Chapter 7
CLDN18.2 expression in mucinous ovarian cancer; not only a therapeutic target but also a diagnostic tool

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Abstract

Purpose Claudins (CLDN) are important components of tight junctions, of which expression is tightly regulated and restricted to specific tissue types. In cancer, however, expression is often dysregulated. CLDN18.2, a splice variant of CLDN18, has previously been shown to be upregulated in various solid tumor types, whereas it is absent in the vast majority of normal tissues. This membrane protein might therefore represent a target for targeted antibody treatment strategies.

Methods By use of immunohistochemistry, we screened large, well-defined data-sets of gynecological malignancies for the expression of CLDN18.2 in order to analyze its diagnostic and therapeutic potential.

Results Cervical, endometrial, and serous ovarian cancer samples showed to be largely negative for this protein. In mucinous ovarian cancer, however, CLDN18.2 expression was abundantly present (76.7%). Importantly, also ovarian metastases from gastrointestinal primary sites (colorectal, ileum, stomach, appendix, and pancreas) were CLDN18.2 negative. Since it is diagnostically challenging to differentiate these metastases from primary mucinous ovarian tumors, the predictive value of CLDN18.2 was analyzed and matched with currently used diagnostic markers and markers under consideration for this purpose. Highest sensitivity and specificity for prediction of primary mucinous tumors was attained with the markers CLDN18.2, CK7, and DPC4.

Conclusion CLDN18.2 might serve as an interesting target for specific antibody treatment in mucinous ovarian cancer and shows potential as a diagnostic marker for this tumor type.
Introduction

Gynecological malignancies of the ovary, endometrium, and cervix yearly represent 12% of new cases of cancer in women in the Western World (1). The most common subtype is endometrial cancer (EC), which, as cervical cancer (CXCA), is most often diagnosed at early stage with a favorable prognosis. The most deadly gynecological cancer is epithelial ovarian cancer (OC), which has a 5-year survival rate of approximately 40%. This is mainly a result of diagnosis at advanced stage of disease due to lack of early symptoms and screening methods (1,2). EC, CXCA, and OC can be differentiated into several histological subtypes with variable clinicopathological characteristics and course of disease (2,3). Furthermore, the ovaries are a common site of metastases from multiple primary locations (4). These metastases require different treatment strategies compared to OC (5). Proper diagnostics to differentiate subtypes and primary from metastatic disease are essential to select patients for the right type of treatment in order to improve outcome in these patients.

Since effective therapeutic strategies for these types of cancers are limited, new therapeutic approaches are being developed to improve outcome. Targeted antibody therapy in which an immune response is evoked towards a tumor target causing antibody-dependent cell cytoxicity (ADCC) or complement-dependent cytotoxicity (CDC)-directed effects has great potential in this regard. The search for specific tumor-associated antigens expressed at the cell surface to use for this approach is therefore highly warranted. A group of proteins that is of interest as targetable tumor antigen is the claudin (CLDN) family of proteins. CLDNs are essential components for the formation of tight junctions expressed at the cell surface and as such, have crucial roles in the control of paracellular transport and maintenance of cell polarity (6,7). The 27 family members of the CLDN family have a tightly regulated expression pattern (8,9). Gene and protein expression profiling has revealed differential expression patterns between normal and cancerous tissue for several CLDNs (10–13).

One of the CLDNs that has been shown to be upregulated in certain cancer types is CLDN18, which has two splice variants; CLDN18.1 and CLDN18.2 (14). While in healthy normal tissues CLDN18.2 is only expressed on short-lived differentiated gastric epithelial cells, gene profiling and protein expression studies have revealed its expression in various tumors (10,15). Since CLDN18.2 is a surface protein, it may serve as a perfect target for directed therapy strategies. IMAB362, a tumor cell-selective therapeutic antibody directed against CLDN18.2, is currently analyzed in clinical trial as a single agent (clinicaltrials.gov: NCT00909025 and NCT01197885), or combined with standard chemotherapy (NCT01630083).

