HE4: Clinical applications in gynaecological cancer
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HE4 immunostaining of malignant ascites differentiates cancer of Mullerian origin from gastrointestinal cancer

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Abstract

**Background** An accurate diagnosis of epithelial ovarian cancer (EOC) is required before initiation of treatment. An overlap in clinical presentation and cytological, histological or imaging studies, with other non-gynaecological tumors does occur. Therefore, immunocytochemistry markers are used to determine tumor origin. HE4 is overexpressed in tissue of EOC. It has shown to be a sensitive and specific serum marker for EOC and to be of value for the differentiation between EOC and ovarian metastases of gastrointestinal origin.

**Aim** To evaluate HE4 immunocytochemistry in malignant ascites for the differentiation between EOC and adenocarcinomas of the gastrointestinal tract.

**Methods** Cytologic specimens of 115 different adenocarcinomas (EOC (n=45), gastric (n=46) and colorectal cancer (n=24)) were stained with HE4, PAX8 and other specific markers.

**Results** The ascites samples from patients with EOC stained HE4 and PAX8 in 91% for both. The four samples without HE4 staining were a clear cell carcinoma, a low grade serous adenocarcinoma, an undifferentiated adenocarcinoma and a neuroendocrine carcinoma. All high grade serous adenocarcinomas (n=37, 100%) stained with HE4, compared with 94% positivity for PAX8. In gastric or colorectal cancer 24% and 21% respectively stained with HE4. No PAX8 staining was seen in colorectal or gastric adenocarcinoma.

**Conclusion** HE4 staining in ascites is feasible and has a high sensitivity for high grade serous ovarian cancer. HE4 is a useful addition to the current panel of immunocytochemistry markers for the diagnosis of EOC and for the differentiation with gastrointestinal adenocarcinomas.
Introduction

An accurate initial diagnosis of epithelial ovarian cancer (EOC) is necessary for tumor staging and determination of optimal treatment strategy. The majority of ovarian cancer patients present with nonspecific abdominal symptoms and is therefore not diagnosed until the disease has extended to the upper abdomen or spread beyond the abdominal cavity.(1,2) The high mortality rate of patients with EOC is a consequence of this late manifestation of the disease. In advanced stage disease EOC metastasizes to the abdominal cavity and causes malignant peritoneal and/or pleural effusions. Performing a paracentesis of ascites to obtain a sample for diagnosis is a common and easy procedure. Immunohistochemistry (IHC) or immunocytochemical analysis is helpful in the differential diagnosis between mesothelial cells and a variety of tumor types, and often involves the use of a panel of antibodies instead of individual markers (3); Ber-Ep4 (usually adenocarcinomas and glandular epithelium), Wilms tumor gene-1 (WT1) (high grade serous adenocarcinoma, mesothelium), estrogen receptor (ER) (gynaecological, breast), cytokeratin (CK)7 (gynecological, upper gastrointestinal, lung, mesothelial cells), CK20 (lower gastrointestinal, mucinous adenocarcinoma) and CDX2 (lower and upper gastrointestinal, mucinous adenocarcinoma).(4,5) Other IHC markers that are commonly used are Cancer Antigen-125 (CA125) which mainly identifies epithelium of gynecological origin while carcinoembryonic antigen (CEA) mainly identifies epithelium of gastrointestinal origin. However, both are too nonspecific for an accurate diagnosis as a single marker. A relatively new marker is PAX8, a member of the pair box family of tissue-specific transcription factor genes. Expression of PAX8 is present in the female genital tract derived from the Müllerian ducts.(6) This supports the recent believe that EOC originates from the fallopian tubes instead of from the ovarian surface epithelium.(7) PAX8 can be used to distinguish gynecologic cancers from non-Müllerian malignancies including cancer of gastrointestinal origin and from mesothelial cells. (8,9) Recently, Human Epididymis protein 4 (HE4) was found to be overexpressed in tissue of EOC.(10) The gene encoding for HE4, WFDC2, is located on chromosome 20q12-13.(11) In normal human tissue HE4 expression is limited to the epithelia of the (uro)genital and respiratory tract in both men and women.(12,13) Besides EOC, some endometrial carcinomas, breast cancers and pulmonary adenocarcinomas have shown HE4 expression. Adenocarcinomas of the gastrointestinal tract show HE4 expression less commonly.(13) Over the last years, HE4 has developed into a relatively specific and sensitive serum marker for EOC. (14,15)

