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Assessing the function of homologous recombination DNA repair in malignant pleural effusion (MPE) samples

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Background: Patients with malignant pleural effusions (MPEs) generally have advanced disease with poor survival and few therapeutic options. Cells within MPEs may be used to stratify patients for targeted therapy. Targeted therapy with poly(ADP ribose) polymerase inhibitors (PARPi) depends on identifying homologous recombination DNA repair (HRR)-defective cancer cells. We aimed to determine the feasibility of assaying HRR status in MPE cells.

Methods: A total of 15 MPE samples were collected from consenting patients with non-small-cell lung cancer (NSCLC), mesothelioma and ovarian and breast cancer. Primary cultures were confirmed as epithelial by pancytokeratin, and HRR status was determined by the detection of γ H2AX and RAD51 foci following a 24-h exposure to rucaparib, by immunofluorescence microscopy. Massively parallel next-generation sequencing of DNA repair genes was performed on cultured MPE cells.

Results: From 15 MPE samples, 13 cultures were successfully established, with HRR function successfully determined in 12 cultures. Four samples – three NSCLC and one mesothelioma – were HRR defective and eight samples – one NSCLC, one mesothelioma, one sarcomatoid, one breast and four ovarian cancers – were HRR functional. No mutations in DNA repair genes were associated with HRR status, but there was probable loss of heterozygosity of FANCG, RPA1 and PARP1.

Conclusions: HRR function can be successfully detected in MPE cells demonstrating the potential to stratify patients for targeted therapy with PARPi.

Malignant pleural effusions (MPEs), characterised by the accumulation of pleural fluid containing malignant cells, usually indicate advanced or disseminated disease with a poor median survival of between 3 and 12 months, depending on the type and stage of cancer (Roberts *et al*, 2010). The most common cause of MPE is lung cancer, which accounts for ~30–40% of cases, followed by breast and ovarian cancers and lymphoma, with mesothelioma being the predominant cause of primary pleural neoplasm associated with pleural effusions (Roberts *et al*, 2010; Kastelik, 2013; Zarogoulidis *et al*, 2013). Modern cancer therapy depends on

patient stratification using predictive biomarkers. The cancer cells in MPEs may be used to stratify patients for appropriate therapy. For example, those from patients with non-small-cell lung cancer (NSCLC) can be used to assess the mutational status of epidermal growth factor receptor (EGFR) for EGFR-targeted therapy with gefitinib (Hung *et al*, 2006).

Targeted therapy with poly(ADP ribose) polymerase inhibitors (PARPi), which are novel agents selective for cancers with dysfunctional homologous recombination DNA repair (HRR) (Bryant *et al*, 2005; Farmer *et al*, 2005), depends on the

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identification of HRR dysfunction. PARPi have promising anti-cancer activity with minimal toxicity in clinical trials in patients carrying mutations in *BRCA1/2*, which encode key proteins in the HRR pathway (Fong *et al*, 2009; O'Shaughnessy *et al*, 2011; De Bono *et al*, 2013; Kaufman *et al*, 2013; Ledermann *et al*, 2013; Michie *et al*, 2013). However, HRR is a multifactorial process, and screening for BRCA mutations alone is likely to underestimate the proportion of cancers with HRR defects that could potentially benefit from PARPi therapy (McCabe *et al*, 2006).

There is a need to develop indicators of HRR defects that may be used as predictive biomarkers for PARPi sensitivity. Different approaches to develop these are currently underway, such as integrated genomic analysis of HRR genes and aCGH to identify genomic instability (CGRN, 2011; Vollebergh *et al*, 2011, 2012). Alternatively, HRR function may be assessed by the ability to form RAD51 foci. RAD51 is a key protein in HRR, and its relocation onto damaged DNA is necessary for the obligatory strand-invasion step of HRR. The RAD51 focus assay correctly identified cells defective in HRR owing to *BRCA1/2* mutation and *BRCA1* epigenetic silencing (Drew *et al*, 2011b). Further application of this assay to primary cultures of epithelial ovarian cancer derived from ascitic fluid demonstrated that ~50% had dysfunctional HRR. Importantly, RAD51 focus formation correlated with *ex vivo* PARPi sensitivity in >90% of cases and was an independent prognostic indicator of survival following platinum-based therapy (Mukhopadhyay *et al*, 2010, 2012). The RAD51 focus assay has also identified HRR defects in other patient-derived tissues: cultured breast and NSCLC biopsies, and acute myeloid leukaemia cells (Willers *et al*, 2009; Birkelbach *et al*, 2013; Gaymes *et al*, 2013). It has even been extended to FFPE blocks of breast tumour biopsies from patients undergoing neoadjuvant chemotherapy (Graeser *et al*, 2010).

