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Edmondson RJ, Curtin NJ.

[Advanced Ovarian Cancer Displays Functional Intratumor Heterogeneity That
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**Advanced ovarian cancer displays functional intra-tumour heterogeneity
which correlates to ex vivo drug sensitivity**

Word count: 4246

Abstract

Introduction Epithelial ovarian cancer (EOC) is recognised to be heterogeneous but is currently treated with a single treatment strategy. Successful patient stratification of emerging chemotherapy agents is dependent upon the availability of reliable biomarkers indicative of the entire tumour.

Aim To evaluate inter- and intra-tumour heterogeneity within a series of EOC using homologous recombination DNA repair (HR) status.

Methods Primary cultures generated from ascites and solid tumour from multiple intra-abdominal sites were characterised by their morphology and expression of protein markers. Results were compared with FFPE tissue pathology.

HR function was determined by quantification of nuclear Rad51 foci. Growth inhibition (SRB) assays were used to calculate the GI₅₀ for cisplatin and rucaparib.

Results Ascites with matched solid tumour were cultured from 25 patients.

Concordance in functional HR status between ascites and solid tumour subcultures was seen in only 13/25 (52%) patients. Heterogeneity in HR status was seen even in patients with homogeneous histological subtype. HR defective cultures were significantly more sensitive to cisplatin and rucaparib.

Additionally inter- and intra-tumour heterogeneity was seen between the expression of epithelial and ovarian markers (EpCAM, cytokeratin, CA125, MOC-31 and vimentin). There was no relationship between heterogeneity of HR functional status and antigen expression.

Conclusions Inter- and intra-tumour functional HR heterogeneity exists that cannot be detected using histological classification. This has implications for biomarker directed treatment.

Word count: 222

Keywords:

Ovarian cancer, Intra-tumoural heterogeneity, Inter-tumoural heterogeneity, Homologous recombination, Functional assessment, PARP inhibitor

Introduction

The term ovarian cancer describes a set of distinct and heterogeneous diseases, all of which are currently treated with a single treatment regimen. Despite advances in surgery and the addition of taxane to platinum chemotherapy, the 5-year survival has remained low at 30-40%. Improved outcomes will require the use of targeted agents and novel cytotoxic agents exploiting the molecular pathology of the tumour.

Reliable molecular predictive biomarkers are still lacking for ovarian cancer. The classification of epithelial ovarian cancer into histological types (high grade serous, endometrioid, clear cell, mucinous and low grade serous), associated with different driver mutations, is well established but more recent work has taken this further and subdivided high grade serous cancer using genomic [1], gene expression [2] and functional [1, 3] techniques. This has generated at least four distinct gene expression subgroups [2, 4] with diverse prognostic behaviour. This approach however has not yet successfully identified a reliable gene signature capable of predicting actual response to cytotoxic agents. Subdivision into groups according to overall function of a DNA repair pathway (homologous recombination) [3] is not related to histological subgroup and has potential to enable stratification of patients according to differences in their sensitivity to conventional and novel chemotherapy agents [5, 6].

To date, systemic treatment for epithelial ovarian cancer has been platinum based, irrespective of histological subtype or other biological markers. An increased understanding of the disease combined with the development of new, targeted, agents is changing this model and allowing the development of personalised medicine. However, effective delivery of novel agents and the correct selection of patients will require the use of accurate biomarkers capable of predicting response and there is great interest in developing these from both academia [7] and industry [8]. Ovarian cancer typically presents at a disseminated stage with multiple sites of disease within the peritoneal cavity and elsewhere. Unless tumours are homogeneous it is likely that biopsies from a single site of a

tumour may not be representative of the rest of the tumour and therefore be unable to predict response accurately. In particular, most biopsies used for clinical diagnosis are taken from the omentum under radiological guidance. It is rare for disease on the diaphragm or nodal disease to be sampled. Given that there may be biological differences between disease which disseminates within the peritoneal cavity, through direct spread, compared to disease that disseminates by a classical process using lymphatic or haematogenous spread [9] it is likely that there could be a systematic bias in reporting of biopsies. There is no published evidence demonstrating radiological variable response to chemotherapy within an individual patient across different tumour sites but this is likely to reflect a lack of studies. RECIST criteria take into account measured response in both target and non-target lesions giving an overall response rather than documentation of individual lesions by anatomical site. As molecular data taken from biopsies start to be used to direct therapy both in clinical trials and in clinical practice, knowledge of heterogeneity between intra-abdominal sites becomes increasingly important. Genetic intra-tumoural heterogeneity has been demonstrated but the question of functional heterogeneity and subsequent response to therapy has not been addressed in any prospective study.

