Improving the cytological diagnosis of high-grade serous carcinoma in ascites with a panel of complementary biomarkers in cell blocks

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Introduction: Precise cytological diagnosis of pelvic high-grade serous carcinoma (HGSC) in ascites is important for tumour staging, therapeutic decision-making and prognostic evaluation. However, it can often be difficult to distinguish metastatic HGSC cells from reactive mesothelial cells based on morphology alone. Immunocytochemical analysis of ascites cell blocks has been used to obtain accurate diagnosis and provide a reliable basis for treatment decisions in the clinic. This study was performed to determine whether a panel of antibodies is necessary to achieve high specificity and sensitivity for the identification of HGSC cells.

Methods: Ascites samples from 70 cases (70/253, 27.7%) of histologically confirmed HGSC were postoperatively collected from 2012 to 2015 and were immunocytochemically analysed.

Results: The sensitivity and specificity of Ber-EP4 (a marker of HGSC) for detecting HGSC was 85.7% and 82.1%, respectively, whereas the sensitivity and specificity of HBME-1 for identifying mesothelial cells was 100% and 68.3%, respectively. To improve the rate of detection further of HGSC, 29 cases of ascites were also stained for E-cadherin (a marker of HGSC) and calretinin (a marker of mesothelial cells). The combination of Ber-EP4 and E-cadherin as markers of adenocarcinoma cells increased the sensitivity and specificity for HGSC detection to 100% and 88.9%, respectively. Meanwhile, the sensitivity and specificity for mesothelial cell identification increased to 100% and 90%, respectively, when HBME-1 and calretinin were combined.

Conclusion: This panel of complementary biomarkers is valuable and ideal for the differential diagnosis of HGSC based on ascites cytology.

Keywords
ascites, cytology, pelvic high-grade serous carcinoma, immunocytochemistry, complementary markers
1 INTRODUCTION

Epithelial ovarian cancer (EOC) is the fifth-most common cause of cancer death in women and a common cause of death due to gynecological tumours. High-grade serous carcinoma (HGSC) is the most common subtype encountered in patients with EOC. Being largely asymptomatic, over 70% of patients are diagnosed at an advanced stage of disease (stage III/IV) with metastasis throughout the peritoneal cavity and a substantial amount of ascites. The primary cytology of ascites is an important for diagnosis, therapeutic decision and prognostic prediction. The results of secondary cytology after treatment are also important independent prognostic indicators that are highly correlated with optimal surgery outcomes, recurrence and overall survival rate.

However, an increasing number of studies have shown that the morphological examination of cytological samples is not a highly sensitive diagnostic tool to distinguish metastatic adenocarcinoma from reactive mesothelial cells in ascites. Immunocytochemistry is commonly used in cytopathological diagnosis. This study was performed to establish whether a panel of antibodies, including two markers for HGSC and two markers for mesothelial cells, can increase the rate of detection of metastatic tumour cells.

2 MATERIALS AND METHODS

2.1 Case selection

All the samples were selected from the Department of Pathology at Tianjin Central Hospital of Gynecology Obstetrics (TCHGO), China. Consent was obtained from all patients to use their ascites samples and clinical data in this study. The ethics review board at the TCHGO approved the study. Ascites or peritoneal washings from 253 patients with histologically confirmed HGSC (pelvic/ovarian/ fallopian tube/peritoneal) were collected postoperatively from 2012 to 2015 and retrospectively analysed. Among these, 183 cases (183/253, 72.3%) were excluded from the immunocytochemical study due to typical tumour morphologies. Finally, 70 atypical cases (70/253, 27.7%) of ascites cell blocks were analysed immunocytochemically.

2.2 Immunohistochemical staining

Cell blocks were fixed in 10% neutral-buffered formalin and processed in a standard tissue processor. Immunocytochemistry was performed on 4-μm paraffin sections from cell blocks corresponding to each case. The sections were deparaffinized in xylene and rehydrated through a graded alcohol series. The sections were treated with 3% H2O2 for 10 min to inactivate endogenous peroxidase.

