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ORIGINAL ARTICLE

# Cytopathological assessment is an accurate method for identifying immunophenotypic features and *BRCA1/2* mutations of high-grade serous carcinoma from ascites

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## Abstract

**Background:** High-grade serous carcinoma (HGSC) is the most common and aggressive type of ovarian cancer, and it is often associated with ascites at presentation. The objective of this study was to evaluate the accuracy of cytopathology to identify immunophenotypic features of HGSC and *BRCA1/2* mutations from ascites.

**Methods:** The study included 45 patients with histologically confirmed primary HGSC and malignant ascites. Immunocytochemistry (ICC) staining for PAX8, WT1, P53, P16, and Ki-67 was performed on cytospins and cytoblocks prepared from ascites. Next-generation sequencing (NGS) was used to detect germline/somatic *BRCA1/2* mutations in the ascites. Both ICC and NGS results were compared with immunohistochemistry (IHC) and NGS results from tissue blocks of primary tumor. Cronbach α and $\chi^2$ statistics, respectively, were used.

**Results:** ICC/IHC results for PAX8, WT1, P53, and P16 showed good reliability between cytospins, cytoblocks, and tissue blocks ($\alpha > 0.75$), whereas poor reliability and significant differences were observed for Ki-67 between ascites and tissue blocks ($\alpha < 0.26$; $p < .001$ [Kruskal–Wallis]). For germline *BRCA1/2* mutations, 100% concordance was confirmed, but only 14% concordance was confirmed for somatic mutations.

**Conclusions:** These results demonstrate that cytopathology is an accurate method for identifying immunophenotypic features of HGSC and detecting germline *BRCA1/2* mutations from ascites. However, further investigation is required for assessing the proliferation activity of HGSC in ascites and for detecting somatic *BRCA1/2* mutations.

KEYWORDS
ascites, *BRCA1/2* mutations, cytopathology, high-grade serous carcinoma, immunocytochemistry/immunohistochemistry, immunophenotype

## INTRODUCTION

High-grade serous carcinoma (HGSC) is the most common and deadliest type of ovarian cancer. HGSC appears to arise from the surface epithelium of the ovaries, fallopian tubes, or peritoneum.[1] It is characterized by rapid growth and early spread to the peritoneal cavity; there, it forms malignant ascites, which is often the first sign of the disease.[2] The standard treatment consists of debulking surgery followed by paclitaxel and carboplatin chemotherapy or neoadjuvant chemotherapy if optimal surgical debulking cannot be performed.[3] With the introduction of the poly(adenosine diphosphate ribose) polymerase inhibitor olaparib into the first-line maintenance treatment of *BRCA1/2*-mutated epithelial ovarian carcinoma, improvements in progression-free survival have been achieved.[4] Consequently, the detection of *BRCA1/2* mutations has become a standard procedure for therapy decisions in the first-line treatment of HGSC. Biopsy of the primary tumor is an invasive but essential approach for identifying immunophenotypic features of HGSC and detecting *BRCA1/2* mutations.[3] However, this approach is not favorable when invasive diagnostic surgical procedures cannot be performed. Cytopathological examination of ascites is an appealing minimally invasive method for assessing immunophenotypic features of HGSC and *BRCA1/2* mutations.[5,6] Cytospin and cytoblock immunocytochemistry (ICC) has been proven to be highly sensitive and specific for distinguishing HGSC cells from reactive mesothelial cells and other malignant cells in ascites.[7] Yet, it is not clear whether cytoblock ICC can provide an accurate assessment of the immunophenotypic features of HGSC from ascites, and studies on cytospin ICC have not yet been reported. As for the *BRCA1/2* status, our group has recently reported that cytopathological specimens (ascites, pleural effusions, and lymph node fine-needle aspirates) of HGSC can be alternatives to primary tumor biopsy for the detection of *BRCA1/2* mutations.[8] However, only tumor cell–rich samples, mainly of patients with recurrent HGSC, were included in the study.[8] Hence, the continuation of *BRCA1/2* mutation validation on ascites from primary HGSC, including paucicellular specimens, is required. The objective of this study was to investigate the accuracy of cytopathology for identifying the immunophenotypic features of HGSC and germline/somatic *BRCA1/2* mutations in ascites.

## MATERIALS AND METHODS

### Patients

Patients who were diagnosed with primary HGSC in the period between January 2019 and May 2021 at the Institute of Oncology Ljubljana or University Medical Centre Ljubljana were included in the study. The inclusion criteria were as follows: age >18 years, World Health Organization performance status of 0–1, histologically confirmed HGSC, presence of malignant ascites, and indication for first-line systemic treatment with platinum agents.