Intra-tumoural heterogeneity can currently be assessed using morphological classification or analysis of genetic mutations. The histological subtypes of epithelial ovarian cancer have distinct and different pathogenetic processes [10] with variable response to therapy but true morphological heterogeneity is relatively rare in ovarian cancer and it has been suggested that this is overdiagnosed [11]. This suggests that histology alone is not sensitive enough to detect changes in underlying tumour biology. . There is general consensus that most cases of ovarian cancer are monoclonal in origin with a high degree of genomic parsimony but significant heterogeneity exists within microsatellites and SNPs [12] and copy number changes and driver mutation status [13] from

anatomically distinct regions. This does not appear to translate into a significant degree of heterogeneity in terms of gene expression [14].

Other possible mechanisms of heterogeneity, including epigenetic changes in methylation, which is known to be important in determining functional status of the tumour, have not been studied.

There is therefore evidence that significant ITH is likely to exist but how this knowledge can be employed to provide prediction to treatment is less clear.

Cancers develop heterogeneity as a result of a process of somatic evolution but many of the mutations that occur have no functional effect and are termed passenger mutations. It may be more clinically relevant to examine the functional effects of heterogeneity rather than just relying on genomic variation. For this reason we have developed functional assays for homologous recombination DNA repair (HR) [15, 16].

The aim of this study was to evaluate the extent of intra- and inter-tumour heterogeneity within a series of ovarian cancers using homologous recombination DNA repair status as a biomarker, and consider the potential impact of heterogeneity upon response to treatment and the subsequent management of such disease.

Materials and Methods

Reagents

Rucaparib was a gift from Clovis (Boulder, USA) and is a potent inhibitor of PARP-1 and -2 proteins ($K_i < 5$ nM). Rucaparib was dissolved in DMSO to give a stock solution of 10 mM, which was stored at -20 °C for in vitro studies. Cisplatin (Alexis Biochemicals, California USA) is a potent antineoplastic drug which forms inter- and intra-strand DNA adducts. Cisplatin was dissolved in water to give stock

solution of 2 mM, which was stored at -20 °C. All other chemicals and tissue culture reagents were from Sigma-aldrich (Sigma-aldrich, UK), unless otherwise stated.

Cell Culture

Sample collection

Ethical approval was granted (12/NW/0202) for the collection of ascites and solid tissue from consented patients undergoing surgery for ovarian cancer at the Queen Elizabeth Hospital, Gateshead, UK. Samples were collected between 02/2012 and 08/2013 from patients recruited with radiological evidence of pelvic masses suspicious for or confirmed to be an ovarian malignancy. Clinical details were recorded and specimens registered and handled in accordance with the Human Tissue Act. Samples were assigned a PCO (Primary Culture Ovary) reference number to retain anonymity. All samples were handled separately with their own reagents to prevent cross-contamination.

Sample transport and preparation

Ascitic fluid was aspirated directly from the patient into a sterile suction bottle. Solid tumour specimens of approximately 1 cm³ were excised from surgical specimens and from any areas of irresectable tumour intra-operatively. The sample site was carefully documented and the solid tumour was placed into a sterile universal containing culture medium (RPMI 1640 medium supplemented with 10% FCS, 20mM L-glutamine and 1% penicillin and streptomycin) pre-warmed to 37°C. Samples were transported from the hospital to the lab immediately in compliance with UK Category B regulations UN3373.

Ascitic cell culture

Cell culture was performed using an aseptic technique in a containment level II laminar flow microbiological safety cabinet, as previously described [17]. Briefly, 20 ml of ascitic fluid was added

to 20 ml of warmed culture medium (RPMI 1640 medium supplemented with 10% FCS, 20 mM L-glutamine and 1% penicillin and streptomycin) in T75 flasks (Corning, NY) and incubated at 37°C, 5% CO₂, 95% humidified air. The medium was aspirated and 13 ml of warmed fresh medium was replaced on day 3 to 5. When confluence was approached cells were passaged, frozen and thawed as previously described [18].