Because of its overexpression in various tumor types and the fact that it has targeting potential without expected off-target effects due to its restricted expression, CLDN18.2 might represent an interesting target in gynecological malignancies as well. In fact, previous work suggests expression in gynecological malignancies
Therefore, we screened large, well-defined datasets of gynecological malignancies for the expression of CLDN18.2, to analyze its potential as a therapeutic and diagnostic marker.
Methods

Patient set
Five cohorts of CXCA, EC, mucinous OC, serous OC, and a cohort of gastrointestinal ovarian metastases were analyzed for expression of CLDN18.2. All tumors were classified by a gynecologic pathologist and subsequently graded and staged according to World Health Organization (WHO) criteria and International Federation of Gynecology and Obstetrics (FIGO) guidelines.

Cervical cancer
The cervical cancer cohort consisted of 318 patients diagnosed with early stage cervical carcinomas in the University Medical Center Groningen (UMCG) between January 1981 and December 2006. 69.9% of patients had a planocellular carcinoma, with the remaining 30.1% presenting with adenocarcinoma. 33.5% of tumors were of differentiation grade 3. Most patients received cytoreductive surgery as primary treatment, which was in 50% of patients supplemented with radio- and/or chemotherapy. A minority of patients (22.5%) presented with recurrences after treatment.

Endometrial cancer
The EC cohort consisted of 359 patients diagnosed at the UMCG between January 1980 and December 2004. A gynecologic pathologist determined histological subtype post-operatively. Most patients had a tumor of the adenocarcinoma subtype (84.1%), 18 patients had a serous tumor and 26 a tumor of clear cell subtype. 71.5% had early stage disease, and 28.3% of patients presented with high grade (G3) disease. Most patients received surgical treatment, which was often (55.4%) supplemented with radiotherapy and in 2.5% of patients with adjuvant chemotherapy.

Serous ovarian cancer
The serous ovarian cancer (sOC) cohort consisted of patients that had received surgery (Primary Surgery (PS) cohort; n=107) or neo-adjuvant chemotherapy (NACT cohort; n=115) as the primary treatment strategy. All patients were diagnosed in the University Medical Center Groningen (UMCG) between January 2000 and December 2012. Patients that underwent PS subsequently received six cycles of platinum-based chemotherapeutic regimen with or without paclitaxel. Patients that were selected for NACT first received three cycles of chemotherapy, followed by interval cytoreduction and three more cycles of chemotherapy. The PS cohort consisted of 107 patients of which the majority (63.6%) of patients had stage IIIC disease. Most tumors (76.6%) were of high grade (G3). In the NACT cohort all tumors were clinically staged as advanced stage, determination of the differentiation grade of tumors after chemotherapeutic treatment can be considered difficult and
not directly comparable to grading of primary tumor samples. As a result, from 16 patients grade could not be determined; of the remaining most tumors (67.8%) were of high grade (G3).

**Mucinous ovarian cancer**

The mucinous ovarian carcinoma (mOC) cohort was created on basis of inclusion of those patients diagnosed with mucinous ovarian carcinoma at the UMCG between January 1985 and December 2010. All 43 patients received primary surgery, in cases of more advanced stage disease followed by platinum-based chemotherapy, which was combined with taxanes from 1995 onwards. Most patients presented with early stage disease (55.8%, FIGO stage IA), and 39.3% of patients had a low grade tumor.

**Ovarian metastases**

Metastases were selected if primary location of the tumor was confirmed by a pathologist through histological analysis of surgically obtained tissue from the primary location or when this location was confirmed by diagnostic imaging. The cohort consisted of a total of 43 patients with tumors that had histological confirmed primary localization in the pancreas (n=4), stomach (n=2), ileum (n=3), appendix (n=4), and from colorectal origin (n=18). The majority of these tumors were adenocarcinomas.

**Ethical review**

For all cohorts, patient data were retrieved from the institutional database into a new anonymous database, in which patient identity was protected by unique patient codes. According to Dutch law no approval from our institutional review board was needed.

