Targeting BRCA1-BER deficient breast cancer by ATM or DNA-PKcs blockade either alone or in combination with cisplatin for personalized therapy

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ABSTRACT

BRCA1, a key factor in homologous recombination repair may also regulate base excision repair (BER). Targeting BRCA1-BER deficient cells by blockade of ATM and DNA-PKcs could be a promising strategy in breast cancer. We investigated BRCA1, XRCC1 and pol β protein expression in two cohorts (n=1602 sporadic and n=50 germ-line BRCA1 mutated) and mRNA expression in two cohorts (n=1952 and n=249). Artificial neural network analysis for BRCA1-DNA repair interacting genes was conducted in 249 tumours. Pre-clinically, BRCA1 proficient and deficient cells were DNA repair expression profiled and evaluated for synthetic lethality using ATM and DNA-PKcs inhibitors either alone or in combination with cisplatin. In human tumours, BRCA1 negativity was strongly associated with low XRCC1, and low pol β at mRNA and protein levels ($p<0.0001$). In patients with BRCA1 negative tumours, low XRCC1 or low pol β expression was significantly associated with poor survival in univariate and multivariate analysis compared to high XRCC1 or high pol β expressing BRCA1 negative tumours ($ps<0.05$). Pre-clinically, BRCA1 negative cancer cells exhibit low mRNA and low protein expression of XRCC1 and pol β. BRCA1-BER deficient cells were sensitive to ATM and DNA-PKcs inhibitor treatment either alone or in combination with cisplatin and synthetic lethality was evidenced by DNA double strand breaks accumulation, cell cycle arrest and apoptosis. We conclude that XRCC1 and pol β expression status in BRCA1 negative tumours may have prognostic significance. BRCA1-BER deficient cells could be targeted by ATM or DNA-PKcs inhibitors for personalized therapy.

INTRODUCTION

Breast Cancer Susceptibility Gene 1 (BRCA1) facilitates the efficient resolution of DNA double-strand breaks (DSBs) through homologous recombination (HR) (Caestecker and Van de Walle, 2013; Huen et al., 2010). Cells lacking functional BRCA1 protein have impaired HR, and thus dependent on the more error-prone non-homologous end joining (NHEJ) pathway leading to chromosomal instability that drive breast cancer development (Huen et al., 2010). In women, BRCA1 germ line mutation is associated with a 60%-70% lifetime risk of developing breast cancer (O'Donovan and Livingston, 2010). In the more common sporadic breast cancers, epigenetic silencing of the BRCA1 promoter has been reported in up to 11%-14% of tumours (Turner et al., 2004) and a dysfunctional BRCA pathway may also contribute to a BRCAness phenotype in about 25% of cancers (Turner et al., 2004). Base excision repair (BER) is critical for processing DNA damage caused by alkylation, oxidation, ring saturation, single strand breaks and base deamination. DNA polymerase β (pol β) and XRCC1 are key BER factors. PARP1 (poly [ADP-ribose] polymerase 1) may play an essential role in single strand break repair (SSBR), a BER-related pathway (Langelier and Pascal, 2013). The DNA repair intermediates generated during BER/SSBR, if unrepaired, may get converted to toxic double strand breaks (DSBs) (Dianov and Hubscher, 2013).

Emerging studies suggest a cross talk between BRCA1 and BER factors. BRCA1 mutated and basal-like breast cancer cells were found to be sensitive to oxidative DNA damage induced by H_2O_2 treatment. The increased sensitivity was associated with defective BER as assessed by cell based BER assay in BRCA1 deficient cells (Alli et al., 2009). In a more recent study, BRCA1 deficient cells were sensitive to methyl methane sulfonate (alkylating agent) and functional interaction between polβ and BRCA1 was demonstrated in that study

(Masaoka et al., 2013) implying a potential role for polβ in BRCA1 mediated DSB repair (Masaoka et al., 2013). In addition, BRCA1 has also been shown to be involved in the transcriptional regulation of BER factor such as OGG1, NTH1 and APE1 (Saha et al., 2010).

Synthetic lethality is a promising strategy for personalized cancer therapy. PARP [poly-(ADP-ribose) polymerase] inhibitors induce synthetic lethality in germ line BRCA1-deficient breast cancers and demonstrate clinical benefit in patients (Lord and Ashworth, 2008). The data provides compelling reasons to investigate other potential synthetic lethal interactions targeting DNA repair for clinical application. Cells that are BRCA1 deficient as well as BER impaired may be reliant upon other back-up repair pathways to maintain genomic integrity and survival. ATM and DNA-PKcs play essential roles in the DNA damage response (DDR) and link DNA damage sensing to DDR effectors that regulate cell cycle progression and DNA repair (Shiloh and Ziv, 2013). ATM, a member of the phosphatidylinositol-3-kinase-like protein kinase (PIKK) family, is a key sensor and transducer of DNA damage signalling during homologous recombination (HR) (Lee and Paull, 2007; Shiloh and Ziv, 2013). ATM recruitment at sites of DNA damage may be dependent upon functional BRCA1 in cells (Lee et al., 2010). DNA-PKcs is another key member of the PIKK family and a critical component of non-homologous end-joining (NHEJ) pathway required for repair of DSBs generated throughout the cell cycle(Hill and Lee, 2010). BRCA1 through a role in DNA end-processing may also be involved in the regulation of NHEJ (Durant and Nickoloff, 2005).

Our hypothesis is that impaired BER in BRCA1 deficient tumours may influence prognosis. BRCA1-BER deficient cells may be reliant upon ATM or DNA-PKcs mediated back-up pathways for cellular survival and could be targeted by synthetic lethality using inhibitors of ATM or DNA-PKcs.

