Next-generation sequencing-based BRCA testing on cytological specimens from ovarian cancer ascites reveals high concordance with tumour tissue analysis

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ABSTRACT

Background With the approval of the poly (ADP-ribose) polymerase (PARP) inhibitor olaparib for newly diagnosed, breast cancer gene *(BRCA)1/2* mutated, ovarian cancer women, the assessment of *BRCA1/2* tumour status will be shortly required at the time of diagnosis.

Aim To investigate the feasibility of next-generation sequencing (NGS)-based BRCA tumour test on cytological specimens from ovarian cancer ascites.

Methods We evaluated the *BRCA1/2* status on neoplastic ascites and corresponding tumour tissue of 11 patients with ovarian cancer, using the NGS 'Oncomine BRCA Research Assay'.

Results The NGS-based BRCA test on cytological samples had a success rate of 100%, with 11 of 11 concordant *BRCA1/2* results between ascites and tumour tissues analyses, including two wild type samples and nine cases harbouring somatic or germline variants.

Conclusion BRCA test may be performed on ovarian cancer ascites, reproducing *BRCA1/2* tumour status and representing a useful tool for clinical decision-making.

INTRODUCTION

Epithelial ovarian cancer (EOC) is an aggressive disease with poor clinical outcome,[1] frequently diagnosed at an advanced stage with evidence of widespread peritoneal carcinomatosis and malignant ascites.[2] poly (ADP-ribose) polymerase (PARP) inhibitor (PARPi) therapy has improved the clinical outcome of women affected by platinum-sensitive recurrent ovarian cancer, in particular for those harbouring *BRCA1/2* mutations.[3–5] Moreover, according to the recent results of SOLO1 trial, the Food and Drug Administration approved the PARPi olaparib for patients with newly diagnosed BRCA-mutated (germline or somatic) advanced EOC who are in complete or partial response to first-line platinum-based chemotherapy.[6 7] Therefore, the assessment of the *BRCA1/2* status at the time of diagnosis of ovarian cancer and the early identification of *BRCA1/2* alterations are pivotal steps for clinical decision-making.

Peritoneal washing is routinely performed during ovarian cancer surgery for staging purposes. Moreover, malignant ascites can be drained before surgery to relieve patient pain and discomfort . These fluids may represent a source of ovarian cancer cells that could be characterised not only phenotypically but also evaluated for molecular alterations, including *BRCA1/2* mutations.

BRCA testing is feasible on whole blood samples, evaluating germline *BRCA1/2* status and on tumour tissue, investigating both germline and somatic *BRCA1/2* variants.[8] In this study, we (1) assessed the feasibility of *BRCA1/2* analysis on cytological specimens from malignant ascites, using next-generation sequencing (NGS) and (2) retrospectively evaluated the concordance of *BRCA1/2* status between cytological and matched tumour tissue samples.

PATIENTS AND METHODS

Patient population

This feasibility study included 11 patients with EOC, who underwent surgery and ascites drainage or washing prior to or during the surgical procedure at the European Institute of Oncology. Tumour *BRCA1/2* status was available for all the patients, as tumour BRCA test was required by gynaecologic oncologists after the histological diagnosis of non-mucinous and non-borderline EOC, according to the test guidelines.[9] Each patient gave written informed consent to perform tumour BRCA test. The clinicopathological characteristics of the patients were summarised in table 1.

Ascitic fluid sample preparation and DNA extraction

Cytological specimens were retrospectively retrieved from the archives of the Division of Pathology of European Institute of Oncology. Ascitic fluid samples were prepared with cytocentrifuge (cytospin smears) and stained with May-Grunwald/Giemsa (figure 1). The identification of ovarian malignant cells in peritoneal fluids was performed by cytopathologist. The most representative cytological slide for each case was selected for the analysis. The slide was unmounted, and the stained smear was scraped in a tube containing 100% alcohol. The DNA was then extracted automatically with the Promega Maxwell instrument (Promega, Madison, Wisconsin, USA) and quantified with the Quantus fluorimeter (Promega).