Primary culture from solid tumour

Solid culture was performed using techniques previously described [17]. Briefly, 1 cm³ solid tumour collected from intra-abdominal sites during cytoreductive surgery and transported to the lab in warmed culture media was dissected into ~3 mm³ pieces and incubated with collagenase/dispase (Roche, UK) solution (1 mg/ml in full medium) for 2 hours at 37°C on an orbital shaker (IKA-Vibrax-VKR) at 2xg. The cell suspension was transferred to a universal container, centrifuged at 400xg for 5 minutes, PBS washed, re-suspended in full medium and placed in a T25 flask for 30 minutes to allow fibroblast seeding. The remaining cell suspension containing an epithelial rich cell suspension was transferred into a T25 flask for on-going cell culture, in RPMI medium containing 10% (v/v) fetal calf serum. When approaching confluence cells were trypsinised, PBS washed and seeded onto sterile coverslips for characterisation and Rad51 HR assay and plated for cell proliferation assays described below.

Characterisation

A single marker is insufficient to reliably differentiate epithelial ovarian cancer cells from all histological subtypes from other cell types sampled from solid tissue or ascites. A characterisation panel consisting of cell culture morphology, immunofluorescent staining of fixed cells, as well as standard pathological and immunohistochemistry examination of paired FFPE samples was combined to ensure accurate characterisation of every culture.

Morphology

Morphological features of cells in culture were studied under an Olympus CK40 inverted microscope at 20X magnification and images were captured using VisiCam® software (VWR, USA). Cultures were classified as having cobblestone, spindle or mesenchymal morphology.

Immunofluorescence

Standard techniques for immunofluorescence were used to stain for six epithelial, mesenchymal, and ovarian markers, **Error! Reference source not found.** Cells were grown on coverslips, fixed with ice cold methanol prior to incubation with primary antibody. All cultures were assessed for antigen expression at passage 1. A panel of markers was used in the absence of a single specific epithelial ovarian marker. Cultures were deemed to be non-epithelial if they failed to demonstrate expression of either cytokeratin, MOC31 or EpCam and were discarded.

Formal histopathology

Formal cytological and histopathological examination of matched ascites and solid tumour specimens from every patient was performed and using standard techniques used to further characterise the cultures. This was performed by an independent expert pathologist blinded to the results of the antigen expression studies. Specimens were assigned to an ovarian histological subtype according to universal World Health Organisation criteria [19].

When all characteristics were in keeping with epithelial ovarian origin, the samples were then used in subsequent experiments, at either passage 1 or 2. Cultures were discarded after passage 2. Where results were inconsistent with epithelial origin, cultures were discarded.

Growth and Cell Proliferation assays

A routine sulforhodamine B (SRB) assay was used to assess cytotoxicity and cell growth as previously described [20]. Briefly, cells were seeded at a density of 1000 cells/well and after adherence, treated with various concentrations of rucaparib (0, 0.1, 1, 10, 50, 100 μ M) or cisplatin (0, 0.01, 0.03, 0.1,

0.3, 1, 3, 10 μ M) for 10 days before fixation. For assessment of growth, cells were seeded, as above, then fixed at 24 hour intervals before staining and spectrophotometer assessment of cell density. The equation $(t_2 - t_1)/3.32 \times (\log n_2 - \log n_1)$ was used to calculate doubling time, where t = time and n = cell density.

Homologous recombination assay

HR functional status was determined using a previously described method [16]. This assay quantifies Rad51, a crucial downstream protein involved in HR repair, which is relocalised within the nucleus in response to DNA damage to form distinct foci that can be visualized by immunofluorescent microscopy. Quantification of Rad51 in response to induction of DNA damage serves as a marker of HR function to distinguish between HR-proficient and HR-deficient cell lines. As part of the validation of this assay, a panel of cell lines with known HR function underwent Rad51 quantification in independent experiments [15, 16].