MATERIALS AND METHODS

Clinical study

BRCA1 and BER protein expression analysis in Nottingham Tenovus Primary Breast Carcinoma cohort: The study was performed in a consecutive series of 1650 patients with primary invasive breast carcinomas who were diagnosed between 1986 and 1999 and entered into the Nottingham Tenovus Primary Breast Carcinoma series described previously (Sultana et al., 2013). Supplemental Table S1 summarizes patient demographics. Supplemental treatment data 1 summarizes various adjuvant treatments received by patients in this cohort.

BRCA1 and BER protein expression analysis in germ line BRCA1 deficient breast cancer: The demographics of a cohort of 50 germ-line BRCA1 mutated breast cancers confirmed by genetic testing is shown in supplementary table S6. All patients received surgery, adjuvant chemotherapy and radiotherapy according to our institutional policy (supplementary treatment data 1).

Tissue Microarrays (TMAs) and immunohistochemistry (IHC): Tumours were arrayed in tissue microarrays (TMAs) and immunohistochemically profiled for BRCA1, APE1, XRCC1, POLB, and other biological markers (Supplementary Table S2) as previously described (Sultana et al., 2013). Supplementary Table S2 summarizes immunohistochemistry protocols for the markers tested using the Bond Max automated staining machine and Leica Bond Refine Detection kit (DS9800) according to manufacturer instructions (Leica Microsystems). We have recently published optimisation and specificity of XRCC1 and pol β antibody used in the current study (Abdel-Fatah et al., 2014; Sultana et al., 2013). To validate the use of

TMAs for immunophenotyping, full-face sections of 40 cases were stained and protein expression levels of the different antibodies were compared. The concordance between TMAs and full-face sections was excellent (k = 0.8). Positive and negative (by omission of the primary antibody and IgG-matched serum) controls were included in each run.

Evaluation of immune staining: The tumour cores were evaluated by specialist pathologists blinded to the clinicopathological characteristics of patients, in two different settings. There was excellent intra and inter-observer agreements ($k > 0.8$; Cohen's κ and multi-rater κ tests, respectively). Whole field inspection of the core was scored and intensities of nuclear staining were grouped as follows: 0 = no staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining. The percentage of each category was estimated (0-100%). H-scores (range 0-300) were calculated by multiplying intensity of staining and percentage staining as previously described (Sultana et al., 2013). Supplementary Table S2 summarizes cut-offs for individual markers.

Statistical analysis: Data analysis was performed using SPSS (SPSS, version 17 Chicago, IL). Where appropriate, Pearson's Chi-square, Fisher's exact, Student's t and ANOVA one way tests were used. Cumulative survival probabilities were estimated using the Kaplan–Meier method, and differences between survival rates were tested for significance using the log-rank test. Multivariate analysis for survival was performed using the Cox proportional hazard model. A p value < 0.05 considered significant.

Transcript levels in the METABRIC (Molecular Taxonomy of Breast Cancer International Consortium) cohort: Investigation of the mRNA expression was performed in METABRIC cohort which refers to a set of 1980 breast cancer samples with a minimum of 5 years of clinical follow up where mRNA expression data was available (Curtis et al.). Patient demographics are summarized in supplementary Table S9. ER positive and/or lymph-

node negative patients did not receive adjuvant chemotherapy. ER negative and/or lymph-node positive patients received adjuvant chemotherapy. All the samples were analysed as triplicates. A sliding window analysis was used to identify a cut-off in gene expression values such that the resulting subgroups have significantly different survival courses.

Artificial neural network (ANN) analysis in Uppsala cohort: The demographics of the Uppsala cohort is summarized in supplementary Table S10 and mRNA analysis has been described previously (Bergh et al., 1995). All microarray data are accessible at National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/, accession number: GSE4922) (Pawitan et al., 2005). All data were normalized using the global mean method (MAS5), and probe set signal intensities were natural log transformed and scaled by adjusting the mean signal to a target value of log 500. The expression levels of the BRCA1, for the probe 204531_s_at located on the HG-U133A chip was utilized to generate the ANN based model as described previously (30) (Lancashire et al., 2010) (Lemetre, 2009). A non-linear, ANN modelling based, data mining approach was utilised to identify the best gene probes for sample classification as described previously (30). 47,293 probes were screened for each sample in the test set (n=249). The data mining algorithm comprised a three layer multilayer perception architecture modified with a feed forward back-propagation algorithm and a sigmoidal transfer function, as previously described (Lancashire et al., 2010). The network momentum and learning rate were respectively set as 0.1 and 0.5. Two hidden nodes were utilised. The output node was coded as 0 if a case was low BRCA1 expression (<the median) and 1 if high BRCA1 expression (>median). Inputs were ranked in ascending order based on their classification error. The top 50 predictive genes identified were merged with 150 gene probes involved in the DNA repair process and then applied to an ANN based network inference algorithm as described in earlier studies (Lemetre, 2009). This model predicted a weighted link (direction

and magnitude) between each of the gene probe markers. The 100 strongest interactions were then visualised as a map with Cytoscape (Smoot et al., 2011). In a second bioinformatics analysis step, we sought to obtain a robust ranking of genes that are differentially expressed between the mRNA BRCA1+ cases and the mRNA BRCA1- and have high predictive power, by applying an ensemble sample classification method within a leave-one-out cross-validation scheme. For this purpose, the 249 patient samples were first grouped into 249 different training/test set partitions, using 248 samples for the training sets and the remaining sample as the test set. For each of the 248 training sets differentially expressed genes were selected independently with the "Empirical Bayes moderated t-statistic" (Smyth, 2004) and used to train a machine learning model, which was evaluated based on the left-out sample (a procedure known as "external cross-validation"). To classify the left-out sample, the prediction results of four algorithms (Support Vector Machine, Random Forest, kNN and Prediction Analysis for Microarrays, with all parameters being optimised by using a grid search within a nested cross-validation) (Tibshirani et al., 2002) were combined to a majority-vote ensemble classifier as to compensate for the inevitable inherent biases and variances that exists amongst each of these machine learning algorithms. In order to rank the genes based on the cross-validation results, their frequency of occurrence in the list of significantly differentially expressed genes (p-value < 0.05) across different cross-validation cycles was recorded, and genes received higher scores the more often they had been selected. All steps of the analysis were conducted using an in-house web-application for microarray analysis, available at www.arraymining.net.