### Study design

Ascites samples were collected during laparoscopy or laparotomy before the biopsy was performed, and they were immediately sent to the Department of Cytopathology at Institute of Oncology Ljubljana. Each ascites sample was used for the preparation of cytospins and cytoblocks for further ICC staining (MOC31, calretinin, PAX8, WT1, P53, P16, and Ki-67), *BRCA1/2* testing, and additional flow cytometry analysis, which was not the part of this study. The results of ICC staining on cytospins and cytoblocks were compared with the results of immunohistochemistry (IHC) staining on corresponding tissue slides from the primary HGSC tumor. Hematoxylin–eosin and IHC slides were retrieved from the pathology archives and were re-evaluated by one experienced gynecological pathologist (Snježana Frković Grazio). The specificity of the ICC staining on cytospins and cytoblocks was additionally tested on non-HGSC malignant effusions from 20 patients with carcinomas of other origins (breast, lung, and gastrointestinal adenocarcinomas) and on reactive effusions from 20 patients with nonmalignant diseases (heart failure, cirrhosis, and inflammation). The results of germline and somatic *BRCA1/2* mutations detected in the ascites samples were compared with *BRCA1/2* mutations detected in primary tumor and blood samples retrieved from the patient electronic medical record (EMR) database. The experimental design of the study is summarized in Figure 1. The study was approved by the National Ethics Committee in Ljubljana, Slovenia (0120-33/303/2018/3 and 0120-33/303/2018/6). All samples from patients diagnosed with HGSC in this study were obtained after appropriate informed written consent.

### Cytological sample preparation

Ascites samples were centrifuged at 2700 rpm for 10 min. The sediment was used for preparation of Giemsa- and Papanicolaou-stained smears, and cell suspension was used for cytospins, cytoblocks, *BRCA1/2* testing, and flow cytometry analyses. Hemacolor rapid-stained (HRS) cytospin was also prepared from the cell suspension to assess the percentage of tumor cells before *BRCA1/2* mutation testing and for the optimization of the cytospin quality.

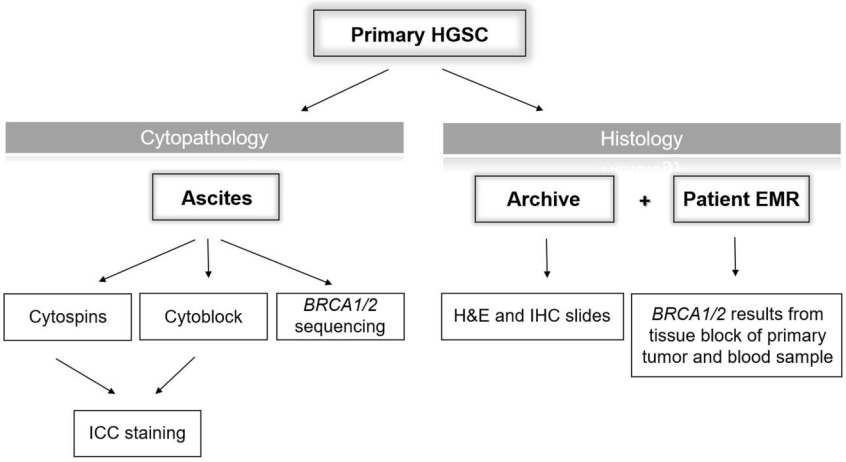


**FIGURE 1** Study design scheme. EMR, electronic medical record; ICC, immunocytochemistry; IHC, immunohistochemistry; H&E, hematoxylin–eosin; HGSC, high-grade serous carcinoma.

Cytospins were prepared with a cytocentrifuge (Shandon Cytospin 4 cytocentrifuge; Thermo Scientific, Waltham, Massachusetts) at 1500 rpm for 4 min, and they were immediately fixed in methanol for at least 30 min. The residual cell suspension was centrifuged at 2700 rpm for 10 min, and the sediment was fixed in 10% neutral buffered formalin for 4 hours (exceptionally overnight), and solidified with HistoGel (Thermo Scientific); this was followed by paraffin embedding according to the standard formalin-fixed, paraffin-embedding (FFPE) protocol at Institute of Oncology Ljubljana. Four-micrometer-thick sections were cut from each cytoblock for the hematoxylin–eosin and ICC staining. The control samples from the reactive and non-HGSC malignant effusions were processed equally but without BRCA analysis.

## ICC staining

For the ICC staining, mouse and rabbit monoclonal antibodies against MOC31, calretinin, PAX8, WT1, P53, P16, and Ki-67 were used. The staining was performed with iView and OptiView detection kits on a BenchMark Ventana Ultra automated immunostainer (Roche Diagnostics, Tucson, Arizona). Cytospins were stained according to the standard ICC protocols at Institute of Oncology Ljubljana; a detailed description of the used antibodies and protocols is given in Table 1. The presence of the antigens was detected with diaminobenzidine as a chromogen, and a positive reaction resulted in a brown precipitate. Negative control slides omitting the primary antibody and appropriate in-house positive control slides for each antibody in the panel were included in all batches.

## Microscopic evaluation

An experienced cytopathologist (V.K.-P.) evaluated Giemsa, Papanicolaou, hematoxylin–eosin, and ICC staining of the cytospins and cytoblocks. The evaluation of the ICC reactions was semiquantitative. Cases with MOC31-positive cells were considered positive for malignancy. The result was given as the percentage of positive cells per all cells in the sample. Cases with positive cell percentages below 5% were quantified as <5% positivity. The expression of PAX8, WT1, P53, P16, and Ki-67 was evaluated on tumor cells only; the result was given as the percentage of positively stained cells per the total number of tumor cells positively stained for MOC31. The Ki-67 proliferation index was defined as the percentage of positively stained tumor cells for Ki-67 per the total number of tumor cells in the sample. Furthermore, the P53/P16 index proposed by Köbel et al.[9] was determined. If the P53 staining was ≥70% or completely absent and P16 was ≥90%, the HGSC immunophenotype was considered.