BRCA1/2 NGS analysis

The *BRCA1/2* status was evaluated using the NGS panel 'Oncomine BRCA Research Assay' (Thermo Fisher Scientific, Waltham, Massachusetts, USA), following the manufacturer's instructions. Both library preparation and the subsequent chip

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Table 1 Clinicopathological characteristics of the patients included in the study

	No of cases (%)
Histological subtype	
High-grade serous carcinoma	10 (90.9)
Low-grade serous carcinoma	1 (9.1)
FIGO staging	
II B	1 (9.1)
III B	1 (9.1)
IV B	1 (9.1)
III C	7 (63.6)
NA	1 (9.1)
Familiar history	
Suggestive for HBOC syndrome	3 (27.3)
Non-suggestive for HBOC syndrome	8 (72.7)

FIGO, International Federation of Gynecology and Obstetrics; HBOC, Hereditary Breast and Ovarian Cancer; NA, not available.

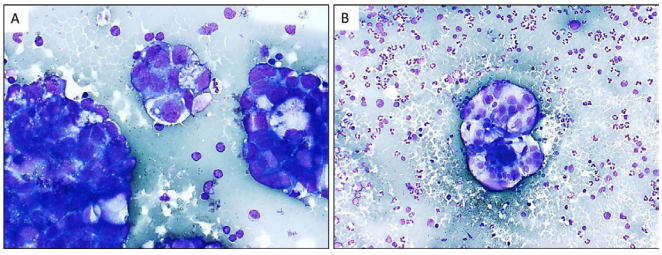

Figure 1 Cytospin smears from malignant ascitic fluid stained with May-Grunwald/Giemsa. (A) Clusters of malignant tumour cells (tumour cell percentage more than 50%); (B) isolated tumour cells spherule in an inflammatory background (tumour cell percentage less than 20%).

loading were automatically performed on the Ion Chef System (Thermo Fisher Scientific). The sequencing was run on the Ion S5 System (Thermo Fisher Scientific), and data were analysed on the Ion Reporter Analysis software (v. 5.10) using Oncomine BRCA (5.10) bioinformatic pipeline (Thermo Fisher Scientific), covering single-nucleotide variants and insertions/deletions (indels). Each variant was then visually inspected using the Integrative Genomics Viewer software. The variants were classified according to five class system of the IARC (International Agency for Research on Cancer) clinical classification, as pathogenic, likely pathogenic, variant of uncertain significance (VUS), likely benign and benign variant. The classification of each variant was defined according to the ENIGMA consortium revision on the BRCA Exchange database (https://brcaexchange.org/).[10] If the variant was labelled as 'not yet reviewed' by ENIGMA consortium, other public databases as ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) or Leiden Open source Variation Database (https://databases.lovd.nl/shared/variants/BRCA1/unique) were consulted. BRCA test results on DNA from cytological specimens were compared with the results obtained from the BRCA test previously performed on DNA extracted from the matched formalin-fixed paraffin-embedded (FFPE) tumour tissue.

RESULTS

BRCA test performance on malignant ascites

The percentage of tumour cells in the sample and the concentration of DNA recovered from the most representative cytological smear of each case were reported in table 2. The median concentration of extracted DNA was 0.9 ng/µL (range 0.1–1.9 ng/µL), eluted to a final volume of 60 µL.

The NGS run metrics obtained for each cytological specimen were reported in table 2. All the 11 cytological cases reached the quality parameter in terms of *on target* (>85%), *mean depth* (>1200) and *uniformity* (>85%) of mapped reads, despite the starting quantity of DNA of four samples (3, 4, 9, 11) was below the amount suggested from the NGS panel (10 ng of DNA in 15 µL for the library preparations). The turnaround time from DNA extraction to the BRCA test result was 5 days.