Pre-clinical study

Compounds and reagents: ATM inhibitors (KU55933 and KU60019) and DNA-PKcs inhibitors (NU7441 and NU7026) were purchased from Tocris Bioscience, UK. The

compounds were dissolved in 100% DMSO and stored at - 20ºC. Cisplatin was obtained from Nottingham University Hospitals.

Cell lines and culture media: BRCA1 deficient HeLa SilenciX® cells and control BRCA proficient HeLa SilenciX® cells were purchased from Tebu-Bio (www.tebu-bio.com). SilenciX cells were grown in DMEM medium (with L-Glutamine 580mg/L, 4500 mg/L D19 Glucose, with 110mg/L Sodium Pyruvate) supplemented with 10% FBS, 1% penicillin/streptomycin and 125 μg/ml Hygromycin B. MDA-MB-436 (BRCA1 deficient human breast cancer cells) was grown in DMEM (Sigma, UK) and MCF7 (BRCA1 proficient human breast cancer cells) was grown in RPMI1640 (Sigma, UK). All media used to culture human cancer cell lines were supplemented with 10% FBS (PAA, UK) and 1% penicillin/streptomycin.

Clonogenic survival assay: 200-500 hundred cells per well were seeded in six-well plates. Cells were allowed to adhere for 4 hours. Compounds (ATM inhibitors or DNA-PKcs inhibitors) were added at the indicated concentrations. For cisplatin combination studies, cells were initially treated with cisplatin for 16 hours and then gently washed twice with 1X phosphate buffered saline and incubated in fresh media with or without ATM or DNA-PK inhibitors at indicated concentration. The plates were left in the incubator for 12-14 days. After incubation, the media was discarded, fixed (with methanol and acetic acid mixture) and stained with crystal violet and counted. Surviving Fraction = [No. of colonies formed/ (No. of cells seeded x Plating efficiency)] x100. All clonogenic assays were done in triplicate.

Evaluation of drug interaction (Combination index): To investigate synergistic and additive activity, combination index was calculated as described previously (Berenbaum, 1981). If D (combination index) is <1 the effect of the combination is synergistic, whereas if D=1 or D is >1 the effect is additive or antagonistic respectively.

γH2AX immunocytochemistry: This assay was performed as described previously(Sultana et al., 2013). Briefly, cells were incubated in medium containing ATM inhibitor or DNA-PKCS inhibitor for 48 hours. For cisplatin combination studies, cells were initially treated with cisplatin for 16 hours and then gently washed twice with 1X phosphate buffered saline and incubated in fresh media with or without ATM or DNA-PK inhibitors at indicated concentration for 48 hours.

Flow cytometric analyses (FACS): Cells grown to sub-confluence were exposed to ATM or DNA-PKcs inhibitors either alone or in combination with cisplatin for 48 hours and collected by trypsinization and centrifugation (1000 rpm for 5 minutes). FACS was performed as described previously (Sultana et al., 2013).

Annexin V flow cytometric analyses: Cells grown to sub-confluence were exposed to ATM or DNA-PKcs inhibitors either alone or in combination with cisplatin for 48 hours and collected by trypsinization and centrifugation (1000 rpm for 5 minutes). The assay was performed as described previously (Sultana et al., 2013).

Quantitative real time PCR: RNA was extracted from cell lines using the RNeasy Mini Kit (Qiagen) and quantified using a microvolume spectrophotometer. cDNA synthesis was performed using the RT^2 First Strand Kit (Qiagen). Primers used for RT-PCR for BER genes are shown in supplementary table S9. Quantitative PCR was performed on an ABI prism 7700 (Applied Biosystems) using SYBR green detection (Applied Biosystems®, UK). The housekeeping gene GAPDH was used to standardise the samples.

RT² Profiler™ PCR Array for global DNA Repair expression analysis: To evaluate the expression of 84 DNA repair genes simultaneously, real-time PCR was performed using the RT² Profiler™ PCR Array for global DNA Repair expression analysis in technical triplicates

(ABI 7500 Fast Block Detection System; Applied Biosystems, Foster City, USA) and the data analysed as per manufacturer's recommendation. GAPDH was used for normalization of the data. A 2 fold change or greater change in expression was considered significant.

RESULTS

Clinical studies

BRCA1 negativity is associated with impaired XRCC1 and pol β protein expression in human sporadic breast cancers: A total of 1602 breast tumours were suitable for BRCA1 expression analysis. 1085/1602 (67.7%) of tumours were BRCA1 positive and 517/1602 (32.3%) were negative for BRCA1 expression (Figure 1A). As shown in supplementary Table S3, BRCA1 negative tumours were highly significantly associated with low XRCC1 ($p<0.00001$) and low polβ ($p<0.000001$). In the BRCA1 negative cohort we then evaluated clinicopathological associations of XRCC1 and pol β protein expression (Figure 1A). The data for XRCC1 and polβ are summarized in supplementary tables S4 and S5 respectively. Although no significant associations were seen with stage, tumour grade, tumour types or pleomorphism, BRCA1 negative/ polβ low tumours were more likely to be Bcl2 negative ($p=0.001$) and BRCA1 negative/ XRCC1 low tumours were more likely to be p53 negative ($p= 0.015$).