## *BRCA1/2* mutation determination

A suspension of each ascites aliquot was tested via next-generation sequencing at the Department of Molecular Diagnostics at Institute of Oncology Ljubljana for *BRCA1/2* mutations. Before the DNA isolation, the percentage of tumor cells was determined with HRS cytospins, and this was later compared with the percentage of MOC31-positive tumor cells in the cytospins in order to evaluate the accuracy of rapid evaluations with HRS cytospins. The isolation of DNA and testing for somatic and germline *BRCA1/2* mutations were performed as described previously by our group.[8] The results from the ascites were compared with the *BRCA1/2* mutations detected in the primary tumor tissue blocks and blood samples retrieved from the patient EMR.

## Statistical analysis

Descriptive statistics were used to describe basic features of the data. The Cronbach α test was used to calculate the internal reliability between cytospins, cytoblocks, and tissue blocks; α ≥ 0.7 was

**TABLE 1** Description of the Abs and ICC staining protocols used

| Primary Ab | Clone | Vendor | Reaction type | Antigen retrieval (100°C) | | Ab dilution | | Ab incubation time, min | | ICC detection kit |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Cytospin | Cytoblock | Cytospin | Cytoblock | Cytospin | Cytoblock | |
| Calretinin | SP65 | Ventana | Nucleus/ cytoplasm | 1×Tris–EDTA, 3 min | CC1, 88 min | RTU | RTU | 32 | 60 | iView |
| EpCAM | MOC31 | Dako | Membrane | 1×Tris–EDTA, 3 min | CC1, 56 min | 1:500 | 1:20 | 32 | 32 | iView |
| PAX8 | EP331 | Epitomics | Nucleus | CC1, 8 min | CC1, 88 min | 1:200 | 1:200 | 32 | 60 | OptiView |
| WT1 | 6F-H12 | Cell Marque | Nucleus | CC1, 16 min | CC1, 88 min | 1:100 | 1:200 | 32 | 60 | OptiView |
| P53 | DO-7 | Dako | Nucleus | CC1, 8 min | CC1, 88 min | 1:300 | 1:100 | 32 | 60 | OptiView |
| P16 | E6-H4 | Ventana | Nucleus | 1×Tris–EDTA, 3 min | CC1, 48 min | RTU | RTU | 16 | 16 | OptiView |
| Ki-67 | MIB-1 | Dako | Nucleus | 1×Tris–EDTA, 3 min | CC1, 48 min | 1:200 | 1:200 | 32 | 60 | iView |

Abbreviations: Ab, antibody; CC1, cell conditioning solution; EpCAM, epithelial cell adhesion molecule; ICC, immunocytochemistry; RTU, ready to use.

considered to indicate good reliability.[10] Wilcoxon rank test and Kruskal–Wallis test were additionally used to determine whether there were significant differences between cytospins and cytoblocks and between cytospins, cytoblocks, and tissue blocks, respectively. A $\chi^2$ test was used for differences in *BRCA1/2* mutation results from ascites, tissue blocks, and blood samples. A $p$ value $< .05$ was considered to be significant. The statistical analysis was performed with IBM SPSS Statistics software.

## RESULTS

Forty-five patients with primary HGSC met the criteria to be included in the study. The mean age at the time of diagnosis was 65 years (range, 42–86 years). Clinical characteristics of the patients are presented in Table 2.

### Assessment of tumor cells in cytospins and cytoblocks

Among the 45 patients with primary HGSC and malignant ascites, the percentage of tumor cells in the ascites, as evaluated by MOC31 positivity, ranged from <5% to 100% with medians of 20% and 40% in the cytospins and the cytoblocks, respectively, which indicated no difference ($p = .39$ [Wilcoxon rank]); however, there was strong reliability between the two cytological techniques ($\alpha > 0.8$). The median calretinin positivity showed <5% mesothelial cells in the ascites with no difference ($p = .21$ [Wilcoxon rank]) and strong reliability ($\alpha = 0.96$) between cytospins and cell blocks as well. Representative images of calretinin and MOC31-positive stained cells are shown in Figure 2.

**TABLE 2** Clinical characteristics of the patients with high-grade serous carcinoma ($N = 45$)

| | |
|---|---|
| Age at diagnosis, years | |
| Mean | 65 |
| Range | 42–86 |
| FIGO stage, No. (%) | |
| I | 0 (0) |
| II | 3 (6) |
| IIIA | 0 (0) |
| IIIC | 30 (67) |
| IVA | 7 (16) |
| IVB | 4 (9) |
| Unknown[a] | 1 (2) |
| Positive family history, No. (%) | 12 (27) |

Abbreviation: FIGO, International Federation of Gynecology and Obstetrics.

[a]The patient continued the treatment abroad; therefore, definitive follow-up data were lost.