The aim of this feasibility study was to extend the application of the RAD51-based HRR functional assay to primary cultures derived from MPEs to estimate the frequency of HRR defects in this population and to potentially identify those patients for whom PARPi therapy could be an option. We show that it is a viable assay for this tissue type and identified HRR defects in NSCLC and mesothelioma samples from patients. In addition, we have undertaken genetic analysis of a selection of HRR-competent and HRR-defective MPE samples; however, this did not reveal any trend in pathogenic/rare genetic variants or probable loss of heterozygosity (pLoH).

MATERIALS AND METHODS

Chemicals and reagents. All routine and tissue culture chemicals and reagents were obtained from Sigma Aldrich (Poole, UK) unless otherwise stated. Rucaparib was a kind gift from Zdenek Hostomsky, Pfizer GRD (La Jolla, CA, USA).

Tissue collection and development of primary cultures. Ethical approval and specific consent were obtained for the collection of clinical material and patient data (REC 12/NW/0202). Primary cultures were derived from MPEs from cancer patients undergoing routine thoracentesis at hospitals in Newcastle upon Tyne and Gateshead. MPE samples were transported to the laboratory and processed immediately and in accordance with the regulations of the Human Tissue Act 2004 (UK) and local guidelines.

Briefly, 50 ml of MPE was centrifuged and the cells and debris were concentrated in 5 ml of the supernatant, mixed with 5 ml of RPMI-1640, supplemented with 20% fetal bovine serum (FBS) and 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin, and cultured in a 25-cm³ tissue culture flask at 37 °C with 5% CO₂ for 3–12 days. Culture medium was replenished every 7 days, and cultures were passaged at a confluency of 60–80%; further

culturing was carried out with RPMI-1640 (10% FBS, 100 U ml⁻¹ penicillin/streptomycin) alone. All experiments were carried out on early-passage cultures (<4).

Immunofluorescence assays. Cells were seeded onto coverslips at a density of 0.25–1 × 10⁵ cells ml⁻¹ depending on cell numbers and incubated for 24–48 h in a 37 °C incubator with 5% CO₂. To confirm epithelial cell origin, cells were fixed and permeabilised with ice-cold methanol, washed with PBS with 0.4% Triton-X-100, blocked with PBS containing 2% (w/v) bovine serum albumin (BSA) and then incubated with 1:100 FITC-conjugated anti-pancytokeratin antibody (Merck Millipore, Watford, UK). Coverslips were mounted onto slides with mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) (Vectashield, Peterborough, UK).

For the HRR functional assay, DNA replication fork collapse was induced with 10 µM of the PARPi rucaparib (AG-014699) for 24 h before fixation and permeabilisation, as described previously (Mukhopadhyay *et al*, 2010). Coverslips were blocked with PBS containing 2% BSA (w/v), 10% skimmed milk powder (w/v) and 10% goat serum (v/v), and then incubated overnight at 4 °C with 1:100 anti-RAD51 antibody (PC130 Calbiochem, Merck Millipore). Coverslips were washed in PBS and then incubated with anti-phospho-Histone H2A.X (Ser139) (clone JBW301, Merck Millipore) diluted to 1:1000 in PBS containing 2% BSA (w/v). After washing with PBS containing 0.4% Triton-X-100, coverslips were incubated with secondary antibodies Alexa Fluor 546 Goat anti-mouse and Alexa Fluor 488 Goat anti-rabbit antibodies (Invitrogen, Life Technologies, Paisley, UK), diluted to 1:1000 and incubated in the dark to visualise γH2AX and RAD51, respectively. Coverslips were mounted as described earlier.

Fluorescence microscopy was conducted using a Leica DMR microscope (Leica microsystems GmbH, Wetzlar, Germany) or a confocal microscope. The number of γH2AX and RAD51 foci was analysed in >50 cells per condition by using the ImageJ software with the PZFociEZ macro (www.pzfociez.com).

Next-generation sequencing. Genomic DNA extracted from cancer cells cultured from MPEs was sheared to a mean length of 500 bp using nitrogen nebulisation. A custom-made Nimblegen SeqCap EZ library (Nimblegen, Madison, WI, USA) was used to enrich for the sequence of interest. This comprised the exonic regions of >180 DNA repair genes and limited intronic material. Captured sequence was subjected to massively parallel next-generation DNA sequencing by using the Roche 454 GS FLX platform (454 Life Science, Branford, CT, USA). A bespoke analysis pipeline was used to identify pathogenic changes and potential variants of interest (single nucleotide polymorphisms with <1% population prevalence) across our target genes. pLoH – that is, loss of genetic variation, rather than copy number – was evaluated by plotting the percentage reads for every detected variant across individual genes. Tracts of contiguous variants detected at >80% reads were considered pLoH.