BRCA1 negative/low XRCC1 or BRCA1 negative/low polβ tumours are associated with poor breast cancer specific survival (BCSS): BRCA1 negativity was significantly associated with poor BCSS compared to BRCA1 positive tumours ($p<0.000001$) (Figure 1B) and is consistent with previous studies showing poor prognostic significance of BRCA1 silencing in sporadic breast tumours (Hsu et al., 2013; Wu et al., 2013). In the BRCA1 negative group we investigated the prognostic influence of XRCC1 and pol β. As shown in Figure 1C1, BRCA1 negative/ low XRCC1 tumours had worse BCSS compared to BRCA1 negative/ high XRCC1 tumours ($p=0.001$). Similarly, BRCA1 negative/ low polβ tumours had worse BCSS compared to BRCA1 negative/ high polβ tumours ($p=0.008$) (Figure 1D1). As BRCA1 negativity is likely to be associated with ER negative tumours we conducted

further analysis. In the BRCA1 negative/ER negative subgroup, low XRCC1 or low polβ remains associated with poor survival (p=0.033 and p=0.034 respectively, Figure 1C2 and 1D2). In the BRCA1 negative/ER positive subgroup, similarly, low XRCC1 was associated with poor survival (p=0.003, Figure 1C3) and although not significant there was trend with low polβ (p=0.121, Figure1D3). In multivariate cox regression analysis (Table 1), low XRCC1 (p=0.005) and low pol β (p=0.036) were independently associated with poor survival.

XRCC1 and pol β expression in germ-line BRCA1 mutated breast cancers: To investigate whether XRCC1 and polβ would also influence outcomes in germ-line BRCA1 deficient breast cancer we investigated a cohort of 50 germ-line BRCA1 mutated breast cancers. Demographics are summarised in supplementary table S6. No significant clinicopathological correlations were observed (supplementary tables S7 and S8). In this small exploratory cohort, low polβ (5/34 tumours) was significantly associated with poor survival (p=0.007) in germ line BRCA1 mutated breast cancers (Figure 2A) compared to high polβ (29/34 tumours). Low XRCC1 expression did not influence survival in this cohort (Supplementary Figure S1A)

Low *XRCC1* and low *pol β* transcript levels have prognostic significance in *BRCA1* mRNA low sporadic breast cancers: To confirm whether the association between BRCA1 and BER also operated at the mRNA level we investigated the Metabric cohort (n=1920, demographics summarized in supplementary table S9) and the Uppsala cohort (n=249, demographics summarized in supplementary table S10) cohorts where mRNA expression data was available. In ER+ tumours (n=1485, metabric), low BRCA1 (n=81) was associated with poor survival compared to high BRCA1 mRNA expressing tumours (n= 1404) (p= 0.0226, Figure 2B1). In the low BRCA1/ER+ group, low polβ (n=66) or low XRCC1 (n=42) remains associated with poor survival (ps= 0.038 and 0.0321 respectively) compared to high

polβ (n=14) or high XRCC1 (n=38) mRNA expressing tumours (Figures 2B1 and 2B2). In the ER- tumours (n=435, metabric), high BRCA1 (n= 385) was associated with poor survival compared to low BRCA1 mRNA expressing tumours (n=50) (p= 0.0365, Supplementary Figure S1B). In the low BRCA1/ER- group, low polβ (n=5) or low XRCC1 (n=17) remains associated with poor survival compared to high polβ (n=43) or high XRCC1 (n=31) mRNA expressing tumours [ps= 0.0224 and 0.0206 respectively) (Figures 2C1 and 2C2). In the Uppsala cohort, low polβ mRNA (36/175 tumours) was associated with poor survival in BRCA1 low mRNA breast cancers (p=0.03, Supplementary Figure S1C) compared to high polβ mRNA tumours (139/175 tumours). XRCC1 mRNA expression levels did not influence survival in the Uppsala cohort (Supplementary Figure S1D).

Artificial neural network (ANN), ensemble classification and cross-validation analysis for *BRCA1* interacting DNA repair genes: The top 100 strongest are shown in Figure 2D. The biological functions of *BRCA1* interaction genes are summarized in supplementary Table S12. The predominant interactions with genes involved in base excision repair, nucleotide excision repair, homologous recombination, non-homologous end joining, inter-strand crosslink repair, mismatch repair and transcription is not only consistent with the previously described functions of BRCA1 (Caestecker and Van de Walle, 2013; Huen et al., 2010; Silver and Livingston, 2012) but also reveals new BRCA1 interacting genes.

Pre-clinical studies

BRCA1 deficient cancer cells exhibit impaired BER expression: BRCA1 deficient HeLa SilenciX cells, control BRCA1 proficient HeLa SilenciX cells, BRCA1 deficient MDA-MB-436 breast cancer cells and BRCA1 proficient MCF7 breast cancer cells were initially examined for the expression of BRCA1, XRCC1 and pol β proteins. BRCA1 deficiency was first confirmed at the protein level in BRCA1 deficient HeLa SilenciX and MDA-MB-436

cells compared to control HeLa SilenciX cells and MCF7 cells (Figures 3A1, 3A2, 3B1, 3B2). The relative expression of XRCC1 and pol β was also found to be low in BRCA1 deficient cells compared to BRCA1 proficient cells at the protein level (Figures 3A1, 3A2, 3B1, 3B2). Low mRNA expression of BRCA1, XRCC1 and pol β was confirmed by qRT-PCR in MDA-MB-436 cells compared to MCF-7 cells and BRCA1 deficient HeLa SilenciX compared to control HeLa SilenciX cells (Figure 3A3, 3B3 respectively). The data is also summarized in supplementary Tables S14 and S15.