### Comparison of the assessments of PAX8, WT1, P53, P16, and Ki-67 on cytospins, cytoblocks, and tissue blocks

Table 3 and Figure 3 show ICC/IHC results for PAX8, WT1, P53, and P16 assessments on cytospins, cytoblocks, and tissue blocks of primary tumor. No significant difference was observed among the three methods ($p > .05$ [Kruskal–Wallis]). More precisely, PAX8 was positive in 44 of 45 cases on cytospins and cytoblocks; only one case with

<10% tumor cells in the ascites was discordant with the result of the primary tumor tissue block (α = 0.96). WT1 was positive in 43 of 45 cases, and the presence/absence of positive staining assessed from the cytospin, cytoblock, and tissue block matched 100% when they were compared for each patient. However, the median positivity of WT1 was 90% in the cytospins and cytoblocks (α > 0.8) and 100% in the tissue blocks, which resulted in lower reliability when the assessment of cytospins and cytoblocks was compared with tissue blocks (α < 0.6). WT1 positivity was also detected in reactive mesothelial cells. P53 overexpression and null expression were detected in 35 of 45 cases and in 10 of 45 cases, respectively. Only one case had an inconclusive result from the cytoblock assessment (α > 0.9; a sample with <10% tumor cells in the ascites). P53 overexpression was also observed in two control malignant effusions (both lung adenocarcinoma effusions), whereas only wild-type expression was present in the rest of the malignant and reactive effusions. P16 was positive in 44 of 45 cases; the presence/absence of positive staining with all three methods matched 100%, and this indicated very good reliability (α > 0.7). Low expression of P16 was observed in both reactive and non-HGSC malignant samples.

According to the P53/P16 index calculation, four cases were discordant with the primary tumor tissue block results; they were mainly cases with <10% MOC31-positive cells in cytospins and cytoblocks (Table 4). However, P53/P16 index successfully differentiated all cases of reactive and non-HGSC malignant effusions from HGSC ascites samples. The Ki-67 proliferation index was similar between cytospins and cytoblocks ($p_W$ > .05 [Wilcoxon rank]; α > 8), but it was significantly higher in tissue blocks; this resulted in unsatisfactory reliability between cytopathology and histology ($p$ < .001; α < 0.26). Ki-67 ranged from <5% to 100% with medians of 60% and 50% in the cytospins and cytoblocks, respectively, and from 30% to 100% with a median of 90% in the tissue blocks. Ki-67 positivity, assessed on the control reactive and non-HGSC malignant effusions, was very low (the median values were <5% and 10%, respectively). Representative images of ICC/IHC staining are shown in Figure 4.

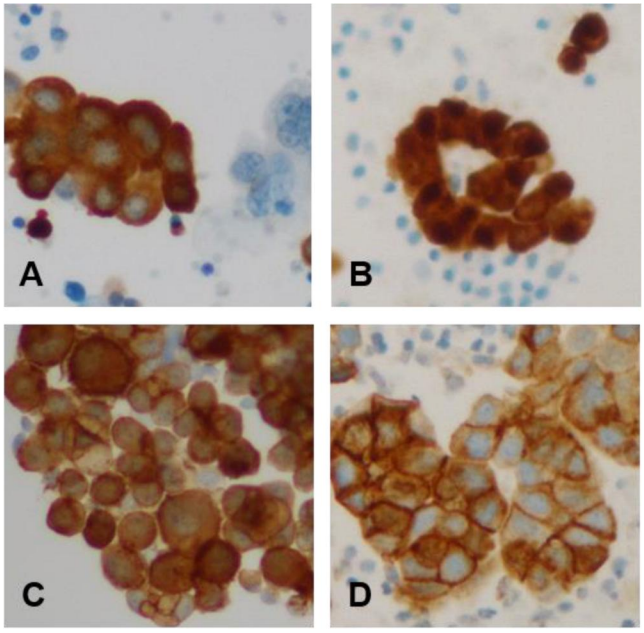


**FIGURE 2** Immunocytochemistry staining on (A,C) cytospins and (B,D) cytoblocks for (A,B) calretinin and (C,D) MOC31 (×200)

## *BRCA1/2* mutation analysis

All 45 ascites samples were tested for *BRCA1/2* mutations. Fourteen cases had missing data for the *BRCA1/2* status from the primary tissue blocks and/or blood samples in the EMR. Missing data were due to DNA fragmentation or patients' irresponsiveness to genetic counselling, respectively. These cases were excluded from the analysis. That is why only 31 of 45 cases were further analyzed. Eighteen cases (58%) had a *BRCA1/2* wild-type status (no mutation), and 13 cases (42%) carried a mutation in the *BRCA1/2* genes. More precisely, six of these mutations matched the mutations from the blood samples and were considered to be germline mutations; the other seven were considered to be somatic mutations. All germline mutations were also detected in the ascites samples, even in the cases with <10% tumor cells; this indicated 100% concordance with primary tumor and blood samples. However, only one of the seven somatic mutations was detected in the ascites (14% concordance). A detailed

**TABLE 3** ICC/IHC results of MOC31, calretinin, PAX8, WT1, P53, P16, and Ki-67 on cytospins, cytoblocks, and tissue blocks

| | Cytospin | | Cytoblock | | Tissue block | |
|---|---|---|---|---|---|---|
| Antigen | No. (%) | Median (range), % | No. (%) | Median (range), % | No. (%) | Median (range), % |
| PAX8 | 44 (98) | 100 (<5–100) | 44 (98) | 100 (<5–100) | 45 (100) | 100 (70–100) |
| WT1 | 43 (96) | 90 (0–100) | 43 (96) | 90 (0–100) | 43 (96) | 98 (0–100) |
| P53 A | 35 (78) | 100 (<5–100) | 34 (76) | 100 (<5–100) | 35 (78) | 100 (80–100) |
| P53 A0 | 10 (22) | 0 (0) | 11 (24) | 0 (0) | 10 (22) | 0 (0) |
| P16 | 44 (98) | 100 (0–100) | 44 (98) | 100 (0–100) | 44 (98) | 100 (0–100) |
| Ki-67 | 45 (100) | 60 (<5–100) | 45 (100) | 50 (<5–100) | 45 (100) | 90 (30–100) |

Abbreviations: A, aberrant overexpression of P53; A0, aberrant null expression of P53; ICC, immunocytochemistry; IHC, immunohistochemistry; No., number of patients with positive immunocytochemistry/immunohistochemistry staining for the corresponding antigen.