**Tissue microarrays (TMA)**

TMAs were constructed as described previously (16–19). In brief, each patient was selected for inclusion on the TMA if enough formalin-fixed paraffin-embedded (FFPE) tumor tissue was available. A gynecologic pathologist selected representative areas from each tissue block on basis of H&E staining and cores of either 0.6 mm (mOC, EC, cervical, metastases) or 1.0 mm (sOC) diameter were taken from selected regions of each paraffin block and placed into a recipient block using a microarrayer (Beecher Instruments, Silver Spring, USA). From each TMA block, sections of 4 µm were cut and applied to APES-coated slides (Starfrost, Braunschweig, Germany). The presence of tumor in the arrayed samples was confirmed by H&E staining.
CLDN18.2 immunohistochemical (IHC) staining

Slides were deparaffinized in xylene and rehydrated through an alcohol gradient. Antigen retrieval was performed by boiling the slides for 15 minutes in a Tris-EDTA buffer (pH 9.0) containing 15 mM NaN$_3$. After cooling down the slides were quenched for endogenous peroxidase in a 3.0% H$_2$O$_2$ in 15.0 mM NaN$_3$ for 5 minutes. Slides were then incubated with a monoclonal antibody directed against human CLDN18.2 (clone: 43-14A mono, 0.8 µg/ml) for 30 minutes at RT. Subsequently, antibody binding was visualized by use of the visualization Reagent CLAU-DETCT18.2 ready-to-use for 30 minutes at room temperature (RT) followed by incubation with the substrate 3,3′-diaminobenzidine (DAB) for 5 minutes. Counterstaining was performed with Mayer’ hematoxylin for 2 minutes, after which slides were dehydrated and mounted.

Immunohistochemistry for CK7, CK20, CA125, CEA, β-catenin, CDX2 and DPC4

The expression patterns on tumors of cytokeratin-7 (CK7), cytokeratin-20 (CK20), carcinoembryonic antigen (CEA), CA125, β-catenin, CDX2, and DPC4 were routinely stained by IHC by use of a Ventana Benchmark Immunostainer (Ventana, Tucson, USA). Primary antibodies and concentrations used are depicted in Supplementary Table 1. The staining of CDX2 and DPC4 was performed manually. After deparaffinization and rehydration, slides were subjected to heat-induced antigen retrieval in an EDTA (pH=8.0) or citrate (pH=6.0) buffer for CDX2 and DPC4, respectively. Endogenous peroxidase was blocked in a 0.3% H$_2$O$_2$ solution for 30 minutes. Slides were incubated for 1 hour with primary mouse antibodies anti-human CDX2 (1:50, clone DAK-CDX2, DAKO, Heverlee, Belgium) and anti-human DPC4 (1:100, clone: B8, Santa Cruz Biotechnology, Santa Cruz, USA) diluted in PBS/1% BSA. The antibodies were detected using HRP-labeled secondary (rabbit anti-mouse) and tertiary (goat anti-rabbit) antibodies for 30 minutes at RT (1:100, DAKO), and visualized with 3,3-diaminobenzidine. Counterstaining was performed by hematoxylin after which slides were dehydrated and mounted.

Scoring and statistical analyses

For all markers, a tumor was considered positive for that marker if at least 5% of tumor cells expressed the marker. Slides were scanned and analyzed for CLDN18.2 expression by two individuals without prior knowledge of patient characteristics. Scoring for CK7, CK20, CA125, CEA, β-catenin, CDX2 and DPC4 was performed by a gynecological pathologist. Cytoplasmic staining of CK7, CK20, CEA and DPC4 was evaluated, while for β-catenin, CDX2, and DPC4 nuclear staining was considered specific, as well as membranous expression of CA125. Data analysis was performed by use of IBM SPSS version 22 (SPSS Inc., Chicago, USA). A p-value <0.05 was considered significant and all tests were performed two-tailed. Differences of CLDN18.2 expression between different patient characteristics and tumor types were assessed by use of χ$^2$ tests.
Results