The sections were immunostained with primary antibodies against the following targets: Ber-EP4 (Epitomics, USA; EPR677(2), rabbit monoclonal, 1:50), E-cadherin (ZSGB-Bio, China; EP6, ZA-0565, rabbit monoclonal), HBME-1 or mesothelial cell marker (ZSGB-Bio, HBME-1; ZM-0386, rabbit monoclonal), calretinin (ZSGB-Bio; EP93, ZA-0026, rabbit monoclonal), P53 (ZSGB-Bio; DO-7, ZM-0408, mouse monoclonal), and PAX-8 (ZSGB-Bio; MRQ-50, ZM-0468, mouse monoclonal). Immunohistochemical staining was manu-
2.3 Data analysis

We used the results from haematoxylin and eosin (HE) staining as gold standard and those of immunostained markers as new findings to construct a 4 × 4 table to calculate both sensitivity and specificity of each marker as shown in Tables 1 and 2. Sensitivity (proportion of true-positive cases) was calculated by dividing the number of true-positive cases (a) by the sum of true-positive (a) and false-negative cases (c). Specificity (proportion of true-negative cases) was calculated by dividing the number of true-negative cases (d) by the sum of false-positive (b) and true-negative cases (d).

3 RESULTS

3.1 Ber-EP4

In total, 70 cases of ascites cell blocks were stained for Ber-EP4. Thirty-six cases were positive for Ber-EP4 among 42 ascites-positive cases based on HE staining. The sensitivity of Ber-EP4 was therefore 85.7% (36/42). However, five ascites-negative cases were positive for Ber-EP4 expression in mesothelial cells. The specificity of Ber-EP4 was thus 82.1% (23/28; Table 1).

3.2 Mesothelial cell marker (HBME-1)

Subsequently, 69 available cases of ascites cell blocks were stained for HBME-1. Mesothelial cells in 28 ascites-negative cases based on HE staining were positive for HBME-1. The sensitivity of HBME-1 was thus 100% (28/28). However, 13 ascites-positive cases based on HE staining exhibited were positive for HBME-1 expression in tumour cells; thus, the specificity of HBME-1 was 68.2% (28/41; Table 2).

3.3 E-cadherin

In total, 29 cases of ascites cell blocks were stained for E-cadherin. Among 20 ascites-positive cases based on HE staining, 19 were positive for E-cadherin. Therefore, the sensitivity of E-cadherin was 95% (19/20). Nevertheless, six ascites-negative cases based on HE staining also showed E-cadherin expression in mesothelial cells. Therefore, the specificity of E-cadherin was 33.3% (3/9; Table 1).

3.4 Calretinin

Twenty-nine available cases of ascites cell blocks were stained for calretinin. Mesothelial cells in eight ascites-negative cases based on
HE staining were positive for calretinin. The sensitivity of calretinin was thus 88.9% (8/9). Two ascites-positive cases also exhibited calretinin-positivity in tumour cells; the specificity of calretinin was therefore 90% (18/20; Table 2).

3.5 | Panel of these complementary markers

For more precise diagnosis, 29 cases of ascites cell blocks were stained for all four markers. Among these cases, five were falsely negative for Ber-EP4, despite being positive for E-cadherin, whereas one case was falsely negative for E-cadherin, despite being positive for Ber-EP4. Meanwhile, only one case showed false-positivity for both Ber-EP4 and E-cadherin (Table 1). Thus, Ber-EP4 combined with E-cadherin can increase the sensitivity and specificity for HGSC detection to 100% and 88.9%, respectively (Table 1). In addition, because of the reduction in the false-positive rate of HBME-1 and false-negative rate of calretinin, the sensitivity and specificity of HBME-1 and calretinin also increased to 100% and 90%, respectively (Table 2).

3.6 | Application of the panel of complementary biomarkers in ascites cytology

These four biomarkers were used to construct a panel of complementary markers for the differential diagnosis of HGSC based on ascites cytology. As shown in Figure 1, the morphology of cells in the ascites samples were relatively uniform, making it difficult to identify the cells as epithelial tumour cells or mesothelial cells based solely cytological observation after HE staining. The presence of Ber-Ep4 and E-cadherin indicated that the most of these cells were epithelial tumour cells, while nuclear positivity of calretinin and membrane positivity of HBME-1 were detected in other cells that were identified as benign mesothelial cells.