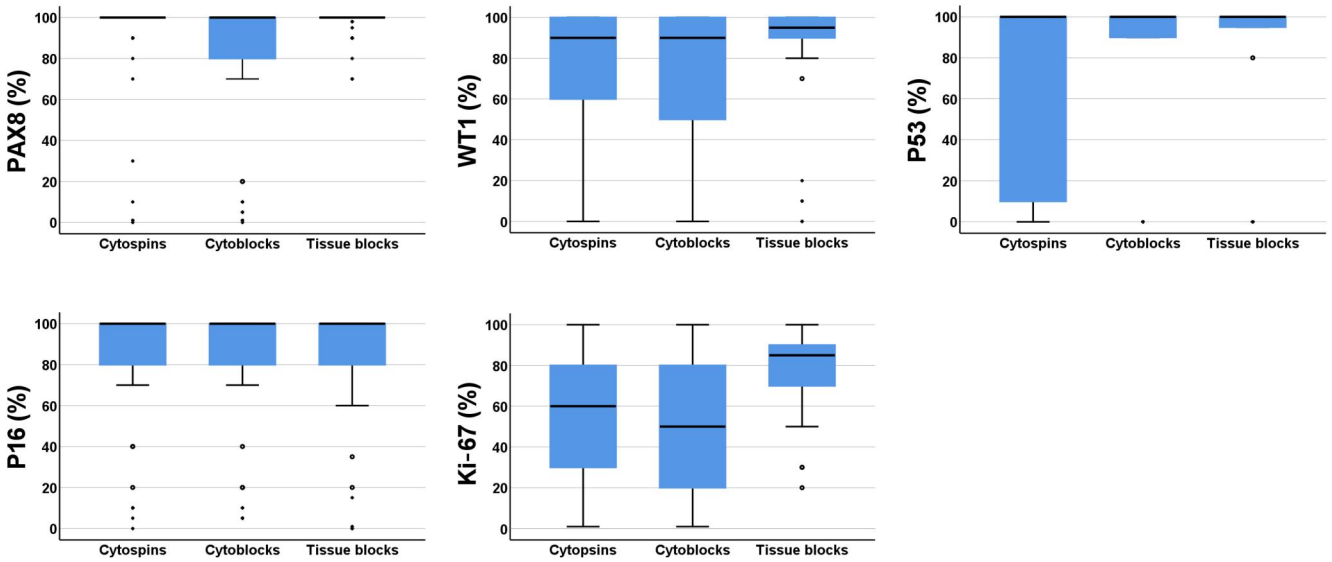


**FIGURE 3** Box plots showing medians, quartiles, ranges, outliers (circles), and extremes (asterisks) for PAX8, WT1, P53, P16, and Ki-67 positivity on cytospins, cytoblocks, and tissue blocks

**TABLE 4** Cronbach α correlation values of the ICC/IHC result comparisons for cytospins, cytoblocks, and primary tumor tissue blocks

| Antigen | Cytospin/cytoblock (ICC/ICC) | Cytospin/tissue block (ICC/IHC) | Cytoblock/tissue block (ICC/IHC) |
|---|---|---|---|
| Calretinin | 0.96 | NA | NA |
| MOC31 | 0.90 | NA | NA |
| PAX8 | 0.96 | 0.75 | 0.74 |
| WT1 | 0.93 | 0.57 | 0.54 |
| P53 | 0.91 | 0.94 | 0.91 |
| P16 | 0.90 | 0.77 | 0.75 |
| Ki-67 | 0.83 | 0.26 | 0.19 |

Abbreviations: ICC, immunocytochemistry; IHC, immunohistochemistry; NA, not applicable.

description of *BRCA1/2* mutations detected in the primary tumor, blood, and ascites samples is shown in Table 5.

Before *BRCA1/2* testing was performed, the percentage of tumor cells in ascites samples was instantly assessed with HRS cytospins, and it ranged from <5% to 20%. No significant difference was observed when these results were compared with the assessment of tumor cell percentages by MOC31 ICC cytospins ($p = .38$ [Wilcoxon rank]); moreover, strong reliability was confirmed ($\alpha = 0.82$). The percentages of tumor cells in the ascites suspensions are also given in Table 5.