Expression of CLDN18.2 in gynecological malignancies

CLDN18.2 expression was detected by use of immunohistochemistry on tissue microarrays containing tumor samples of patients with gynecological malignancies. Patient characteristics of these datasets are depicted in Table 1 and 2, Supplementary Tables 2, 3, and 4. Expression of CLDN18.2 was not found on any of the serous ovarian cancer samples before or after chemotherapeutic treatment, and only 4 of 324 (1.2%) cervical and 3 of 379 (0.8%) of endometrial cancer samples expressed this marker. Fig. 1A shows a representative image of a positively stained mucinous ovarian tumor. In this cohort most tumors demonstrated membranous CLDN18.2 staining (76.7%; Fig. 1B, Table 1). All samples with at least 5% of tumor cells showing CLDN18.2 expression were considered positive. Most cells demonstrated a high intensity varying between 5-100% of cells having any expression. 69.8% of patients showed at least 70% of tumor cells positive for CLDN18.2.

Furthermore, a cohort with samples from metastases found in the ovary that were of confirmed gastrointestinal origin was analyzed for expression of CLDN18.2. Fig. 1C shows a representative image of a negative colorectal metastasis. Most metastases demonstrated to be negative for this protein (77.4%, Fig. 1D, Table 2). Out of a total of 31 samples, one colorectal, one stomach and one appendix originating sample showed positivity (Table 2). All 4 samples from the pancreas as the primary site showed expression of CLDN18.2.

Expression of diagnostic markers in primary mucinous versus metastatic ovarian tumors

The described expression pattern led us to hypothesize that CLDN18.2 might be of great use to differentiate primary mucinous ovarian tumors from gastrointestinal metastases found in the ovary. To determine its potential as a diagnostic marker, the expression of CLDN18.2 in these datasets was compared with that of markers that are currently used, or are being under consideration as diagnostic tools for this purpose. We analyzed the expression patterns on tumors of CK7, CK20, CEA, CA125, β-catenin, CDX2, and DPC4 by IHC in both cohorts. Tumors were considered positive when expression was visible in at least 5% of tumor cells. Staining results of all markers are depicted in Table 3. Highly expressed on primary mucinous OC tumors were CK7 (100%), DPC4 (97.6%), and CLDN18.2 (78.6%). Most metastases showed expression of CDX2 (96.8%), CK20 (93.5%), and CEA (77.4%). None of the markers stained specifically a single tumor type. In fact, most markers showed rather high positivity in both tumor types. For instance, CK20 was very highly expressed in metastases (93.3%), but also in primary mucinous tumors (64.3%). None of the highly expressed markers in metastases had a low expression rate in primary mucinous. On the other hand, CK7 and CLDN18.2 showed a high expression in primary tumors, but relatively low expression in metastases.
Diagnostic potential of IHC markers

To further analyze the diagnostic potential of all markers, we determined their sensitivity, specificity, predictive values, and likelihood ratios for predicting primary mucinous OC tumors. The obtained results for all markers are depicted in Table 4. While DPC4 and CK20 showed a high sensitivity, also a high percentage of metastases displayed expression and therefore specificity was low (32.3% and 6.5%, respectively). CA125 and β-catenin expression was highly specific, but sensitivity was low. On the other hand, CK7 staining represented a high sensitivity (100%) as well as CLDN18.2 (78.6%), and both had a relatively high specificity (64.5% and 77.4%, respectively). Therefore, these markers are of interest for diagnostic purposes.

Table 1: Patient characteristics mucinous tumors

<table>
<thead>
<tr>
<th>Grade</th>
<th>All patients N (%)</th>
<th>CLDN18.2+ N (%)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>17 (39.5)</td>
<td>15 (93.8)</td>
</tr>
<tr>
<td>2</td>
<td>16 (37.2)</td>
<td>14 (87.5)</td>
</tr>
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<td>3</td>
<td>8 (18.6)</td>
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<td>2 (4.7)</td>
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FIGO stage