BRCA1 deficient cells have deregulated gene expression of multiple DNA repair pathways: To investigate whether down regulation of DNA repair is restricted to polβ and XRCC1 or also includes additional DNA repair pathways, we profiled a panel of 84 DNA repair genes in BRCA1 deficient and BRCA1 proficient cells using the RT^2 Profiler DNA Repair PCR array. All experiments were done in triplicates and DNA repair expression was compared between BRCA1 deficient and BRCA1 proficient cells [BRCA1 deficient HeLa SilenciX versus control BRCA1 proficient HeLa SilenciX cells and MDA-MB-436 versus MCF7 cells]. The data is summarized in Figure 3C (BRCA1 deficient and proficient HeLa SilenciX cells), supplementary table S16 (BRCA1 deficient and proficient HeLa SilenciX cells), Figure 3D (MDA-MB-436 and MCF7 cells), and supplementary table S17 (MDA-MB-436 and MCF7 cells). In MDA-MB-436 cells as well as in BRCA1 deficient HeLa SilenciX cells, we observed a consistent down regulation of several BER genes as well as genes involved in other pathways including base excision repair, nucleotide excision repair, homologous recombination, non-homologous end joining, inter-strand crosslink repair and mismatch repair.

BRCA1 deficient cancer cells are sensitive to ATM inhibitors either alone or in combination with cisplatin: KU55933 (2-morpholin-4-yl-6-thianthren-1-yl-pyran-4-one) is an ATP-competitive potent ATM inhibitor with an IC_{50} of 13 nmol/L (Hickson et al., 2004).

For additional validation we also tested KU60019 [(2*R*,6*S*-rel)-2,6-Dimethyl-*N*-[5-[6-(4-morpholinyl)-4-oxo-4*H*-pyran-2-yl]-9*H*-thioxanthen-2-yl-4-morpholineacetamide] another ATP-competitive potent ATM inhibitor (Golding et al., 2009). Treatment with KU55933 resulted in reduced survival of BRCA1 deficient HeLa SilenciX cells compared to BRCA1 proficient HeLa SilenciX cells (Figure 4A1). Similarly, MDA-MB-436 cells were sensitive to KU55933 compared to MCF7 cells respectively (Figures 4B1). As an additional validation we investigated KU60019. As shown in supplementary Figures S3A and S3E, BRCA1 deficient HeLa SilenciX cells and MDA-MB-436 cells were also sensitive to KU60019 compared to BRCA1 proficient cells.

To provide mechanistic evidence that ATM inhibition leads to a synthetic lethality effect in BRCA1 deficient cells, we investigated the functional consequence of ATM inhibition in BRCA1 proficient and BRCA1 deficient cells. Double strand breaks (DSBs) induce phosphorylation of H2AX at serine 139 (γH2AX), and accumulation of γH2AX foci in the nucleus is a marker of DSBs. Therefore, γH2AX immunocytochemistry was performed in BRCA1 deficient HeLa SilenciX cells and MDA-MB-436 cells and compared to BRCA1 proficient control SilenciX or MCF7 cells (Supplementary Figure S2A). Nuclei containing more than six γH2AX foci were considered positive. Cells were treated with KU55933 (10μM) for 48 hours. The percentage of cells with more than six γH2AX foci was significantly higher in BRCA1 deficient cells in comparison to BRCA1 proficient cells (Figures 4A2, 4B2). Similar results were observed with KU60019 (Supplementary Figures S3B and S3F). The data provides evidence that BRCA1 deficient cells accumulate DSBs at an increased rate after treatment with an ATM inhibitor relative to BRCA1proficient cells. Accumulation of DSBs may delay cell cycle progression. In BRCA1 deficient and BRCA1 proficient cells, cell cycle progression was monitored after 48 hours of treatment with KU55933 (10μM) (Supplementary Figure S2B). BRCA1 deficient cells were shown to be

significantly arrested in G2/M phase of the cell cycle compared to BRCA1 proficient cells (Figures 4A3, 4B3). Similar results were observed with KU60019 (Supplementary Figures S3C and S3G). Accumulation of DSBs may result in eventual induction of apoptosis. Apoptosis detection by FITC-annexin V flow cytometric analysis was therefore performed in cells treated with KU55933 (10μM) for 48 hours (Supplementary Figure S2C). The percentage of cells undergoing apoptosis following ATM inhibitor treatment was significantly higher in BRCA1 deficient cells in comparison to BRCA1 proficient cells (Figures 4A4, 4B4). Similar results were observed with KU60019 (Supplementary Figures S3D and S3H). The functional studies together provide evidence that ATM inhibition can induce synthetic lethality in BRCA1 deficient cells by causing accumulation of DSBs, G2M cell cycle arrest and induction of apoptosis. However the level of synthetic lethality effect seen with ATM inhibitor was modest compared that demonstrated previously using PARP inhibitors in BRCA1 deficient cells (Lord and Ashworth, 2008).

Cisplatin hypersensitivity has been well established in BRCA1 deficient cells (Tassone et al., 2009). We investigated whether low dose cisplatin could potentiate synthetic lethality induced by KU55933. Cells were treated with a combination of low dose cisplatin (0.00001μM – 0.1 μM) and KU55933 (5μM). As shown in Figure 4C1 and 4D1, KU55933 treatment increased cytotoxicity of cisplatin in BRCA1 deficient HeLa SilenciX as well in MDA-MB-436 compared to BRCA1 proficient control SilenciX and MCF7 cells. The interaction was synergistic [combination index= 0.6 (BRCA1 deficient HeLa SilneciX) and 0.7 (MDA-MB-436), supplementary Figure S5A]. In BRCA1 deficient cells treated with a combination of cisplatin and KU55933, the observed increased cytotoxicity was associated with accumulation of DSBs (Figure 4C2 and 4D2), G2/M cell cycle arrest (Figure 4C3 and 4D3) and increased apoptosis (Figure 4C4 and 4D4).

BRCA1 deficient cancer cells are sensitive to DNA-PKcs inhibitors either alone or in combination with cisplatin: NU7441 (2-N-morpholino-8-dibenzothiophenyl-chromen-4-one) is a potent and a specific inhibitor of DNA-PKcs with an IC_{50} of 14 nmol/L for DNA-PK inhibition (Tavecchio et al., 2012). NU7026 (2-(morpholin-4-yl)-benzo[h]chromen-4-one) is another DNA-PKcs inhibitor (Nutley et al., 2005).