However, because of its high specificity but relatively poor sensitivity, Ber-EP4 staining appeared falsely negative in some cases (Figure 2). Nevertheless, these Ber-EP4-negative cells were positive E-cadherin, negative for calretinin, and weakly positive for HBME-1. By comprehensive analyses, these cells were confirmed as metastatic epithelial tumour cells. By contrast, because of its high sensitivity
and poor specificity, HBME-1 gave false-positive results in some cases (Figure 3). In one preoperative case, Ber-Ep4 and E-cadherin were positive in the membrane of some cells. However, some parts of the membrane in these cells were also positive for HBME-1 (D) but negative for calretinin (E), indicating that HBME-1 likely cross-reacted with tumour cells in this case. P53- (F) and PAX-8-positivity (G) confirming the diagnosis of HGSC. (A, haematoxylin and eosin ×200; B-G, immunohistochemistry ×200)

FIGURE 3 False-positive staining for mesenchymal cell markers in parts of tumour cells in one preoperative case. Cell block sections stained by haematoxylin and eosin (A). Ber-Ep4 (B) and E-cadherin (C) were positive in the membrane of these cells. However, some parts of the membrane in these cells were also positive for HBME-1 (D) but negative for calretinin (E), indicating that HBME-1 likely cross-reacted with tumour cells in this case. P53- (F) and PAX-8-positivity (G) confirming the diagnosis of HGSC. (A, haematoxylin and eosin ×200; B-G, immunohistochemistry ×200)

Among 16 patients with preoperative ascites, three simultaneously had pleural effusion. Among these cases, 15 (93.7%, 15/16) were confirmed to be positive for tumour cells in ascites. The immunocytochemical staining results are summarized in Table 3. The rate of false-negativity for Ber-EP4 and E-cadherin in tumour cells was 33.3% (5/15) and 12.5% (1/8), respectively, whereas the rate of false-negativity for HBME-1 and calretinin in mesothelial cells was 0% and 11.1% (1/9), respectively. Nevertheless, the rate of positivity for P53 and PAX-8, the markers of HGSC, was 54.5% (6/11) and 58.3% (7/12), respectively. After comprehensive analyses, six cases were diagnosed as HGSC prior to surgery.

4 | DISCUSSION

Precise cytological diagnosis of pelvic HGSC from ascites is important for tumour staging and therapeutic decision-making, especially for patients who need accurate tumour typing by preoperative
ascites cytology before receiving neoadjuvant chemotherapy. However, based on published studies, the sensitivity of peritoneal cytology ranges from 50%-70%. Zivadinovic et al showed that the false-negativity in ascites cytology for ovarian carcinoma was 30.2%, whereas the false-positivity was 6.38% for all histological types of malignant ovarian tumours. For serous carcinoma, the false-negativity was 27.77% and the false-positivity was 5.12%.

Such a high percentage of false-negative cytological detection of ascites may arise from an uneven distribution of cells in the sampled ascites, poor preparation, such as high background and weak staining, or insufficient cell extraction. Since cytological interpretation can be subjective, some errors can also arise from wrong interpretation of findings by pathologists. The reason for false-positive cytological results is often an over-interpretation of reactively altered mesothelial cells. These reactive mesothelial cells are usually enlarged, with dense cytoplasm and large nuclei in the presence of nucleoli and occasional vacuoles.

Because a precise diagnosis based on ascites cytology is critical, it is necessary to use specific immunocytochemical markers to reduce cytological errors, which can result in wrong tumour grading and inappropriate treatment. The antibody against Ber-EP4 can recognize two glycoproteins (weighing 34 and 39 kDa) on the surface or in the cytoplasm of most epithelial cells except for superficial squamous cells and liver cells. Approximately 32%-100% metastatic adenocarcinomas in serous effusion were shown to express Ber-EP4, and the specificity of Ber-EP4 in adenocarcinomas was as high as 83%-100%. The antibody against the mesothelial cell marker HBME-1, which has been isolated from a human malignant epithelial mesothelioma cell suspension, can bind to an antigen on the surface of mesothelial cells. HBME-1 has been reported to be expressed in 76%-100% of mesothelioma cells, 77%-100% of reactive mesothelial cells, and 15%-100% of adenocarcinoma cells. In this study, the sensitivity and specificity was 85.7% and 82.1% for Ber-EP4 and 100% and 68.3% for HBME-1, respectively. Thus, our results are consistent with other reports.