<table>
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<th>FIGO stage</th>
<th>All patients N (%)</th>
<th>CLDN18.2+ N (%)</th>
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<tr>
<td>IA</td>
<td>24 (55.8)</td>
<td>21 (91.3)</td>
</tr>
<tr>
<td>IC</td>
<td>8 (18.6)</td>
<td>8 (100)</td>
</tr>
<tr>
<td>IIC</td>
<td>1 (2.3)</td>
<td>1 (100)</td>
</tr>
<tr>
<td>IIIC</td>
<td>9 (20.9)</td>
<td>3 (33.3)</td>
</tr>
<tr>
<td>IV</td>
<td>1 (2.3)</td>
<td>0 (0)</td>
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</table>

Chemotherapy

<table>
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<th>Chemotherapy</th>
<th>All patients N (%)</th>
<th>CLDN18.2+ N (%)</th>
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</thead>
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<tr>
<td>platinum-containing</td>
<td>18 (41.9)</td>
<td>10 (55.6)</td>
</tr>
<tr>
<td>other</td>
<td>2 (4.7)</td>
<td>1 (50.0)</td>
</tr>
<tr>
<td>no chemotherapy</td>
<td>21 (48.8)</td>
<td>20 (95.2)</td>
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<td>missing</td>
<td>2 (4.7)</td>
<td>-</td>
</tr>
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</table>

Table 2: Patient characteristics ovarian metastases

<table>
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<tr>
<th>Primary tumor</th>
<th>Colorectal N=18</th>
<th>Ileum N=3</th>
<th>Stomach N=2</th>
<th>Appendix N=4</th>
<th>Pancreas N=4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histological subtype</td>
<td>Adenocarcinoma</td>
<td>16</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Mucinous adenocarcinoma</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Carcinoid</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Grade</td>
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<td>1</td>
<td>0</td>
<td>0</td>
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<tr>
<td>2</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
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<tr>
<td>missing</td>
<td>11</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>

| CLDN18.2 | Positive | 1 | 0 | 1 | 1 | 4 |
| Negative | 17 | 3 | 1 | 3 | 0 |
Figure 1: Staining pattern of Claudin18.2 in mucinous ovarian cancer and intestinal metastases in the ovary. A) Representative image of a CLDN18.2-expressing mucinous ovarian tumor sample. B) Bar graph depicting the percentage of CLDN18.2 positive tumors in mucinous ovarian cohort. C) A representative image of a colorectal (CRC) metastasis in the ovary, stained for CLDN18.2. D) Bar graph depicting the percentage of CLDN18.2 positive tumors in cohort consisting of ovarian metastasis from intestinal primary origin.

Table 3: Expression of predictive markers in primary mucinous OC and gastrointestinal metastases found in ovary cohort

<table>
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<tr>
<th>Markers</th>
<th>OC %</th>
<th>All %</th>
<th>colorectal %</th>
<th>ileum %</th>
<th>stomach %</th>
<th>pancreas %</th>
<th>appendix %</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLDN18.2+</td>
<td>33</td>
<td>78.6</td>
<td>7 22.6</td>
<td>1 5.6</td>
<td>0 0</td>
<td>1 50.0</td>
<td>4 100</td>
</tr>
<tr>
<td>CK7+</td>
<td>42</td>
<td>100</td>
<td>11 35.5</td>
<td>2 11.1</td>
<td>1 33.3</td>
<td>2 100</td>
<td>4 100</td>
</tr>
<tr>
<td>CK20+</td>
<td>27</td>
<td>64.3</td>
<td>29 93.3</td>
<td>18 100</td>
<td>1 33.3</td>
<td>2 100</td>
<td>4 100</td>
</tr>
<tr>
<td>CEA+</td>
<td>22</td>
<td>52.4</td>
<td>24 77.4</td>
<td>16 88.9</td>
<td>1 33.3</td>
<td>1 50.0</td>
<td>3 75.0</td>
</tr>
<tr>
<td>CA125+</td>
<td>10</td>
<td>25.0</td>
<td>1 3.2</td>
<td>1 5.6</td>
<td>0 0</td>
<td>0 0</td>
<td>0 0</td>
</tr>
<tr>
<td>DPC4+</td>
<td>41</td>
<td>97.6</td>
<td>21 67.7</td>
<td>12 66.7</td>
<td>1 33.3</td>
<td>2 100</td>
<td>3 75.0</td>
</tr>
<tr>
<td>β-catenin+</td>
<td>0</td>
<td>0</td>
<td>5 16.1</td>
<td>5 27.8</td>
<td>0 0</td>
<td>0 0</td>
<td>0 0</td>
</tr>
<tr>
<td>CDX2+</td>
<td>21</td>
<td>50.0</td>
<td>30 96.8</td>
<td>18 100</td>
<td>2 66.7</td>
<td>2 100</td>
<td>4 100</td>
</tr>
</tbody>
</table>