Treatment with NU7441 resulted in reduced survival of BRCA1 deficient HeLa SilenciX cells compared to BRCA1 deficient HeLa SilenciX cells (Figure 5A1). MDA-MB-436 cells were modestly sensitive to NU7441 compared to MCF7 cells respectively (Figures 5B1). As an additional validation we investigated NU7026. As shown in supplementary Figures S6A and S6E, BRCA1 deficient HeLa SilenciX cells and MDA-MB-436 cells were also sensitive to NU7026 compared to BRCA1 proficient cells. To provide mechanistic evidence that DNA-PKcs inhibition leads to a synthetic lethality effect in BRCA1 deficient cells, we investigated the functional consequence of DNA-PKcs inhibition in BRCA1 proficient and BRCA1 deficient cells. Cells were treated with NU7441 (1.5μM) for 48 hours. The percentage of cells with more than six γH2AX foci was significantly higher in BRCA1 deficient cells in comparison to BRCA1 proficient cells (Figures 5A2, 5B2). Similar results were observed with NU7026 (supplementary Figures S4B and S4F). In BRCA1 deficient and BRCA1 proficient cells, cell cycle progression was monitored after 48 hours of treatment with NU7441 (1.5μM). BRCA1 deficient cells were shown to be significantly arrested in G1 phase of the cell cycle compared to BRCA1 proficient cells (Figures 5A3, 5B3). Similar results were observed with NU7026 (supplementary Figures S4C and S4G). Apoptosis detection by FITC-annexin V flow cytometric analysis was therefore performed in cells treated with NU7441 (1.5μM) for 48 hours. The percentage of cells undergoing apoptosis following DNA-PKcs inhibitor treatment was significantly higher in BRCA1 deficient cells in comparison to BRCA1 proficient cells (Figures 5A4, 5B4). Similar results were observed

with NU7026 (Supplementary Figures S4D and S4H). The functional studies together provide evidence that DNA-PKcs inhibition can induce synthetic lethality in BRCA1 deficient cells by causing accumulation of DSBs, G1 cell cycle arrest and induction of apoptosis. We then investigated whether low dose cisplatin could potentiate synthetic lethality induced by NU7441. Cells were treated with a combination of cisplatin (0.00001µM – 0.1 µM) and NU7441 (0.75µM). As shown in Figure 5C1 and 5D1, NU7441 treatment substantially increased cytotoxicity of low dose cisplatin in BRCA1 deficient HeLa SilenciX as well as in MDA-MB-436 compared to BRCA1 proficient control SilenciX and MCF7 cells. The interaction was synergistic [combination index= 0.5 (BRCA1 deficient HeLa SilneciX) and 0.6 (MDA-MB-436), supplementary figure S5B]. Increased cytotoxicity was associated with accumulation of DSBs (Figure 5C2 and 5D2), G2/M cell cycle arrest (Figure 5C3 and 5D3) and increased apoptosis (Figure 5C4 and 5D4).

DISCUSSION

We have shown, for the first time, that BRCA1 negative tumours have significantly lower expression of XRCC1 and Polβ, a feature that was also seen in BRCA1 deficient cancer cell lines. Patients with BRCA1 negative/ low XRCC1 or BRCA1 negative /low Polβ breast tumours also have worse breast cancer specific survival including in ER+ and ER- sub-groups. These new observations suggest that polβ/XRCC1 based sub-stratification may refine prognostication in BRCA1 deficient phenotypes. In addition, poor prognostic significance of pol β in a small cohort of germ-line BRCA1 mutated tumours is also consistent with a recent preclinical study linking BRCA1 and pol β in cancer cell line models (Masaoka et al., 2013). Moreover, the data presented here is also consistent with our previous clinical studies demonstrating poor prognostic and predictive significance of low XRCC1 (Sultana et al., 2013) and low pol β (Abdel-Fatah et al., 2014) expression in breast cancers. The DNA repair profiling in BRCA1 proficient and deficient cell lines demonstrated impairment in additional DNA repair pathways such as NHEJ, NER and MMR. This suggests that genomic instability in BRCA1 null cells may over a period of time eventually lead to acquisition of new defects in other DNA repair pathways. In addition, recent studies implicating a role for BRCA1 in transcriptional regulation of BER (Saha et al., 2010) and NER(Hartman and Ford, 2002) suggests additional mechanisms may also operate for the observed genomic instability in BRCA1 deficient cells.

We have demonstrated that a potential synthetic lethality relationship also exists between BRCA1 deficiency and blockade of ATM or DNA-PKcs in cells. A model for synthetic lethality is shown in supplementary figure S6. We have concluded synthetic lethality for the following reasons: a) BRCA1 deficient cells have increased sensitivity to ATM or DNA-PKcs inhibitors; b) upon ATM inhibitor treatment, BRCA1 deficient cells accumulate DSBs,