HE4 has never been evaluated in ascites before. Therefore, we investigated whether immunostaining with HE4 in ascites is feasible and could facilitate the differentiation between EOC and adenocarcinomas of gastrointestinal origin.
Materials and methods

Collection of samples and data
Paraffin embedded cell blocks of cytology specimen of patients with EOC or with gastric or colorectal cancer were obtained from the biobank of the Netherlands Cancer Institute (NKI-AVL) following approval of the Institutional Review Board (IRB). In case of gastric and colorectal cancer both men and women were included. Relevant clinical data were collected for all patients and definitive histological diagnosis and tumor subtype were obtained from the pathology reports. In case of doubt, review of the original Giemsa and hematoxylin and eosin (H&E) slides was performed by a dedicated gynecologic pathologist (KV).
Serous adenocarcinomas were divided into low grade and high grade, while mucinous adenocarcinomas were divided into low, moderately of highly differentiated. All specimens were handled in a coded fashion as prescribed by the Dutch national guidelines for secondary use of specimens (“Human tissue and Medical Research: Code of conduct for responsible use”)

Immunohistochemistry
PAX8 and HE4 staining were performed on all slides. HE4 antibody was kindly provided by Fujirebio Diagnostics, Inc. Formalin-fixed and paraffin tissue slides of resection specimen of EOC were used for testing and as control. Immunohistochemistry of samples was performed on a BenchMark Ultra autostainer (Ventana Medical Systems). Briefly, paraffin sections were cut at 3 µm, heated at 75°C for 28 minutes and deparaffinised in the instrument with EZ prep solution (Ventana Medical Systems). Heat-induced antigen retrieval was carried out using Cell Conditioning 1 (CC1, Ventana Medical Systems) for 32 minutes at 95°C (WT1, Cytokeratin 20, cytokeratin 7, CA125, CEA and CDX2) or 64 minutes at 95°C (PAX8, HE4).
WT1 was detected using clone 6F-H2 (1:50 dilution, 32 minutes at 37°C, DAKO), Cytokeratin 20 using a polyclonal antibody (1:6000, 32 minutes at 37°C, ImmunoLogic cat: E16444), PAX8 using clone MRQ-50 (1:100 dilution, 32 minutes at 37°C, Sanbio), HE4 using clone 12A2 (1:2000 dilution, 32 minutes at 36°C, Fujireibo), Cytokeratin 7 using clone OVTL12/30 (1:200 dilution, 32 minutes at 37°C, Monosan), CA125 using clone Ov185:1 (1:40 dilution, 32 minutes at 37°C, Biogenex), CEA using clone Col-1 (1:200 dilution, 32 minutes at 37°C, Genetex), and CDX2 using clone EPR2764Y (1:1600 dilution, 32 minutes at 37°C, Thermo Scientific). For PAX8 and HE4 an additional amplification step (Ventana Medical Systems) was selected in the protocol to amplify this primary antibody, using the OptiView Amplification Kit. Bound antibody was detected using the OptiView DAB Detection Kit (Ventana Medical Systems). Slides were counterstained with Hematoxylin II and Bluing Reagent (Ventana Medical Systems).
Evaluation of immunocytochemistry staining

Evaluation of the slides was performed by two observers (AS/KV) without previous knowledge of the clinical characteristics and the histology report. The percentage of tumor cells staining for HE4 was based on review of the entire cytological section of the cell block. Slides with an extremely low quantity of tumor cells were excluded from this study. Slides with tumor cells that showed HE4 staining were further scored into ‘strong’, ‘moderate’ or ‘weak’. Weak staining in less than 5% of tumor cells was considered as no staining in the final results. The same definitions were applied to the other immunocytochemistry markers (PAX8, WT1, CK7, CK20, ER, CA125, CEA and CDX2).