In order to further improve prediction of tumor type using immunohistochemical markers, use of combinations were analyzed for predictive value. Combination of the two markers, with both markers expressed, of the highly sensitive markers CK7 and DPC4 resulted in a sensitivity of 97.6% and specificity of 77.4% for prediction of primary mucinous tumor. A higher specificity could be achieved when combining CK7 with CLDN18.2 (83.9%), at the expense of a slight reduction in sensitivity (78.6%). Also the combination of CLDN18.2 and DPC4 showed to be a good predictor for primary mucinous with high sensitivity and specificity (Table 4). Predictive values significantly depend on the prevalence of the disease in the population tested. In order to correct for this, likelihood ratios (LR) can be determined. In general, a positive likelihood ratio (LR+) value of above five and a LR- near to zero can be considered a good predictor to confirm a specific disease. The combination of CLDN18.2, CK7, and DPC4 attained the highest LR+. These markers together predicted primary mucinous ovarian tumors with a sensitivity of 76.0%, specificity of 87.1%, high positive and negative predictive values and a LR+ of 5.90 and LR- of 0.27.

Therefore, combinations of the markers CK7, CLDN18.2, and DPC4 can be used as powerful tools to differentiate mucinous ovarian tumors from gastrointestinal metastases. Differentiation between mucinous ovarian tumors and metastases with pancreas as the primary site showed to be most challenging, since for all markers that were highly predictive for mucinous OC (CLDN18.2, CK7 and CK20) the 4 pancreatic samples were positively stained as well.

Table 4: Predictive value of markers differentiating primary mucinous from gastrointestinal metastases in the ovary

<table>
<thead>
<tr>
<th>markers</th>
<th>sensitivity</th>
<th>specificity</th>
<th>PPV</th>
<th>NPV</th>
<th>LR+</th>
<th>LR-</th>
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<tr>
<td>CLDN18.2+</td>
<td>78.6</td>
<td>77.4</td>
<td>0.83</td>
<td>0.73</td>
<td>3.49</td>
<td>0.28</td>
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<tr>
<td>CK7+</td>
<td>100</td>
<td>64.5</td>
<td>0.79</td>
<td>1.00</td>
<td>2.82</td>
<td>0.0</td>
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<td>CK20+</td>
<td>64.3</td>
<td>6.5</td>
<td>0.48</td>
<td>0.12</td>
<td>0.6</td>
<td>5.49</td>
</tr>
<tr>
<td>CEA+</td>
<td>52.4</td>
<td>22.6</td>
<td>0.48</td>
<td>0.26</td>
<td>0.12</td>
<td>2.11</td>
</tr>
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<td>CA125+</td>
<td>25.0</td>
<td>96.8</td>
<td>0.91</td>
<td>0.50</td>
<td>7.81</td>
<td>0.77</td>
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<tr>
<td>DPC4+</td>
<td>97.6</td>
<td>32.3</td>
<td>0.66</td>
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<td>0.66</td>
<td>0.07</td>
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<tr>
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<td>0.00</td>
<td>0.38</td>
<td>0.00</td>
<td>1.19</td>
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<tr>
<td>CDX2+</td>
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<td>3.2</td>
<td>0.41</td>
<td>0.05</td>
<td>0.52</td>
<td>15.63</td>
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<td>64.5</td>
<td>0.71</td>
<td>0.57</td>
<td>1.81</td>
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<td>CK7+ &amp; DPC4+</td>
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<td>77.4</td>
<td>0.85</td>
<td>0.96</td>
<td>4.32</td>
<td>0.03</td>
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<td>100.0</td>
<td>1.00</td>
<td>0.51</td>
<td>=</td>
<td>0.75</td>
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<td>83.9</td>
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<td>0.74</td>
<td>4.87</td>
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<td>77.4</td>
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<td>0.57</td>
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<td>0.55</td>
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<td>4.72</td>
<td>0.28</td>
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<td>12.5</td>
<td>96.8</td>
<td>0.83</td>
<td>0.46</td>
<td>3.88</td>
<td>0.90</td>
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<tr>
<td>CLDN18.2+ &amp; CK7+ &amp; DPC4+</td>
<td>76.2</td>
<td>87.1</td>
<td>0.89</td>
<td>0.73</td>
<td>5.90</td>
<td>0.27</td>
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</table>