Cells were seeded onto uncoated glass cover slips and treated with 2 Gy ionising radiation and rucaparib at 10 μ M concentration for 24 hours to induce double strand breaks (DSB) [15]. All experiments were performed alongside untreated controls with equivalent 0.1% DMSO. Cells were then fixed and rehydrated prior to staining with 1:100 mouse monoclonal anti- γ H2AX (Upstate, Millipore Corp., USA) and 1:100 goat polyclonal anti-Rad51 (Calbiochem, EMD Biosciences, Inc.) antibodies with appropriate secondary fluorochrome conjugated antibodies, as previously described [16].

Image J counting software [21, 22] was used to count γ H2AX and Rad51 nuclear foci across three microscope fields for both treated (DNA damage induced) and control cells. The average number of foci per cell was expressed as percentage of untreated controls. Reliability, reproducibility, and validity of our data were confirmed by repeated tests across different fields, comparison of two counting methods (manual and software), and counting by two independent reviewer. Cells were

classed as HR competent if there was more than a 2 fold increase in Rad51 foci after DNA damage, confirmed by a 2 fold increase in γ H2AX [15].

Results

Patient demographics

Patient demographics for patients with solid and ascitic cultures are summarised in Table 2. In total PCO cultures from ascites with matched solid tumour were cultured from 25 patients. Collection was not restricted by histological subtype or collection at primary surgery only ensuring all patterns of heterogeneity were captured. Solid tumour was sampled from pelvic tumour only in 13 patients and in pelvic and intra abdominal deposits for the remaining 12 patients, giving an overall total of 68 cultures.

Intra-tumour heterogeneity of HR functional status

When considered individually, of the 68 subcultures tested, 32 (47.1%) were HR competent and 36 (52.9%) were HR defective, supplementary Table 2 for raw Rad51 data.

HR function was homogeneous throughout all subcultures from an individual PCO patient in 13 of the 25 patients tested (52%). Of these 13 homogeneous PCO patients, five were universally HR defective and eight were universally HR competent. Conversely there was heterogeneity of HR function between subcultures taken from the same patient in 12/25 (48%) cases.

Combining results from all 25 patients but considering only a single biopsy, reflecting current diagnostic clinical practice, and comparing this to the HR status of the ascites, concordance rates for HR function were 19/25 (76%) cases. Concordance of HR function decreased to 3/12 (27%) when HR results were included from additional tumour sampling from two or more spatially distinct areas of solid tumour. Functional HR heterogeneity was not predictable based upon histological subtype,

immunohistochemistry of FFPE or immunofluorescent detected antigen expression. Intra-tumour heterogeneity of antigen expression is seen even within the same morphological group.

Histological classification or characterisation of protein expression by immunohistochemistry or immunofluorescent microscopy has no clinical relevance in terms of response to therapy

All subcultures were characterised in terms of morphology and classified as cobblestone, spindle cell or mesenchymal phenotype, Figure 1. In addition to morphology of actual cultures formal histopathological examination, supplemented with immunohistochemistry, of matched FFPE samples was also performed. The majority of the cohort (19/25, 76%) had high grade serous disease with a cobblestone monolayer appearance and almost all (23/35, 92%) had homogenous histology.

Inter- and intra-tumour heterogeneity was seen between the expression of epithelial and ovarian markers using the immunofluorescent characterisation panel, 2. The majority of the tumours showed expression of epithelial as well as mesenchymal markers using both techniques. Vimentin expression was universal throughout all subcultures.

PCO samples taken from nine patients showed completely homogeneous staining patterns between all subcultures from the same patient with no difference in expression of any markers between ascitic and solid subcultures for epithelial, mesenchymal or ovarian markers.

However for 16 patients there was non concordance between at least one of the markers. With the exception of vimentin, concordance rates decreased as the number of samples analysed for each patient was increased from two (ascites and one solid sample) to three (ascites and two paired solid samples), Table 3.

Heterogeneity of expression was seen universally amongst all antigen groups (epithelial and ovarian). This heterogeneity was not reflected in the histological classification of the tumours with only 2/16 (12.5%) patients with heterogeneous antigen expression being classified as having mixed histological subtypes on formal pathology, $\kappa = -0.560$ (95%CI -0.780 to -0.340).