BRCA1/2 status on cytological and matched tumour tissue samples

The results of BRCA test on cytological specimens were reported in table 3. The concordance of *BRCA1/2* status between malignant ascites and tumour tissue was 100% (11 of 11 concordant results), including two wild-type samples and cases harbouring *BRCA1/2* pathogenic/likely pathogenic variants (n=7) or variants of uncertain significance (n=2). A slight difference in the variant allele frequencies (VAFs) was observed in cytological specimens as compared with tumour tissue samples. *BRCA1/2* germline status was available for four patients. In two patients with no *BRCA1/2* germline mutations, somatic *BRCA1* variants were pinpointed both in the tumour tissue and cytological samples.

Table 2 Percentage of tumour cells in the sample, concentration of DNA extracted from a single smear and NGS metrics of each cytological sample

Sampl e no	Tumour cell percentage	DNA concentration (ng/µL)	Mapped reads	On target (%)	Mean depth	Uniformity (%)
1	50	1	611 416	96.14	2762	99.63
2	80	0.9	1 433 174	92.67	6397	99.99
3	<20 cells*	0.1	432 728	96.81	1951	99.15
4	<20 cells*	0.1	325 818	96.29	1458	97.17
5	70	1.1	1 336 214	95.45	5924	99.62
6	80	1.8	989 586	94.35	4355	99.59
7	70	0.7	1 247 150	93.38	5320	98.40
8	70	1.9	407 176	93.03	1834	99.32
9	70	0.2	1 873 620	96.53	8350	99.36
10	40	1.7	601 914	95.73	2774	99.98
11	10	0.1	716 788	96.64	3230	100.00

*Less than 20 malignant cells collected in the smear.
NGS, next-generation sequencing.

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Table 3 *BRCA1/2* status in FFPE tumour tissue and cytological specimens from ascites. When available, germline *BRCA1/2* status were indicated

Specimen no	BRCA germline status	BRCA status—malignant ascitic fluid	BRCA status—FFPE tumour tissue	Mutation type	Variant type	Classification
1	NA	*BRCA2* c.7436-4A>G	*BRCA2* c.7436-4A>G	SNV	Splice site	VUS
2	NA	*BRCA1* c.5062_5064delGTT	*BRCA1* c.5062_5064delGTT	Indel	In frame	Pathogenic
3	**WT**	*BRCA1* c.5425G>T	*BRCA1* c.5425G>T	SNV	Missense	Likely pathogenic
4	***BRCA2* mutated**	*BRCA2* c.2808_2811delACAA	*BRCA2* c.2808_2811delACAA	Indel	Frameshift	Pathogenic
5	NA	*BRCA1* c.3626T>G	*BRCA1* c.3626T>G	SNV	Nonsense	Pathogenic
6	NA	*BRCA2* c.9101A>G	*BRCA2* c.9101A>G	SNV	Missense	VUS
7	NA	WT	WT			
8	NA	*BRCA1* c.1088delA	*BRCA1* c.1088delA	Indel	Frameshift	Pathogenic
9	**WT**	*BRCA1* c.3598C>T	*BRCA1* c.3598C>T	SNV	Nonsense	Pathogenic
10	NA	WT	WT			
11	***BRCA1* mutated**	*BRCA1* c.5267_5268insC	*BRCA1* c.5267_5268insC	Indel	Frameshift	Pathogenic
Variant allele frequency (%)	5–20	20–40	40–60	60–80		80–100

Variants were reported according to HGVS nomenclature and NCBI reference sequence NM_007294.3 for *BRCA1* and NM_000059.3 for *BRCA2* and colour-coded based on their variant allele frequency. Bold terms: BRCA germline status available

FFPE, formalin-fixed paraffin embedded; HGVS, Human Genome Variation Society; NA, not available;NCBI, National Center for Biotechnology Information; SNV, single-nucleotide variant; VUS, variant of uncertain significance; WT, wild type.