Statistical analyses. GraphPad Prism (version 6.00, San Diego, CA, USA) was used for analyses. Univariate analysis of overall survival (OS) was carried out by generating a Kaplan–Meier survival curve; statistical significance was derived from Mantel–Cox log-rank tests for statistical significance.

RESULTS

Establishing primary cultures from pleural effusion samples. MPE samples were collected from patients diagnosed with different cancers (ovarian, breast, NSCLC and mesothelioma); patient characteristics are described in Table 1. A total of 15 MPE samples were collected and primary cultures were established (denoted

primary pleural effusion (PPE)) by using an optimised methodology based on that established previously for ascites cells (Mukhopadhyay *et al*, 2010). PPE cultures were established for 13 out of 15 samples; 2 samples (PPE004 and PPE005, ovarian cancers) were contaminated from the outset of culturing and were not assessed further. PPE cultures grew as an adherent monolayer in discreet patches; the majority of cells had a polygonal shape and displayed a cobblestone-like appearance at confluency (Figure 1A). Epithelial cell cultures were confirmed with pancytokeratin immunofluorescence; all PPE cultures had >90% cells with a positive pancytokeratin phenotype (Figure 1B).

Determining HRR status in primary cultures. The HRR status of primary cultures was assessed using the immunofluorescence-based γ H2AX and RAD51 assay. A greater than 2-fold increase in γ H2AX foci was taken as confirmation that stalled/collapsed replication forks and/or DNA double-strand breaks (DSB) had been generated. Cells were defined as HRR competent if a ≥ 2 -fold increase in RAD51 foci was also observed and HRR defective if there was no significant increase in RAD51 foci (Figure 2A). Using these thresholds, DNA damage was induced in 12 out of 13 samples analysed. DNA DSBs were not induced in PPE011 (ovarian cancer). Of the remaining samples, eight were identified as HRR competent (four ovarian cancer, one mesothelioma, one sarcomatoid lung carcinoma and one breast cancer) and four were found to be HRR defective (three NSCLC and one mesothelioma) (Figure 2B, Table 2).

Clinical data. Defects in HRR render cells highly sensitive to platinum agents, and, in our previous studies, patients with ovarian cancer identified as HRR defective had greater than OS following platinum-based therapy (Mukhopadhyay *et al*, 2012). To investigate whether the HRR status of the PPEs in this cohort of patients had any bearing on response to therapy or survival, clinical data were collected. Of the 12 patients in whom tumour HRR status was determined, only the patients with ovarian cancer and breast cancer and one of the patients with mesothelioma received anticancer therapy (Table 2). None of the patients with NSCLC were fit enough to receive platinum-based therapy, but the patient with the HRR-defective mesothelioma received platinum-based therapy. There was no statistical difference in the OS between the HRR functional and dysfunctional patients (data not shown).

Genetic investigation. The exonic regions of >180 DNA repair genes were captured and subjected to massively parallel sequencing (full list of genes studied provided in Supplementary Figure 1). Known pathogenic variants and variants with a reported population prevalence <1% were identified. No pathogenic/rare genetic variants or pLoH were observed recurrently in either HRR-competent or dysfunctional PPE cultures (Figure 3). Our results do not support the role of any single gene in determining HRR status in tumours generally. pLoH was restricted to HRR-defective tumours for FANCG, PARP1 and RPA1. We have previously used this simple approach to identify regions of pLoH in tumour samples that were confirmed by array comparative genomic hybridisation to be monoallelic, as a result of both interstitial duplication and deletion (data not shown). Our chosen sequencing platform does not lend itself to the evaluation of copy number in these tumours, owing to the comparatively modest read depth.

Patient characteristics (n = 12)		Value
Sex – number (%)		
Female		8 (67)
Male		4 (33)
Age (years)		
Mean		74
Range		59–82
Cancer type – number (%)		
NSCLC		4 (33)
Mesothelioma		2 (17)
Other lung		1 (8)
Ovarian		4 (33)
Breast		1 (8)

Abbreviation: NSCLC = non-small-cell lung cancer. Data from patients donating MPE samples; other lung = sarcomatoid lung/??sarcomatoid mesothelioma.