exhibit G2/M cell cycle arrest and induction of apoptosis; and c) upon DNA-PKcs inhibitor treatment, BRCA1 deficient cells accumulate DSBs, exhibit G1 cell cycle arrest and induction of apoptosis. Although the magnitude of synthetic lethality seen in BRCA1 deficient cells treated with ATM or DNA-PKcs inhibitor alone was not as pronounced as that demonstrated for PARP inhibitors (Lord and Ashworth, 2008), we found that low dose cisplatin combination significantly enhanced synthetic lethality. Of note, the dose of cisplatin used in our study was $1/10^{th}$ the dose used in previous preclinical studies investigating cisplatin sensitivity *in vitro* in BRCA1 deficient cells (Husain et al., 1998). We speculate that low dose cisplatin treatment generates low levels of DSBs. In cells with proficient BRCA1 and BER, despite ATM or DNA-PKcs blockade, DSBs may be rapidly repaired in back-up DNA repair pathways and cells continue to survive. The back-up repair could operate at multiple levels including the complex interactions/overlap between HR, NHEJ, alternative NHEJ (B-NHEJ) (Chapman et al., 2012; Schipler and Iliakis, 2013), components of NER and ICL repair pathways. On the other hand, in cells with deficient BRCA1 and low BER, the associated pharmacological blockade with ATM or DNA-PKcs inhibitors may lead to DSB accumulation which beyond a threshold, may severely compromise back-up DNA repair machinery leading to DSB accumulation, cell cycle arrest and apoptosis. We have recently shown that XRCC1 deficient cells are cisplatin sensitive (Abdel-Fatah et al., 2013) and ATM or DNA-PKcs inhibitors are also synthetically lethal in XRCC1 deficient cells (Sultana et al., 2013). Given the potential role of XRCC1 in B-NHEJ (Mladenov and Iliakis, 2011) we speculate that BRCA1 deficient cells that have low XRCC1 could also have compromised B-NHEJ in addition to BER resulting in increased genomic instability and enhanced synthetic lethality with cisplatin treatment seen in the current study. Moreover, ATM or DNA-PKcs modulation has previously been shown to enhance cisplatin cytotoxicity (Dejmek et al., 2009; Yoshida et al., 2008). Taken together, the data suggests that cisplatin combination may be

more successful than ATM or DNA-PK monotherapy in BRCA1 negative tumours and would be consistent with a recent study showing enhancement of synthetic lethality with ABT-888 (PARP inhibitor) in combination with platinum chemotherapy in BRCA deficient cells (Clark et al., 2012).

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Table 1. Multivariate analysis in BRCA1 negative sporadic breast cancers (Nottingham cohort).

Variables	Multivariate Model		
	Exp (B)	**CI 95%**	**P Value**
Pol beta expression	0.810	0.666-0.986	**0.036**
XRCC1 expression	0.831	0.731-0.945	**0.005**
Tumour stage	3.088	2.414-3.950	**<0.0001**
ER receptor status	0.522	0.328-0.833	**0.006**
Chemotherapy status	0.522	0.356-0.764	**0.001**

FIGURE LEGENDS

Figure 1. BRCA1 and BER protein expression in human breast cancer. **A.** Microphotographs of BRCA1 negative, BRCA1 positive, polβ positive and XRCC1 positive breast cancers. **B.** Kaplan Meier curves showing breast cancer specific survival (BCSS) in patients based on BRCA1 expression status. **C1.** Kaplan Meier curves showing breast cancer specific survival (BCSS) in patients with BRCA1 negative tumours based on XRCC1 expression status. **C2.** Kaplan Meier curves showing breast cancer specific survival (BCSS) in patients with BRCA1 negative/ER negative tumours based on XRCC1 expression status. **C3.** Kaplan Meier curves showing breast cancer specific survival (BCSS) in patients with BRCA1 negative/ER positive tumours based on XRCC1 expression status. **D1.** Kaplan Meier curves showing breast cancer specific survival (BCSS) in patients with BRCA1 negative tumours based on polβ expression status. **D2.** Kaplan Meier curves showing breast cancer specific survival (BCSS) in patients with BRCA1 negative/ER negative tumours based on polβ expression status. **D3.** Kaplan Meier curves showing breast cancer specific survival (BCSS) in patients with BRCA1 negative/ER positive tumours based on polβ expression status.

Figure 2. A. Kaplan Meier curves showing overall survival in patients with germ-line BRCA1mutated tumours based on polβ protein expression status. **B1.** Kaplan Meier curves showing breast cancer specific survival (BCSS) in patients based on BRCA1 mRNA expression status in ER+ breast cancer (METABRIC cohort). **B2.** Kaplan Meier curves showing breast cancer specific survival (BCSS) in patients with BRCA1 mRNA low/ER+ based on polβ mRNA status (METABRIC cohort). **B3.** Kaplan Meier curves showing breast cancer specific survival (BCSS) in patients with BRCA1 mRNA low/ER+ based on XRCC1 mRNA status (METABRIC cohort). **C1.** Kaplan Meier curves showing breast cancer specific survival (BCSS) in patients with BRCA1 mRNA low/ER- based on polβ mRNA status

(METABRIC cohort). **C2.** Kaplan Meier curves showing breast cancer specific survival (BCSS) in patients with BRCA1 mRNA low/ER- based on XRCC1 mRNA status (METABRIC cohort). **D.** Artificial neural network analysis. Top pair-wise interactions for gene probe markers associated with BRCA1 expression and the DNA repair process in 249 breast cancers is shown here. Each gene probe is represented by a node and the interaction weight between them as an edge, the width being defined by the magnitude of the weight. Interactions are directed from a source gene to a target gene as indicated by arrows. Red interactions indicate an excitatory interaction and blue indicates an inhibitory interaction. Highly linked genes represent hubs that are indicated to be highly influential or highly regulated in the BRCA1-DNA repair system. See supplementary data x for the biological functions of individual genes.

Figure 3. DNA repair expression in BRCA1 deficient and BRCA1 proficient cells **A1.** Representative Western blots of BRCA1, XRCC1 and polβ in BRCA1 deficient HeLa SilenciX cells and control BRCA1 proficient HeLa SilenciX cells. **A2.** Protein quantification in BRCA1 deficient HeLa SilenciX cells and control BRCA1 proficient HeLa SilenciX cells are shown here. **A3.** mRNA expression in BRCA1 deficient HeLa SilenciX cells and control BRCA1 proficient HeLa SilenciX cells are shown here. **B1.** Representative Western blots of BRCA1, XRCC1 and polβ in BRCA1 deficient MDA-MB-436 cells and BRCA1 proficient MCF7 cells. **B2.** Protein quantification in MDA-MB-436 cells and MCF7 cells are shown here. **B3.** mRNA expression in MDA-MB-436 cells and MCF7 cells are shown here. **C.** Scatter plots indicate up- and down-regulation of DNA repair mRNA expression in BRCA1 deficient HeLa SilenciX cells compared to BRCA1 proficient HeLa SilenciX cells. **D.** Scatter plots indicate up- and down-regulation of DNA repair mRNA expression in MDA-MB-436 cells compared to MCF7 cells are shown here. Green circles show genes that are two-fold or more down-regulated. See also results section and supplementary table S17 and S18.