## DISCUSSION

Histological assessment of primary tumor tissue is a standard approach for confirming the immunophenotype of HGSC and for the genetic analysis of *BRCA1/2* mutations.[11] However, for cases where surgery is delayed or infeasible, the diagnosis of HGSC cannot be made for proper therapy decisions. We have postulated that ICC assessments of PAX8, WT1, P53, P16, and Ki-67 on ascites cytospins and cytoblocks can provide the same information as the primary tumor tissue for the accurate immunophenotyping of HGSC and detection of germline and somatic *BRCA1/2* mutations. So far, the sensitivity and specificity of cytopathology for differentiating tumor cells and mesothelial cells in ascites have been proven,[7] yet only a few studies have focused on immunophenotypic features of HGSC. For instance, Bansal et al.[12] suggested that an accurate and specific diagnosis of HGSC might be achieved by a combined assessment of cytomorphology and cytoblock ICC of PAX8, WT1, and P53. Baransi et al.[5] also showed the high accuracy of cytopathological determination of HGSC for neoadjuvant decisions based on cytokeratin 7, cytokeratin 20, and cancer antigen 125. Essential HGSC markers such as P16, the P53/P16 index, and Ki-67 have been not evaluated.

To our knowledge, studies on cytospins for this practice have not yet been reported. Here we have compared the accuracy of cytospins and cytoblocks for determining the immunophenotypic features of HGSC. The benefit of cytospin ICC over cytoblock ICC is much faster sample processing and immunostaining (up to 3 h for the final assessment and more optimal processing when one is dealing with paucicellular samples and/or low volumes of ascites).[13] We also used

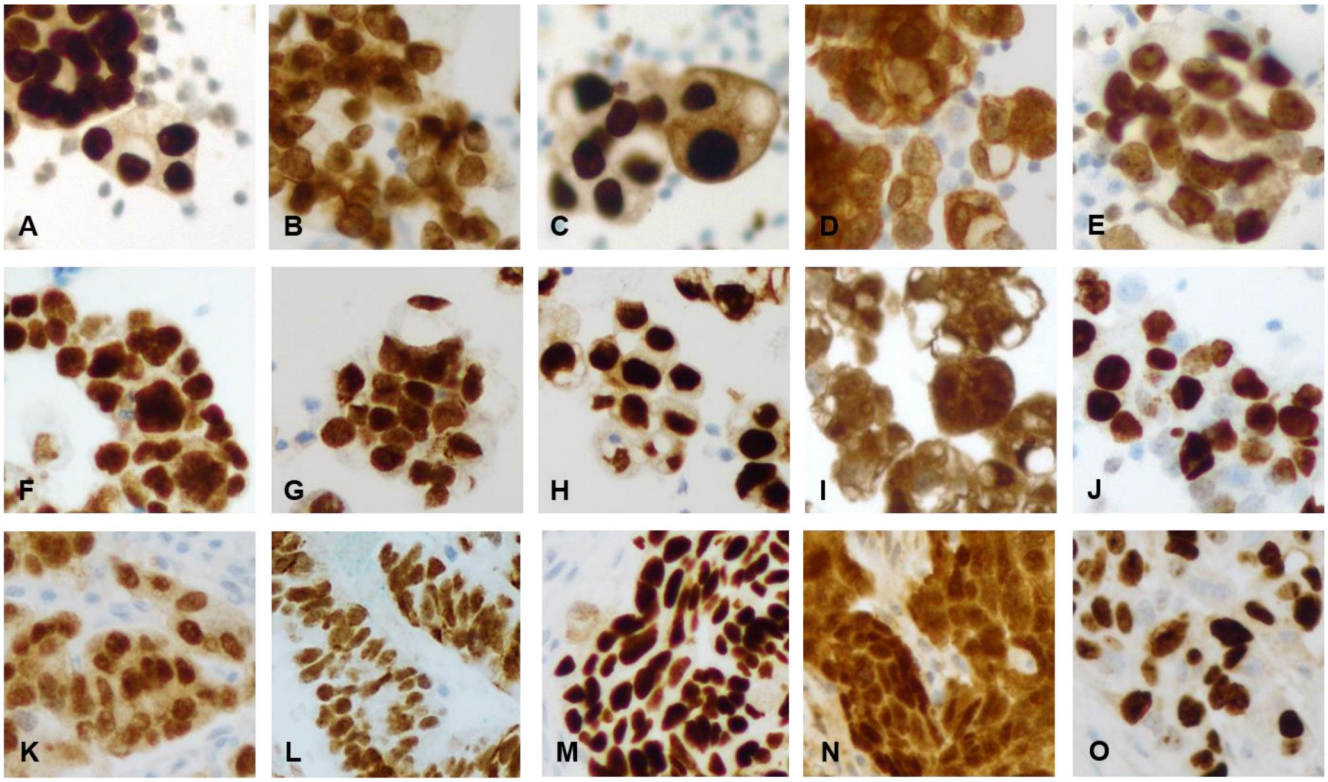


**FIGURE 4** Immunocytochemistry/immunohistochemistry staining on (A–E) cytospins, (F–J) cytoblocks, and (K–O) primary tumor tissue blocks for (A,F,K) PAX8, (B,G,L) WT1, (C,H,M) P53, (D,I,N) P16, and (E,J,O) Ki-67 from the same patient with HGSC (×200)