DISCUSSION

In this feasibility study, we have shown that we are able to culture a variety of different cancer cell types from MPEs with an 80% success rate. Furthermore, we were able to determine the HRR status of 12 out of 13 samples that were successfully cultured. Therefore, cells from MPEs represent a source of patient-derived tumour material that could potentially be used to stratify patients for either PARPi or platinum-based therapy. We believe that MPEs represent a valuable resource that would otherwise be disposed of.

We have established that around 35% of PPE cultures overall were HRR dysfunctional. This relatively high frequency may reflect that the loss of a high-fidelity DNA repair pathway can generate genomic instability that is an enabling characteristic of cancer (Hanahan and Weinberg, 2011). Defective DNA repair may underlie the response of tumours to DNA-damaging anticancer therapy (Curtin, 2012). Indeed, defects in DNA repair, particularly those associated with HRR, are a common feature of adult cancers,

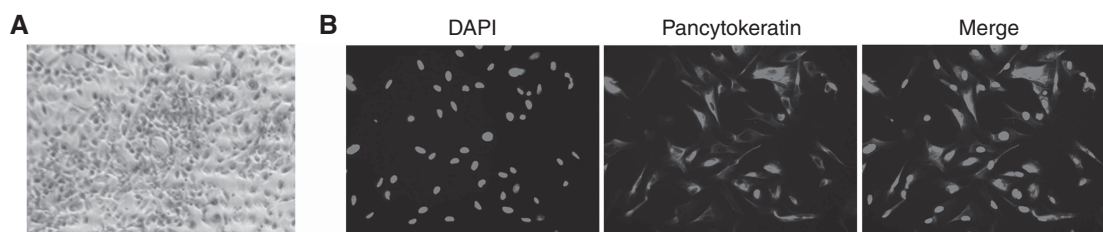


Figure 1. Pleural effusion primary cultures (A) Primary cultures established from pleural effusion samples grew as a monolayer exhibiting a polygonal cell morphology and a cobblestone-like appearance at confluency. (B) Epithelial cell growth in primary cultures assessed by pancytokeratin staining; cell nuclei are visualised with DAPI. Cultures with >90% positive cells were analysed further. Example images are taken from PPE007, a pleural effusion from a breast cancer.

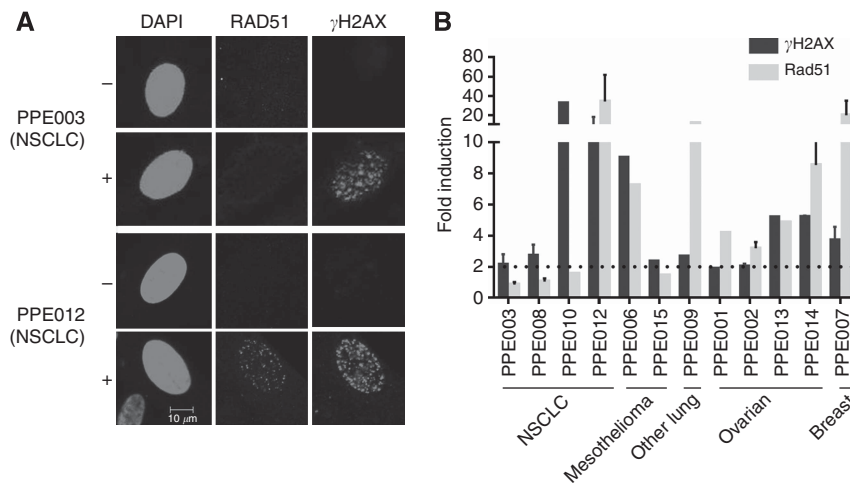


Figure 2. HRR status in primary cultures. (A) Example immunofluorescence microscopy in primary cultures following DSB induction with a 24-h exposure to rucaparib. In HRR-competent cultures (PPE012, NSCLC), increased levels of γ H2AX (indicating DSB) and RAD51 (indicating HRR) foci are seen in the nucleus following rucaparib treatment; however, in cultures with dysfunctional HRR (PPE003, NSCLC), only increased levels of γ H2AX foci are seen following rucaparib treatment (+) compared with untreated control (-). **(B)** HRR status of primary cultures. Average number of γ H2AX and RAD51 foci per cell was determined by foci counting (ImageJ). Foci numbers were normalised to the control and expressed as a fold induction following rucaparib treatment. A 2-fold induction (dashed line) was set as the threshold for γ H2AX and RAD51 induction. Data are representative of 2–3 independent experiments (PPE002, 003, 007, 008, 009, 012, 014), with error bars indicating the s.e.m., or a single experiment (PPE001, 006, 010, 013, 015) where sample sizes were small or cultures stopped proliferating at an early passage.