Discussion

CLDN18.2 is a tight junction protein which is found to be upregulated in various solid tumors (10,15). Here, we analyzed the expression pattern of this surface protein in gynecological tumors in order to define its diagnostic and therapeutic potential. Confirming previous results, we show that mucinous ovarian cancers often express CLDN18.2 (10). This tumor type therefore represents an interesting target for antibody treatment directed against CLDN18.2. This splice variant of CLDN18 is a tissue-restricted marker, for which expression in healthy tissue is only seen in short-lived differentiated cells of the gastric mucosa (10,14). Therefore, antibody treatment targeting this protein will likely not cause major off-target effects. The antibody IMAB362 has, in vitro, been shown to induce ADCC, CDC and direct apoptosis and growth inhibition of several tumor cell lines (personal communication) and is currently analyzed in clinical trials for various tumor types. The results presented here provide rationale to extend these trials with mucinous ovarian cancer patients.

Epithelial ovarian cancer (EOC) entails several histological subtypes, including endometrioid, clear cell, high grade serous, and mucinous carcinomas. The mucinous counterpart represents only 2-5% of all EOC and has a relative good prognosis due to the fact that most mucinous tumors are diagnosed at early stage (2,4). However, women with advanced stage have significantly worse survival as compared to the other histological subtypes (20). These tumors may be misdiagnosed metastases from gastrointestinal primary sites. Diagnostic differentiation of these tumors from primary mucinous ovarian tumors is difficult since symptoms and clinical features are similar. Importantly, the correct diagnosis is crucial for subsequent choice of treatment. Platinum-based chemotherapy supplemented with taxanes, as given to ovarian tumors, is certainly not the first choice of treatment in gastrointestinal malignancies (5). Misdiagnosis may lead to wrong treatment choice with risk of side effects, and delayed proper treatment may affect prognosis of the patient. In general, primary mucinous tumors are larger and usually manifest unilateral, while ovarian metastases are smaller in size and bilateral (4). The combination of these two characteristics may therefore be predictive, however, also with this algorithm a high percentage is still incorrectly predicted, especially for gastrointestinal metastases. Thus, there is an urgent demand for immunohistochemical markers that can differentiate the tumor entities in a fast, easy and cheap approach.

While the expression of CLDN18.2 was abundant in primary mucinous tumors, only 22.6% of all analyzed gastrointestinal metastases expressed this protein. Primary pancreatic tumors as well as metastases in the liver and lymph nodes were previously found to express CLDN18.2 (15). Here, we found all analyzed ovarian metastases from pancreatic origin (N=4) to be CLDN18.2 positive. A high percentage of gastric tumors was previously found to have overexpression of CLDN18.2 as well (10). Here, only two of the metastatic tumors were of gastric origin, of which one showed CLDN18.2 expression. A study in a larger cohort will have to reveal what the
The immune environment in ovarian cancer

The percentage of gastric metastases is that expresses this protein.