Ex vivo growth rate is highly variable and heterogeneous between and within PCOs

The median doubling time for all cultures was 126 hours, range 55 - 303. There was no significant difference between the median doubling times of HR competent (126 hours, 55 - 303) and HR defective tumours (128 hours, 81 - 233), ($p=0.2543$). The median doubling time for solid cultures (120 hours, 82 - 279) was lower than for ascitic cultures (159 hours, 55 - 303), ($p=0.0142$). The inter- and intra-tumour doubling times were highly variable. When comparing all subcultures from each PCO patient, there was no significant difference in the doubling times between PCO patients (ANOVA, $p=0.1425$) but there was a significant difference between the SD of each PCO group of subcultures (Brown-Forsythe, $p = 0.0098$) indicating that intra-tumour heterogeneity is greater than inter-tumour heterogeneity in terms of growth, Figure 2.

Sensitivity to cisplatin and rucaparib correlates with HR functional status

Sensitivity to rucaparib and cisplatin was assessed for 59/68 subcultures generated. There was 84.7% concordance between HR status and rucaparib sensitivity and 75.5% concordance between HR status and platinum sensitivity. When subcultures are grouped together according to HR function, HR defective cultures were sensitive to both cisplatin and rucaparib with mean GI_{50} of 4.02 μM and 9.73 μM respectively; compared to HR competent cultures, with mean GI_{50} of $>10 \mu M$ and $>100 \mu M$, ($p < 0.0001$), 4.

Discussion

In this study we add to current evidence of inter-tumoural heterogeneity in ovarian cancer in terms of antigen expression and proliferation rate, and DNA repair HR function from ascites. Additionally, we provide new evidence that a large proportion of ovarian cancer patients have intra-tumoural heterogeneity in terms of HR function with approximately 50% of tumours demonstrating intra-tumoural heterogeneity in this feature. The correlation of HR status with cytotoxic sensitivity to cisplatin and PARP inhibitors is important as it demonstrates the relevance of the approach of functional analysis. This heterogeneity of the function biomarker HR is not related to or predictable using histological subtype.

Bashashati *et al* have recently described extensive intratumoural mutational diversity in a small panel of high grade serous cancers with TP53 being the only somatic mutation present in all samples and only 51.5% concordance in the presence of other driver mutations across all samples from each patient [13]. This builds upon previous work by Khalique *et al* who suggested that ovarian cancer develops by a non linear clonal evolutionary process [23]. Taken together these findings suggest a model in which early divergence gives rise to clones with different driver mutation phenotypes, the only common feature being mutation of TP53. Given that dysfunction of DNA damage repair pathways are also key driver events [24] it is perhaps unsurprising therefore to see variable DNA repair status in different areas of the same tumour representing these different, early divergent, clones.

By correlating the results of functional analysis of the HR pathway with *ex vivo* response to chemotherapy for each subculture studied we suggest that this divergence and subsequent heterogeneity has an important implication for clinical practice both in terms of partly explaining the variable response to chemotherapy but also in determining the limited benefit of relying on the results of single biopsies to stratify treatment.

The main limitation of this work is the relatively small number of patients included (25 patients contributing 68 successful primary cultures). The impact of intra-tumoural functional heterogeneity upon PFS and OS is difficult to interpret in this small cohort and is additionally complicated by the many variable clinical factors, in particular the timing and end result of cytoreductive surgery. In order to correct analyses for the patient and histological variances the sample size required is large and may in fact require a multi-centre approach. This study does raise the possibility that tumours demonstrating significant intra-tumoural heterogeneity at presentation may be more likely to demonstrate resistance to chemotherapy. The finding of heterogeneity in tumours even following neoadjuvant chemotherapy (commonly 3-4 cycles of carboplatin with or without taxane), demonstrates that the selection pressure of chemotherapy may be less than previously thought and reinforces the importance of thorough sampling in every tumour at surgery irrespective of preceding therapy .

We have previously demonstrated a very strong correlation between HR status and *ex vivo* sensitivity to the PARP inhibitor rucaparib using cells cultured from just one compartment, namely ascites [16] with responses seen in approximately 50% of cases [3]. In clinical practice however, response rates to olaparib in non germline BRCA 1 and 2 patients are only 24% [25]. This may be a result of underlying heterogeneity at presentation and subsequent clonal selection pressure as a result of initial chemotherapy.

