists may occasionally encounter ovarian mucinous tumours that are difficult to classify as either IMBT or EMBT. In such instances, we propose that the best immunohistochemical panel 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 have also shown 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 tumours' and that EMBTs are 'Müllerian-type mucinous borderline tumours'. With regard to the tumorigenesis of ovarian gastrointestinal-type mucinous tumours, future studies of the origin of gastric-type epithelium in the ovary are warranted.

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Conflict of interests

None declared.

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