Results

In total, 123 cell blocks of ascites were collected and stained. After staining, eight cases were excluded because of the absence of tumor cells (n=5) or definitive histological diagnosis (n=3). Of the remaining 115 slides, there were 45 samples from patients with EOC, 46 from patients with gastric cancer and 24 from patients with colorectal cancer. The slides were reviewed based on the diagnostic criteria described above.

The group of EOC mainly consisted of high grade serous adenocarcinomas (n=37, 82%). The most common histological subtype in the gastric cancer group was diffuse type adenocarcinoma (n=38, 83%), and the remaining subtypes were intestinal type adenocarcinoma (n=5, 11%), mixed diffuse/intestinal type adenocarcinoma (n=2, 4%) or mucinous adenocarcinoma (n=1, 2%). The histological subtypes of colon cancers were intestinal type adenocarcinoma (n=8, 33%), adenocarcinoma not otherwise specified (n=9, 38%), mucinous type adenocarcinoma (n=1, 4%) and mixed adenocarcinomas (n=3, 13%). Of three tumors, histological diagnosis could not be confirmed because tumor tissue of the resection specimen was not available for pathology review.

HE4 staining

Results of HE4 staining are summarized in table 1. Ascites from EOC stained HE4 in 91% (n=41) (fig 1a-b) and strong HE4 staining was seen in ≥50% of tumor cells. All high grade serous adenocarcinomas (n=37) stained with HE4 (100%). The four specimens that did not show HE4 staining were a low grade serous adenocarcinoma, a clear cell adenocarcinoma, an undifferentiated adenocarcinoma and a neuroendocrine carcinoma (fig 1c). A mucinous adenocarcinoma stained HE4 as well.
Table 1

HE4 staining in ascites of epithelial ovarian cancer, gastric cancer and colorectal cancer.

<table>
<thead>
<tr>
<th>Diagnosis of primary tumor</th>
<th>No. of cases (n=115)</th>
<th>HE4 staining</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Negative</td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Moderate</td>
<td>Strong</td>
<td></td>
</tr>
<tr>
<td>Ovarian</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High grade serous</td>
<td>37</td>
<td>0</td>
<td>4</td>
<td>33</td>
</tr>
<tr>
<td>Low grade serous</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Mucinous carcinoma</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Clear cell carcinoma</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Undifferentiated carcinoma</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Neuroendocrine carcinoma</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>4 (9%)</td>
<td>41 (91%)</td>
<td></td>
</tr>
<tr>
<td>Gastric</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diffuse type carcinoma</td>
<td>38</td>
<td>29</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Intestinal type carcinoma</td>
<td>5</td>
<td>4</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Mucinous type carcinoma</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Mixed type carcinoma</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>46</td>
<td>35 (76%)</td>
<td>11 (24%)</td>
<td></td>
</tr>
<tr>
<td>Colorectal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intestinal type carcinoma</td>
<td>8</td>
<td>6</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Mucinous type carcinoma</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Mixed type carcinoma</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Adenocarcinoma NOS</td>
<td>9</td>
<td>8</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Unknown</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>19 (79%)</td>
<td>5 (21%)</td>
<td></td>
</tr>
</tbody>
</table>

Negative: no HE4 staining observed or <5% of tumor cells with weak immunoreaction

The ascites samples from patients with gastric cancer stained in 24% (n=11) with HE4 (fig 2a). Among these were 9 diffuse type adenocarcinomas, one intestinal type adenocarcinoma and one mucinous type adenocarcinoma. Among the 24 specimens from colorectal cancer, HE4 immunostaining was seen in only 5 cases (21%): two intestinal type adenocarcinomas, one mucinous adenocarcinoma, one mixed intestinal/mucinous type adenocarcinoma and one adenocarcinoma not otherwise specified. In the remaining 19 specimens (79%) HE4 immunostaining was negative (fig 2b). Immunoreactivity for HE4 in gastrointestinal cancer specimens was not as strong as in EOC. There was no difference in HE4 staining between gastrointestinal carcinomas in men or women.
**Figure 1**

HE4 staining in ascites of epithelial ovarian cancer (*tumor cells are indicated by arrows*)