The demonstration in this study that the results of predictive biomarkers will be affected by intra tumour heterogeneity has ramifications for future translational research and biomarker directed therapy. It is crucial that future studies include multiple biopsies to allow assessment of intra tumour heterogeneity and that protocols which require sequential biopsies taken before and after treatment attempt to collect these biopsies from the same tumour site to minimise the risk of selecting non paired clones.

Further work is required to extend this work. In particular correlation of heterogeneity with disease location (intra peritoneal versus extra peritoneal) would provide insight into the biology of the metastatic process in ovarian cancer.

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Legends to figures

Figure 1: Brightfield microscopy (x10) demonstrating cobblestone (PCO 174 – ascites), mesenchymal (PCO 179 – ascites) and spindle cell morphology (PCO 226 – ovary).

Figure 2: representative images demonstrating intra tumour heterogeneity from a single patient. (A) Cultures were generated from 6 spatially distinct deposits. (B) expression of immunofluorescent markers varied between early passage cultures. (C) rucaparib sensitivity assays revealed two distinct phenotypes. These correlated with HR status.

Figure 3 Growth rate of patient samples shows high intra and inter tumour variability. The doubling time of individual cultures was estimated and is expressed as median (range) for each set of patient samples

Figure 4 Sensitivity to rucaparib and cisplatin by HR function. A total of 59 subcultures were generated and treated with (A) rucaparib or (B) cisplatin. Those cultures deemed HR defective had greater sensitivity to both agents ($p < 0.0001$)

Tables

Table 1 – Characterisation panel antibodies

Marker	Description	Concentration	Company
Pancytokeratin	PCO samples were classified to be epithelial in origin if more than 95% of cells stained with mouse monoclonal anti-pancytokeratin FITC-conjugated antibody. Clone C11	1:100	Upstate Millipore Corp., USA
Epithelial cell adhesion molecule (EpCAM)	Epithelial origin was confirmed with positive staining for 1:100 mouse monoclonal anti-CD326 (EpCAM) Alexafluor® 488-conjugated antibody. Clone 9C4	1:100	Biolegend, USA
Cancer Antigen 125 (CA125)	CA125 is a tumour marker expressed in approximately 80% of epithelial ovarian cancers [29]. Expression was assessed using mouse monoclonal anti-CA125 antibody and Alexafluor® 546 goat anti-mouse secondary antibody. Clone EPR1020(2)	1:100 (Primary) 1:1000 (Secondary)	Abcam, USA Invitrogen, USA
Epithelial related antigen (MOC-31)	Epithelial transmembrane glycoprotein 2 (EGP-2, also known as ESA, GA733-2, KSA) is present on most normal and malignant epithelia [30] enabling discrimination from mesothelial derived tumours. Expression was assessed using mouse monoclonal anti-MOC-31 antibody and Alexafluor® 596 goat anti-mouse secondary antibody. Clone MOC-31	1:100 (Primary) 1:1000 (Secondary)	Dako, Germany Invitrogen, USA
Vimentin	Marker commonly used to detect epithelial-mesenchymal transition (EMT). Expression was assessed using rabbit monoclonal anti-vimentin antibody, clone EPR3776 and Alexafluor® 488 goat anti-rabbit secondary antibody	1:100 (Primary) 1:1000 (Secondary)	Abcam, USA Invitrogen, USA

Table 2 – Summary of patient demographics

Demographic		Median (range) / n (%)
Patient age (years)		63 (43 - 83)
FIGO Stage		
	Stage 2	1 (4)
	Stage 3	19 (76)
	Stage 4	5 (20)
Serum CA125 at presentation (U/I)		600 (57 – 9740)
Histology		
	High grade serous carcinoma	19 (76)
	Clear cell	2 (8)
	Endometrioid	1 (4)
	Other	3 (12)
Surgery		
	Primary	16 (64)
	IDS	9 (36)
Surgical outcome		
	Optimal/Complete	22
	Suboptimal	2

Table 3 – Concordance of antigen expression

Marker	Two sample concordance ¹ (%) (n=25)	Three sample concordance ² (%) (n=12)
CK	92	58
CA125	68	42
EpCam	56	36
MOC31	65	36
Vimentin	100	100
HR status	76	27

¹ concordance between ascitic sample and single paired solid sample

² concordance between ascitic sample and two paired solid samples