To further analyze the diagnostic potential of CLDN18.2, we determined the expression pattern of currently used markers and some markers that are under consideration for diagnostic purposes. Confirming previous data, the expression of CK7 was high in mucinous tumors, while metastases showed a rather low expression (21). The combination of the markers CK7 and CK20 is highly predictive for ovarian cancer in general, with a CK7+CK20- profile. However, primary mucinous tumors often express CK20 (22,23), which was confirmed in the current study. Therefore, other markers that can be used in combination with CK7 which are specific for mucinous ovarian tumors are warranted. Interestingly, combining CLDN18.2 with CK7 improved specificity of diagnosis to 83.9%. Addition of DPC4 improved specificity further as well as the positive likelihood ratios of correct prediction of a case of primary mucinous ovarian cancer. DPC4 is considered an interesting marker for differentiation since it was previously found to be downregulated in a high percentage of pancreatic cancer metastases (21,24). Here, we analyzed 4 samples of pancreatic metastases, and one had lost DPC4 expression. In a larger population a higher percentage of DPC4 loss might be found in these types of tumors, improving the predictive value of the combination CK7, CLDN18.2 and DPC4. While CA125 is expressed on many ovarian tumors in general, mucinous tumors are only partially positive for this marker (25), which was also found in this study (10/40 samples positive). Therefore, sensitivity for detection of mucinous OC samples is low. On the other hand, CEA and CDX2 are specific markers for colorectal samples, and were found to be expressed only in few patients with ovarian cancer. In primary mucinous samples, however, we and others found abundant expression of these markers (21,26,27).

For all markers that were highly predictive for mucinous OC (CLDN18.2, CK7, and CK20) the 4 pancreatic samples were also positively stained. Therefore, it will be of interest to add a marker that is highly predictive for pancreatic tumors, and is not expressed by ovarian tumors. A weakness of this study is the relatively small number of patients with metastatic disease to the ovary included in the analyses. The studied population with metastases was extensively analyzed, with primary site proven by histologic analysis of surgical obtained tissue or diagnostic imaging of the primary site. It will be of interest to validate the value of CLDN18.2 staining for diagnostic purposes in a large independent cohort. Inclusion of more pancreatic and gastric metastases is important in this regard since these might be most difficult to differentiate with the current set of markers.

In conclusion, CLDN18.2 is an interesting marker for primary mucinous ovarian tumors. In our cohort 78.6% of tumors showed expression of this protein, providing rationale for targeted therapy. Furthermore, we have shown that CLDN18.2 can be used in the diagnosis of primary mucinous ovarian tumors, which can differentiate this subtype from metastases from gastrointestinal origin.
References

21. Lagendijk JH, Mullink H, Van Diest PJ, Meijer GA, Meijer CJ. Tracing the origin of adenocarcinomas with unknown primary using immunohistochemistry: differential diagnosis between colonic...

Supplementary data

Supplementary Table 1: Antibodies for immunohistochemistry Ventana Benchmark Immunostainer

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Company</th>
<th>Dilution</th>
<th>Pretreatment</th>
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<tr>
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<td>DAKO</td>
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<td>12M Protease</td>
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<td>CK20</td>
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<td>DAKO</td>
<td>1:100</td>
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<tr>
<td>CEA</td>
<td>Col-1</td>
<td>Zymed</td>
<td>1:50</td>
<td>-</td>
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<tr>
<td>CA125</td>
<td>M11</td>
<td>DAKO</td>
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<td>Ultra CC1 (Ventana)</td>
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<td>B-Catenin</td>
<td>14/Beta-catenin</td>
<td>BD Transduction</td>
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Supplementary Table 2: patient characteristics serous ovarian cancer dataset

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<td>N (%)</td>
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<td>2</td>
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<td>3</td>
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<td>FIGO stage</td>
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<td>IIB</td>
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<td>0 (0)</td>
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<tr>
<td>IIIC</td>
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<tr>
<td>complete (no residual tissue)</td>
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<td>44 (38.3)</td>
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<td>optimal (&lt;1cm residual tissue)</td>
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<td>incomplete (&gt;1cm residual tissue)</td>
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PS: primary surgery. NACT: neo-adjuvant chemotherapy
### Supplementary Table 3: patient characteristics endometrial dataset

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### Supplementary Table 4: Patient characteristics cervical cancer dataset

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<td>IA2</td>
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