In view of the poor sensitivity and a relatively low specificity of Ber-EP4 and its cross-reaction with HBME-1, a panel of antibodies is needed for more precise diagnosis. Therefore, we examined another marker of epithelial cells, E-cadherin, because this marker was expressed in 72%-100% metastatic adenocarcinomas in the serous effusions and because its specificity ranged from 54% to 100%. In addition, the expression of calretinin, an intercellular calcium-binding protein belonging to the troponin C superfamily, was also examined in our study. The cytological examination of serous effusions showed that the rate of positivity for calretinin in mesothelial cells ranged from 58% to 100%, with a specificity of approximately 91%-100%. Based on our results, the combination of Ber-EP4 and E-cadherin as markers of adenocarcinoma cells increased sensitivity and specificity to 100% and 88.9%, respectively. Meanwhile, these values could be further improved to 100% and 90% after combining HBME-1 and calretinin staining to exclude mesothelial cells. In conclusion, a panel of complementary markers including Ber-EP4, E-cadherin, HBME-1.

### Table 3: Immunocytochemical results of preoperative ascites/pleural effusion cytology

| Case | Ascites/pleural effusion | Tumour cells | Mesothelial cells |
|------|-------------------------|--------------|-------------------|
|      | Ber-EP4 | E-cadherin | PS3 | PAX-8 | HBME-1 | Calretinin |
| 1    | +<sup>a</sup> | + | - | - | + | + | - |
| 2    | + | - | + | + | + | + | - |
| 3    | + | + | - | - | + | + | - |
| 4    | + | + | - | - | - | - |
| 5    | + | - | - | - | + | + | - |
| 6    | + | + | - | - | + | + | - |
| 7    | +<sup>a</sup> | - | + | + | - | + | + |
| 8    | +<sup>a</sup> | - | + | + | - | + | - |
| 9    | - | - | - | - | - | - |
| 10   | + | + | - | - | + | - |
| 11   | + | + | - | - | - |
| 12   | + | - | + | - | - |
| 13   | + | + | - | - | + | + |
| 14   | + | + | + | + | + | + |
| 15   | + | + | - | - | + | + |
| 16   | + | + | + | + | + | - |

*<sup>a</sup>Patients had preoperative ascites and pleural effusion.*

76%-100% of mesothelioma cells, 77%-100% of reactive mesothelial cells, and 15%-100% of adenocarcinoma cells. In this study, the sensitivity and specificity was 85.7% and 82.1% for Ber-EP4 and 100% and 68.3% for HBME-1, respectively. Thus, our results are consistent with other reports.

In view of the poor sensitivity and a relatively low specificity of Ber-EP4 and its cross-reaction with HBME-1, a panel of antibodies is needed for more precise diagnosis. Therefore, we examined another marker of epithelial cells, E-cadherin, because this marker was expressed in 72%-100% metastatic adenocarcinomas in the serous effusions and because its specificity ranged from 54% to 100%. In addition, the expression of calretinin, an intercellular calcium-binding protein belonging to the troponin C superfamily, was also examined in our study. The cytological examination of serous effusions showed that the rate of positivity for calretinin in mesothelial cells ranged from 58% to 100%, with a specificity of approximately 91%-100%. Based on our results, the combination of Ber-EP4 and E-cadherin as markers of adenocarcinoma cells increased sensitivity and specificity to 100% and 88.9%, respectively. Meanwhile, these values could be further improved to 100% and 90% after combining HBME-1 and calretinin staining to exclude mesothelial cells. In conclusion, a panel of complementary markers including Ber-EP4, E-cadherin, HBME-1.
and calretinin may be optimal for the cytological diagnosis of ascites, not only because of the high diagnostic sensitivity and specificity but also because this limited panel of stains would reduce the economic burden by removing the need for several antibodies.

In addition, in our study, 16 patients had preoperative ascites, and 15 cases (93.7%, 15/16) were confirmed as metastatic epithelial carcinoma in ascites based on the results of immunocytochemical staining for Ber-EP4, E-cadherin, HBME-1, calretinin, P53 and PAX-8. However, because the rate of positivity for the HGSC markers P53 and PAX-8 was only 54.5% (6/11) and 58.3% (7/12), only six cases were diagnosed as HGSC before surgery. Although several studies have indicated that neoadjuvant chemotherapy does not improve survival in patients with HGSC, it is still important for surgeons to consider the extent of surgery (such as the removal of staging lymph nodes) if the pathology can be preoperatively determined based on cytology. Hence, P53 and PAX-8 are not very sensitive for the cytological diagnosis of HGSC from ascites, and negative staining for these two markers does not entirely exclude the diagnosis of HGSC.

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