Figure 4. ATM inhibitors in BRCA1 deficient and BRCA1 proficient cells. **A1.** Clonogenic survival assays in BRCA1 deficient HeLa SilenciX cells and control BRCA1 proficient HeLa SilenciX cells treated with KU55933. **A2.** yH2AX immunohistochemistry in BRCA1 deficient HeLa SilenciX cells and control BRCA1 proficient HeLa SilenciX cells treated with KU55933. **A3.** FACS analysis in BRCA1 deficient HeLa SilenciX cells and control BRCA1 proficient HeLa SilenciX cells treated with KU55933. **A4.** Annexin V flow cytometric analysis in BRCA1 deficient HeLa SilenciX cells and control BRCA1 proficient HeLa SilenciX cells treated with KU55933.**B1.** Clonogenic survival assays in MDA-MB-436 and MCF7 cells treated with KU55933. **B2.** yH2AX immunohistochemistry in MDA-MB-436 and MCF7 cells treated with KU55933. **B3.** FACS analysis in MDA-MB-436 and MCF7 cells treated with KU55933. **B4.** Annexin V flow cytometric analysis in MDA-MB-436 and MCF7 cells treated with KU55933. Inhibitors were added at the indicated concentrations (see methods for details). **C1.** Clonogenic survival assays in BRCA1 deficient HeLa SilenciX cells and control BRCA1 proficient HeLa SilenciX cells treated with cisplatin alone or in combination with KU55933. **C2.** yH2AX immunohistochemistry in BRCA1 deficient HeLa SilenciX cells and control BRCA1 proficient HeLa SilenciX cells treated with cisplatin alone or in combination with KU55933. **C3.** FACS analysis in BRCA1 deficient HeLa SilenciX cells and control BRCA1 proficient HeLa SilenciX cells treated with cisplatin alone or in combination with KU55933. **C4.** Annexin V flow cytometric analysis in BRCA1 deficient HeLa SilenciX cells and control BRCA1 proficient HeLa SilenciX cells treated with cisplatin alone or in combination with KU55933. **D1.** Clonogenic survival assays in MDA-MB-436 and MCF7 cells treated with cisplatin alone or in combination with KU55933. **D2.** yH2AX immunohistochemistry in MDA-MB-436 and MCF7 cells treated with cisplatin alone or in combination with KU55933. **D3.** FACS analysis in MDA-MB-436 and MCF7 cells treated with cisplatin alone or in combination with KU55933. **D4.** Annexin V flow cytometric

analysis in MDA-MB-436 and MCF7 cells treated with cisplatin alone or in combination with KU55933. * $p<0.05$, ** $p<0.01$.

Figure 5. DNA-PKcs inhibitors in BRCA1 deficient and BRCA1 proficient cells. **A1.** Clonogenic survival assays in BRCA1 deficient HeLa SilenciX cells and control BRCA1 proficient HeLa SilenciX cells treated with NU7441. **A2.** yH2AX immunohistochemistry in BRCA1 deficient HeLa SilenciX cells and control BRCA1 proficient HeLa SilenciX cells treated with NU7441. **A3.** FACS analysis in BRCA1 deficient HeLa SilenciX cells and control BRCA1 proficient HeLa SilenciX cells treated with NU7441. **A4.** Annexin V flow cytometric analysis in BRCA1 deficient HeLa SilenciX cells and control BRCA1 proficient HeLa SilenciX cells treated with NU7441.**B1.** Clonogenic survival assays in MDA-MB-436 and MCF7 cells treated with NU7441. **B2.** yH2AX immunohistochemistry in MDA-MB-436 and MCF7 cells treated with NU7441. **B3.** FACS analysis in MDA-MB-436 and MCF7 cells treated with NU7441. **B4.** Annexin V flow cytometric analysis in MDA-MB-436 and MCF7 cells treated with NU7441. Inhibitors were added at the indicated concentrations (see methods for details). **C1.** Clonogenic survival assays in BRCA1 deficient HeLa SilenciX cells and control BRCA1 proficient HeLa SilenciX cells treated with cisplatin alone or in combination with NU7441. **C2.** yH2AX immunohistochemistry in BRCA1 deficient HeLa SilenciX cells and control BRCA1 proficient HeLa SilenciX cells treated with cisplatin alone or in combination with NU7441. **C3.** FACS analysis in BRCA1 deficient HeLa SilenciX cells and control BRCA1 proficient HeLa SilenciX cells treated with cisplatin alone or in combination with NU7441. **C4.** Annexin V flow cytometric analysis in BRCA1 deficient HeLa SilenciX cells and control BRCA1 proficient HeLa SilenciX cells treated with cisplatin alone or in combination with NU7441. **D1.** Clonogenic survival assays in MDA-MB-436 and MCF7 cells treated with cisplatin alone or in combination with NU7441. **D2.** yH2AX immunohistochemistry in MDA-MB-436 and MCF7 cells treated with cisplatin alone or in

combination with NU7441. **D3.** FACS analysis in MDA-MB-436 and MCF7 cells treated with cisplatin alone or in combination with NU7441. **D4.** Annexin V flow cytometric analysis in MDA-MB-436 and MCF7 cells treated with cisplatin alone or in combination with NU7441. * $p<0.05$, ** $p<0.01$.