**a.** High grade serous adenocarcinoma. Strong HE4 expression

![High grade serous adenocarcinoma](image)

**b.** Low grade serous adenocarcinoma. Moderate HE4 expression

![Low grade serous adenocarcinoma](image)

**c.** Low grade serous adenocarcinoma. No HE4 expression

![Low grade serous adenocarcinoma](image)
Figure 2
HE4 staining in ascites of gastrointestinal cancer (*tumor cells are indicated by arrows*)


b. Intestinal type adenocarcinoma of colon origin. No HE4 expression.
HE4 immunostaining of malignant ascites differentiates cancer of Mullerian origin from gastrointestinal cancer

PAX8 staining
Strong PAX8 immunostaining was seen in 91% (n=41) of ascites from EOC patients and none of the ascites samples from gastrointestinal cancer patients (table 2). 94% of high grade serous adenocarcinomas stained with PAX8. Ascites from a clear cell carcinoma also showed strong PAX8 staining. The four EOC specimens that did not show PAX8 immunostaining were two high grade serous adenocarcinomas, a mucinous adenocarcinoma and a neuroendocrine carcinoma.

Combination of markers
The sensitivity and specificity of HE4 and PAX8 and a combination of markers are listed in table 2. The combination of HE4 and PAX8 staining has a sensitivity of 84% and specificity of 100% for the differential diagnosis between EOC and gastrointestinal carcinomas. Combinations with other markers from the gynecological panel did not further improve the sensitivity or specificity (sensitivity of 93% and specificity of 80%, respectively).

Table 2
Immunostaining results of markers and combinations of markers

<table>
<thead>
<tr>
<th>Marker</th>
<th>EOC cases (n=45)</th>
<th>Gastrointestinal cases (n=70)</th>
<th>Sensitivity for EOC</th>
<th>Specificity for EOC</th>
</tr>
</thead>
<tbody>
<tr>
<td>HE4 +</td>
<td>41 (91%)</td>
<td>16 (23%)</td>
<td>91%</td>
<td>77%</td>
</tr>
<tr>
<td>PAX8 +</td>
<td>41 (91%)</td>
<td>0 (0%)</td>
<td>91%</td>
<td>100%</td>
</tr>
<tr>
<td>Two markers</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HE4+/PAX8+</td>
<td>38 (84%)</td>
<td>0</td>
<td>84%</td>
<td>100%</td>
</tr>
<tr>
<td>HE4+/PAX8-</td>
<td>3 (7%)</td>
<td>16 (23%)</td>
<td>7%</td>
<td>77%</td>
</tr>
<tr>
<td>HE4-/PAX8+</td>
<td>3 (7%)</td>
<td>0 (0%)</td>
<td>7%</td>
<td>100%</td>
</tr>
<tr>
<td>HE4-/PAX8-</td>
<td>1 (2%)</td>
<td>54 (77%)</td>
<td>2%</td>
<td>23%</td>
</tr>
<tr>
<td>Combination of multiple markers</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥1 positive (WT1, ER, CK7)</td>
<td>42 (93%)</td>
<td>14 (20%)</td>
<td>93%</td>
<td>80%</td>
</tr>
<tr>
<td>≥1 positive (CEA, CK20, CDX2)</td>
<td>1 (2%)</td>
<td>62 (89%)</td>
<td>2%</td>
<td>11%</td>
</tr>
</tbody>
</table>

Abbreviations: EOC; epithelial ovarian cancer
Discussion

In the present study, we examined the use of HE4 as immunostaining marker in ascites of patients with EOC and patients with adenocarcinomas of gastrointestinal origin and compared this with PAX8 and other well-known IHC markers. We showed that HE4 staining is strongly present (91%) in ascites of patients with EOC in contrast with HE4 staining in ascites of patients with gastric and colorectal cancer. Furthermore, 100% of the high grade serous adenocarcinomas of the ovary stained with HE4 compared to 94% with PAX8. The sensitivity to differentiate between ascites from EOC and ascites from gastrointestinal cancer was comparable for HE4 and PAX8, while the specificity of HE4 for the total group of samples was lower. Other markers performed less and could be replaced by HE4. As a single marker HE4 is not better than PAX8, but in combination it could be enough in case a (high grade) serous adenocarcinoma is suspected. The amount of markers that are used in panel could thereby be reduced.