**TABLE 5** Percentages of tumor cells and detected *BRCA1/2* mutations in the primary tumor tissue, blood, and ascites

| | % of tumor cells | | *BRCA1/2* mutations | | | | | |
|---|---|---|---|---|---|---|---|---|
| Sample No. | HRS cytospin | MOC31-stained cytospin | Tumor | Ascites | Blood | HGVS coding versus HGVS protein | Type of mutation | VAF ascites, % |
| 1 | <5 | 30 | *BRCA1* | *BRCA1* | *BRCA1* | *c.1687C > T p.(Gln563*)* | *Germline* | *46* |
| 2 | 50 | 20 | *BRCA2* | *BRCA2* | *BRCA2* | p.(Ser1764*) | Germline | 74 |
| 3 | 10 | 30 | *BRCA2* | *BRCA2* | *BRCA2* | c.4284dupT p.(Gln1429Serfs*9) | Germline | 55 |
| 4 | 20 | 10 | *BRCA1* | *BRCA1* | *BRCA1* | c.1687C > T p.(Gln563*) | Germline | 56 |
| 5 | 70 | 10 | *BRCA1* | *BRCA1* | *BRCA1* | c.5377A > T p.(Lys1793*) | Germline | 56 |
| 6 | 90 | 90 | *BRCA1* | *BRCA1* | *BRCA1* | c.1687C > T p.(Gln563*) | Germline | 88 |
| 7 | 5 | 50 | *BRCA1* | *wt* | *wt* | c.1762delA p.(Ser588Alafs*4) | Somatic | NA |
| 8 | 5 | <5 | *BRCA2* | *wt* | *wt* | del ex 14-17 p? | Somatic | NA |
| 9 | <5 | 10 | *BRCA1* | *wt* | *wt* | wg del c.(?_-1)_(5592_?) p? | Somatic | NA |
| 10 | <5 | <5 | *BRCA2* | *wt* | *wt* | del ex 12-13 c.(6841 + 1_6842-1) p? | Somatic | NA |
| 11 | 20 | 30 | *BRCA1* | *BRCA1* | *wt* | c.3108dupT p.(Lys1037*) | Somatic | 56 |
| 12 | 30 | 30 | *BRCA2* | *wt* | *wt* | del ex 12-13 c.(6841 + 1_6842-1) p? | Somatic | NA |
| 13 | 20 | 10 | *BRCA1* | *wt* | *wt* | c.1961dupA p.(Tyr655Valfs*18) | Somatic | NA |

Abbreviations: HRS, Hemacolor rapid-stained; HGVS, Human Genome Variation Society; VAF, variant allele frequency; wt, wild type.

a larger selection of antibodies to assess the immunophenotypic features of tumor cells in ascites in comparison with other published studies.[2,14,15] Using MOC31 as a tumor marker, we could detect down to <5% tumor cells on ascites cytospins and cytoblocks, and this indicates that both methods are equally reliable. Assessments of PAX8, WT1, P53, P16, and Ki-67 are generally used in routine

histology to confirm an HGSC diagnosis, and they also allow the differentiation of HGSC from the other ovarian cancer histotypes.[2,15–17] However, this combination of antibodies has been not validated on cytopathological materials because the incidence of some ovarian cancer histotypes is low. This is also the drawback of our study. Very few of these cases were present for the duration of the study, and they were, therefore, excluded.

Our results demonstrate that assessment of PAX8, WT1, P53, and P16 on both cytospins and cytoblocks provides a reliable identification of the immunophenotypic features of HGSC in comparison with the primary tumor. A combination of PAX8 and WT1 was used to confirm the serous ovarian origin. This approach was similar to the approaches in studies conducted by Zhao et al.[14] and then Bansal et al.[12] WT1 is essential for distinguishing serous carcinomas—both HGSC and low-grade serous carcinoma (LGSC)—from the other epithelial ovarian carcinoma histotypes: endometrioid, clear cell, and mucinous.[15] However, PAX8 has recently emerged as a surrogate marker for carcinomas of Müllerian origin and seems to be more effective than WT1 for the detection of HGSC with detection rates of 87% and 63%, respectively.[14] Our data showed no difference in WT1 between cytospins, cytoblocks, and tissue blocks, but the reliability of cytopathology was lower in comparison with histology. However, no significant difference was seen when the Kruskal–Wallis statistic was additionally calculated. The lower reliability may be due to the lower proportion of WT1-positive tumor cells in the ascites. We also speculate that a possible reason for the lower average expression of WT1 in ascites tumor cells may be associated with the cytopathological sampling procedure or biological diversity of the tumor cells. No other studies reporting similar data were found. Mesothelial cells in ascites were positive for WT1 at a very low percentage; calretinin staining successfully distinguished them from tumor cells. A comprehensive analysis of PAX8 expression in human epithelial tumors has confirmed that lung, breast, and gastrointestinal adenocarcinomas are negative for PAX8, and this indicates that PAX8 is an excellent marker for confirming HGSC from ascites.[18] These data correlate with our findings from control malignant and nonmalignant effusions. The combination of PAX8 and WT1 increases the reliability of the cytopathological detection of the HGSC histotype.

The mutation pattern of the *TP53* gene is universal in HGSC, especially for distinguishing between HGSC and LGSC.[15] HGSC has two unique *TP53* mutation patterns: a missense mutation, which is seen as aberrant overexpression of the P53 protein, and nonsense mutations, which are seen as a complete absence of protein expression.[19] *TP53* is not mutated in LGSC; the expression is wild type (range, <5%–50%). More than 80% of HGSCs are diffusively positive for P16.[20,21] P16 is especially important for cytopathology when the P53 protein is absent in ascites samples with a low number of tumor cells and may lead to wrong assumptions.[22] For this reason, P16 and P53 together are used as key markers for the differential diagnosis of HGSC and LGSC.[2,9] Our analysis of P53/P16 index confirmed 90% concordance between cytopathology and histology. Discrepancies were observed in cytoblock and cytospin samples that contained less than 10% tumor cells.