DISCUSSION

The *BRCA1/2* status is crucial for the clinical management of women affected by EOC. Moreover, the presence of *BRCA1/2* alteration, whether somatic or germline, may indicate a clinical benefit of PARPi treatment even for women with a newly diagnosed tumour. Therefore, an early evaluation of *BRCA1/2* status represents an important clinical need.

The presence of malignant cells in ascitic fluid is a well-known factor of poor prognosis for patients with ovarian cancer that correlates with worse progression-free and overall survival.[11] In the last years, different efforts have been made to exploit peritoneal fluids for the molecular characterisation of primary tumours.[12–14] In particular, cancer cells,[12] cell-free DNA[13] and RNA extracellular vesicle RNA biomarkers[14] from ascites have been thoroughly investigated. However, so far, few studies analysed *BRCA1/2* status on malignant cells recovered from peritoneal/pleural fluids.[15 16] Recently, Barquín *et al* reported on the feasibility of *BRCA1/2* mutation analysis on 10 cytological samples from fresh peritoneal washings[17] and Gornjec *et al* evaluated a large cohort of patients with ovarian cancer using capture-based NGS approaches.[18] However, they assumed that fresh peritoneal liquid was available immediately for DNA extraction[17] or used as a protocol for cytological specimen preparation including a cell medium addition step that is not routinely performed in most diagnostic laboratories.[18] In the last years, our group demonstrated that archival smear slides may represent suitable samples for molecular tests, including NGS analysis.[19 20] In the present study, we showed the feasibility of *BRCA1/2* evaluation on archival stained smears from ascitic liquid drainage or peritoneal washing. The evaluation of each stained cytological specimens by an expert cytopathologist is a crucial preliminary step to assess the ovarian tumour cell content and reduce the risk of cross-reactivity with cells of different origin. Moreover, it may reduce the detection of clonal haematopoiesis-related genetic events, although no data on *BRCA1/2* mutations and this phenomenon have been reported yet. We used an amplicon-based NGS panel and we achieved a BRCA test success rate of 100% despite four cases had a starting DNA yield below the suggested quantity. Indeed, as previously demonstrated higher quality DNA can be extracted from stained smear specimens.[20] The *BRCA1/2* status between FFPE tumour tissue and ascitic fluid specimens was concordant in all cases, including *BRCA1/2* wild-type samples and cases harbouring *BRCA1/2* somatic or germline alterations. Although we observed differences in the VAFs of *BRCA1/2* mutations, these variances may be related to the different tumour cell content of cytological and tumour tissue samples.

Given the limited number of the cases included in this study, these results should be considered preliminary and to be confirmed in a larger cohort. However, these data may lead to the implementation of *BRCA1/2* testing on cytological samples in a routine diagnostic setting. Indeed, the evaluation of *BRCA1/2* status on ascitic fluid specimens may be clinically useful as: (1) peritoneal fluid drainage is a minimally invasive procedure, often prior to the surgery to relieve symptoms associated to the fluid build-up; (2) the *BRCA1/2* status may impact on the surgical procedure, since the *BRCA1/2* positive status was recently associated with a survival benefit in patients with gross residual disease[21] ; (3) peritoneal liquid offers a dynamic snapshot of tumour genomic alterations, enabling to capture intratumour genetic heterogeneity as compared with single-site sample analysis and (4) both somatic and germline variants could be identified in malignant ascite cells as in tumour tissue.

In conclusion, we reported on the *BRCA1/2* assessment on stained cytological smear from ascites using amplicon-based NGS. Although the number of cases is limited, the high success rate, the turnaround time compatible with clinical needs and the high concordance with tumour tissue *BRCA1/2* analysis are promising achievements that suggest the feasibility and reliability of the evaluation of *BRCA1/2* status on cytological preparation from ovarian cancer ascites.

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