Table 2. Pleural effusion sample details and patient demographics

Cancer type	Histological subtype	Sample	HRR status	Prior therapy	OS (days)
NSCLC	Adenocarcinoma	PPE003	-	None	396
	Adenocarcinoma	PPE008	-	None	37
	Adenocarcinoma	PPE010	-	None	61
	Adenocarcinoma	PPE012	+	None	31
Mesothelioma	Not diagnostic, radiologically defined	PPE006	+	None	137
	Epithelioid	PPE015	-	4 cycles carboplatin/pemetrexed	168
Sarcomatoid lung carcinoma/??sarcomatoid mesothelioma	Sarcomatoid lung carcinoma/??sarcomatoid mesothelioma	PPE009	+	None	103
Ovarian	Adenocarcinoma	PPE001	+	6 cycles carboplatin/paclitaxol followed by 3 cycles topotecan	7
	Brenner tumour	PPE002	+	Unknown	321
	Papillary serous carcinoma	PPE013	+	3 cycles carboplatin	NA
	Papillary serous carcinoma	PPE014	+	6 cycles carboplatin/paclitaxol followed by 6 cycles carboplatin	33
Breast	Carcinoma	PPE007	+	None	279

Data from all established PPE cultures with histological subtype, HRR status data (HRR-competent samples denoted as + and dysfunctional HRR samples -), overall survival (OS) calculated in days following sample donation and NA (not available) denotes that the patient is living. Data were used to generate a Kaplan–Meier survival curve; Mantel–Cox log-rank tests showed no significant difference in OS between HRR-competent and dysfunctional patients ($P=0.45$).

as well as paediatric cancer syndromes (Kennedy and D'Andrea, 2006). We may even have underestimated the proportion of HRR-defective tumours, as recent evidence indicates that some NSCLC cell lines have defects in HRR downstream of RAD51 focus formation, and instead fail to resolve these foci leading to their persistence (Kommajosyula *et al*, 2013; Postel-Vinay *et al*, 2013). Adaptation of the current HRR functional assay in future studies will allow detection of defects in the resolution of RAD51.

Our previous studies, and those of others, indicate that around 50% of ovarian cancers are HRR defective (Mukhopadhyay *et al*, 2010; CGRN, 2011). It was therefore somewhat surprising that none of the four PPE cultures derived from ovarian cancers were HRR defective. This may be due to chance in this small sample size. Alternatively, the platinum-based therapy that the patients

received (Table 2) may have led to the restoration of HRR function or selection of HRR functional clones from a heterogeneous tumour. Restoration of HRR function in cancers associated with *BRCA1* and *BRCA2* mutations has been observed in both the laboratory and clinical setting (Patel *et al*, 2012).

Interestingly, three of the four NSCLC samples analysed in this pilot study were found to have dysfunctional HRR. Evidence of HRR dysfunction in lung cancer is emerging. A recent study identified that 4 out of 16 NSCLC cell lines failed to form RAD51 foci following cisplatin-induced DNA damage and that this correlated with olaparib (a PARPi) sensitivity for 14 out of 16 cell lines (Birkelbach *et al*, 2013). Furthermore, this group identifies two tumours with a low RAD51 score, indicative of dysfunctional HRR, in fresh tumour tissues from 13 patients with

NSCLC were too ill to receive conventional chemotherapy. However, the patient with HRR-defective mesothelioma underwent several rounds of carboplatin/pemetrexed therapy (Table 2) before succumbing to the disease. On the basis of only one patient, it is impossible to establish whether this response to therapy was due to an HRR defect, although the data are encouraging.

NSCLC patients are often not eligible for platinum-based chemotherapy owing to concomitant illness. Given that PARPi are selectively toxic to HRR-defective tumour tissue while causing negligible clinical toxicity (Fong *et al.*, 2009, 2010; Schelman *et al.*, 2011; Drew *et al.*, 2011a), treating NSCLC patients with a PARPi may be an attractive option. Excitingly, there are indications that PARPi may be of benefit in the treatment of NSCLC. A phase I dose escalation study of the PARP inhibitor niraparib (MK4827) included two patients with NSCLC. In one patient (carrying a *BRCA2* mutation), stable disease was observed for 175 days, and in the other (with platinum-sensitive disease) for 316 days (Sandhu *et al.*, 2013). A further application of the HRR functional assay using MPEs may be in the selection of SCLC patients for PARPi treatment; a phase I trial for the PARPi BMN 673 is currently underway and recruiting SCLC patients (NCT01286987, clinicaltrials.gov). We propose that this feasibility study may be extended to a larger cohort of cancer patients presenting with MPEs and may be used in stratifying patients, in particular NSCLC patients, to receive PARP inhibitor treatment.

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