HGSC is characterized by a high Ki-67 proliferation index, which is associated with tumor aggressiveness and a poor prognosis and represents an additional tool for differentiation from LGSC.[23] We have found no other studies comparing the proliferation index from ascites and tumor tissue from HGSC. In our study, we observed significantly lower expression of Ki-67 in cytospins and cytoblocks versus primary tumor tissue blocks. Only one study reported 33% positivity of Ki-67 on cytological samples from different ovarian tumors, and it cannot be compared with our results (50%–60%) because HGSC histotype was not part of the study.[24] However, a possible reason for the discrepancy might be the biological diversity between tumor cells in the ascites and the primary tumor. We suspect that tumor cells in the ascites lose their proliferation capacity when they are not attached to the endothelium and blood vessels. This assumption may also lead to lower WT1 expression. We found no data published regarding this issue. It is noteworthy that for HGSC, there is no standardized cutoff value that defines high proliferation with prognostic importance for HGSC from the primary tumor.[25] However, we recommend further analysis of the correlation between Ki-67 expression in ascites and patient survival to find a cytological cutoff. A comparison of our findings with LGSC ascites samples may also be of benefit.

Ascites may also represent a source of tumor cells that not only can be characterized immunophenotypically but also can be evaluated for molecular alterations, including *BRCA1/2* mutations.[26] An early evaluation of the *BRCA1/2* status is important for the clinical management of *BRCA1/2*-mutated HGSC with olaparib.[27] Very few studies have compared the concordance between FFPE and cytopathology specimens (ascites fluid, pleural effusions, and fine-needle aspiration) for *BRCA1/2* testing.[8,26] The overall prevalence of *BRCA1/2* mutations ranges from 11% to 15% for germline mutations and from 5% to 9% for somatic mutations.[28] We detected a much higher prevalence of *BRCA1/2* mutations among the patients because of our strict inclusion criteria, as mentioned in the Materials and Methods section. Moreover, we had to exclude 14 patients from the analysis because of missing data about the *BRCA1/2* status from the primary tumor and/or blood samples. The reason for missing data for the primary tumor FFPE tissue blocks was DNA fragmentation; missing data from the blood samples was related to patients' irresponsiveness to genetic counselling.

The evaluation of tumor cells in ascites for *BRCA1/2* testing is poorly described. Our results have confirmed that the percentage of tumor cells can be determined as accurately with HRS cytospins as with IHC staining with MOC31. These data are mandatory for the decision about whether the sample should be further processed for *BRCA1/2* analysis. Already published data indicate a high concordance of somatic and germline mutations between HGSC tumor tissue and cytopathological specimens.[8] Our results demonstrated 100% concordance of germline *BRCA1/2* mutations between ascites and primary tumor tissue; however, low concordance was found for somatic mutations. Noteworthy, only two studies have included somatic mutations in their analysis. One was our previous study, which detected two somatic mutations from fine-needle aspiration cytology

samples of enlarged lymph nodes, where the concentration of tumor cells was more than 90%. The other was the study by Fumagali,[29] who did not specify the number of somatic mutations over germline mutations or their corresponding percentages of tumor cells. In the current study, we used a 10% limit of detection for single-nucleotide variants and single-nucleotide insertions or deletions (indels) and a 25% limit of detection for indels of 2–11 nucleotides. Because mutated cells might be heterozygous for the mutation, the recommended percentage should be at least 2 times higher to ensure reliable detection.[8] Moreover, the percentage of tumor cells in the ascites samples might have been overestimated. All these factors may explain why we did not detect somatic mutations in the ascites sample, where the estimeted concentration of tumor cells was 30%. We speculate that enriching tumor cells in a sample may improve the detection of somatic *BRCA1/2* mutations. For this matter, further studies are needed. A larger cohort of somatic *BRCA1/2* mutations is also required.

In conclusion, our results demonstrate that cytopathology is an accurate method for identifying immunophenotypic features of HGSC and detecting germline *BRCA1/2* mutations in ascites, and this is especially beneficial when optimal surgical debulking cannot be performed. However, further investigation is required for assessing the proliferative activity of HGSC in ascites and detecting somatic *BRCA1/2* mutations.

## AUTHOR CONTRIBUTIONS

**Simona Miceska:** Methodology, data curation, formal analysis, investigation, statistics, visualization, and writing–original draft. **Erik Škof:** Project administration, conceptualization, and writing–review and editing. **Srdjan Novaković:** Methodology and writing–review and editing. **Vida Stegel:** Methodology. **Anja Jeričević:** Methodology. **Biljana Grčar Kuzmanov:** Methodology. **Špela Smrkolj:** Methodology. **Branko Cvjetičanin:** Methodology. **Sonja Bebar:** Methodology. **Marta Globočnik Kukovica:** Methodology. **Veronika Kloboves-Prevodnik:** Supervision, conceptualization, methodology, and writing–review and editing.

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## CONFLICTS OF INTEREST

The authors made no disclosures.

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