The abdominal cavity is the primary site of metastatic disease in EOC and this often results in the presence of ascites. Metastasized gastrointestinal malignancies and other malignancies such as breast cancer can also present with generalized peritonitis with corresponding ascites. The initial diagnosis of EOC is often based on cytology (72%) compared to histology (17%) or clinical factors alone (10%).(16) Confirming the ovarian origin of metastatic disease in ascites specimens can be difficult based on cell morphology only. The use of immunostaining markers contributes to the accuracy of the correct diagnosis of intra-abdominal malignancies before the onset of treatment. The clinical implication of misdiagnosis varies and depends on the primary tumor origin but may have serious consequences: e.g. a patient with a gastrointestinal tumor will be considered for different chemotherapy regimens and for cytoreductive surgery in combination with hyper thermic intraperitoneal chemotherapy (HIPEC). This highlights the importance of the evaluation of new immunocytochemistry markers that can be used to differentiate between EOC and non-ovarian adenocarcinomas.

At this moment, several markers (WT-1, CK7, CK20, ER, CA125, CEA and CDX2) are often used for immunostaining in addition to morphologic features. Recently, PAX8 is added to this panel of IHC markers. Different results of PAX8 are reported, but overall the sensitivity is high (85-100%).(6,9) In the study of Zhao et al. an overall detection rate of 85% was found. Subgroup analysis showed a sensitivity of 85% in serous subtypes (n=52) and 100% in a small subgroup (n=5) of clear cell carcinoma of the ovary.(9) We report a similar sensitivity of PAX8 and HE4 of 91% respectively. However, in a subgroup of high grade serous adenocarcinomas only, HE4 staining was positive in 100% compared to 94% for PAX8. A combination of HE4 and PAX8 could increase the specificity to 100%.
Notable, we observed some staining of WT1 and PAX8 in the reactive cells which has also been observed by others.(6,9) This can be confusing when mesothelial cells or large B-lymphocytic cells have to be distinguished from cancer cells and highlights the importance of using IHC markers in panels and not separately.

The small numbers of some of the histological subtypes that were included in this study, limits the possibility to draw a definitive conclusion about the HE4 expression in EOC other than serous subtypes. Furthermore, we limited this study to the evaluation of ascites of patients with EOC and adenocarcinomas of gastrointestinal origin and did not include other malignancies that can present with peritonitis and ascites (e.g pancreas carcinoma, breast cancer). A previous study that has performed immunostaining on tissue slides of different tumor types showed variable HE4 staining in breast tumors, staining in most pancreatic tumors but none in gastrointestinal tumors.(13) This suggests that for the differentiation between tumor types, immunostaining with HE4 can not be used alone but should be used in combination with other markers such as PAX8.

The most common histological subtype in the gastric cancer group included in this study was diffuse type adenocarcinoma. This is expected because diffuse type adenocarcinomas of gastric origin tend to metastasize to the peritoneal cavity and cause ascites more often than intestinal type gastric carcinomas.(17)

Serum HE4 was not available for all patients because it is no standard diagnostic tool for gastrointestinal tumors. Therefore, we could not correlate serum concentrations with immunostaining results. However, we have shown in a previous study that serum HE4 can be used for the differentiation between EOC and ovarian metastases of gastrointestinal origin.(18) A combination of these two diagnostic possibilities, serum HE4 and HE4 immunostaining, could improve the differentiation of EOC from other adenocarcinomas with or without ovarian metastases. Despite the previously mentioned limitations, this is the first study evaluating immunostaining with HE4 in ascites of patients with EOC and patients with gastrointestinal cancer.

In conclusion, HE4 staining is feasible in ascites and is a useful addition to the current panel of immunocytochemistry markers for the diagnosis of high grade serous ovarian cancer and for the differentiation with gastrointestinal-derived adenocarcinomas.
Conflict of interest

The authors report no conflict of interest for this study.

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