Targeting XRCC1 deficiency in breast cancer for personalized therapy

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ABSTRACT

XRCC1 is a key component of DNA base excision repair, single strand break repair and backup non-homologous end joining pathway. XRCC1 deficiency promotes genomic instability, increases cancer risk and may have clinical application in breast cancer. We investigated XRCC1 expression in early breast cancers (n=1297) and validated in an independent cohort of ER-α negative breast cancers (n= 281). Pre-clinically, we evaluated XRCC1 deficient and proficient Chinese hamster and Human cancer cells for synthetic lethality application using double strand break (DSB) repair inhibitors [KU55933 (ATM inhibitor) and NU7441 (DNA-PKcs inhibitor)]. In breast cancer, loss of XRCC1 (16%) was associated with high grade (p<0.0001), loss of hormone receptors (p<0.0001), triple negative (p<0.0001) and basal like phenotypes (p=0.001). Loss of XRCC1 was associated with a 2-fold increase in risk of death (p<0.0001) and independently with poor outcome (p<0.0001). Pre-clinically, KU55933 and NU7441 were synthetically lethal in XRCC1 deficient compared to proficient cells as evidenced by hypersensitivity to DSB repair inhibitors, accumulation of DNA double strand breaks, G2/M cell cycle arrest and induction of apoptosis. This is the first study to demonstrate that XRCC1 deficiency in breast cancer results in an aggressive phenotype and that XRCC1 deficiency could also be exploited for a novel synthetic lethality application using DSB repair inhibitors.

Précis: Findings suggest how XRCC1 deficiency in breast cancer can inform choice of targeted chemotherapies for treatment, based on the synthetic lethality that can be achieved with the inhibition of particular mechanisms of DNA double strand break repair.
INTRODUCTION

Impaired DNA repair is a driving force for carcinogenesis and may promote aggressive clinical behaviour in breast cancer (1, 2). Base excision repair (BER) is required for the accurate removal of damaged DNA bases induced by oxidising and alkylating agents. DNA single strand breaks induced by chemotherapeutics are processed by single strand break repair (SSBR), a pathway related to BER. XRCC1 (X-ray repair cross-complementing gene 1) is a critical factor in BER, SSBR and back-up non-homologous end joining pathway (B-NHEJ). XRCC1, a 70-kDa protein, has three functional domains: an N-terminal DNA binding domain, a centrally-located BRCT I domain and a C-terminal BRCT II domain. Although XRCC1 has no known enzymatic activity [reviewed in (3, 4)], it functions as a molecular scaffold protein and is intimately involved in the coordination of DNA repair by interacting with several components of BER/SSBR pathway such as DNA glycosylases, apurinic/apyrimidinic endonuclease (APE1), PARP-1, polynucleotide kinase (PNK) and ligase III [reviewed in (3, 4)].

Pre-clinically, XRCC1 deficiency delays SSB rejoining, induces mutations and results in elevated levels of sister chromatid exchanges, a hallmark of genomic instability. XRCC1 deficiency results in hypersensitivity to ionizing radiation and chemotherapeutics (4, 5). In human association studies, germline polymorphisms in XRCC1 may influence cancer risk (6-9), and also response to radiotherapy (10, 11) and chemotherapy (12) in patients (13, 14).
Given the essential role of XRCC1 in DNA repair and carcinogenesis, we hypothesised that XRCC1 may be dysregulated in breast cancer. In the current study we provide the first evidence that XRCC1 deficiency in tumours is an independent predictor of poor clinical outcome and is associated with an aggressive phenotype. Moreover we also provide the first preclinical evidence that XRCC1 deficiency in cancer can be exploited for synthetic lethality application using DNA double strand break repair inhibitors. Our study has important clinical translational applications in patients.
METHODS

STUDY PATIENTS

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. All patients were treated uniformly in a single institution and have been investigated in a wide range of biomarker studies (15-17). Supplemental Table S1 summarizes patient demographics. Supplemental treatment data 1 summarizes various adjuvant treatments received by patients in this cohort.

We also evaluated an independent series of 281 ER-α negative invasive BCs diagnosed and managed at the Nottingham University Hospitals between 1999 and 2007. All patients were primarily treated with surgery, followed by radiotherapy and anthracycline chemotherapy. The characteristics of this cohort are summarised in supplementary Table S2.

The Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) criteria, recommended by McShane et al (18), were followed throughout this study. This work was approved by Nottingham Research Ethics Committee.

Tissue Microarrays (TMAs) and immunohistochemistry (IHC)

Tumours were arrayed in tissue microarrays (TMAs) constructed with 2 replicate 0.6mm cores from the centre and periphery of the tumours. The TMAs were immunohistochemically profiled for XRCC1 and other biological antibodies (Supplementary-Table S3) as previously described (19-22). Immunohistochemical staining for XRCC1 was performed using the Bond Max automated staining machine.
and Leica Bond Refine Detection kit (DS9800) according to manufacturer instructions (Leica Microsystems). Pre-treatment of TMA sections was performed with citrate buffer (pH 6.0) antigen for 20 minutes. TMA sections were incubated for 15 minutes at room temperature with 1:200 anti-XRCC1 mouse monoclonal antibody (Ab-1, clone 33-2-5, Thermoscientific, Fremont, CA, USA). HER2 expression was assessed according to the new ASCO/CAP guidelines using IHC and fluorescence in situ hybridisation (FISH) (23).

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 three 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-score (range 0-300) was calculated by multiplying intensity of staining and percentage staining as previously described (19-22). Low/negative XRCC1 (XRCC1-) expression was defined by mean of H-score of ≤100. Not all
cores within the TMA were suitable for IHC analysis due to missing cores or absence of tumour cells.

**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. The proportional hazards assumption was tested using standard log-log plots. Hazard ratios (HR) and 95% confidence intervals (95% CI) were estimated for each variable. All tests were two-sided with a 95% CI and a p value < 0.05 considered significant. For multiple comparisons, p values were adjusted according to Holm-Bonferroni correction method (24).

**Sample size and power analysis**

Power of study, sample size and effect size were determined using PASS (NCSS, version 11, USA). Cox regression of the log hazard ratio on XRCC1 covariant (SD=0.73), based on a sample of 1163 observations, achieves 90% power at 0.05 significant level to detect a small regression coefficient equal to 0.25 and 0.28 for risk of recurrence and death, respectively. The sample size was adjusted since a multiple regression of the variable of interest on the other covariates in the Cox regression is expected to have an R squared of 0.3. The sample size was adjusted for recurrence and death event rate of 0.40 and 0.30 respectively.
PRECLINICAL STUDIES

Compounds and reagents: Highly specific and potent ATM inhibitor (KU55933), DNA-PKcs inhibitors (NU7441 and NU7026) were purchased from Tocris Bioscience, UK. The compounds were dissolved in 100% DMSO and stored at -20°C.

Cell lines and culture: Previously well characterized Chinese hamster ovary cells; CHO9 (Wild type), EM-C11 (XRCC1-mutant), EM-C12 (XRCC1-mutant) were provided by Professor MZ. Zdzienicka, Department of Molecular Cell Genetics, Collegium Medicum in Bydgoszcz, Nicolaus-Copernicus University in Torun, Bydgoszcz 85-094, Poland (25, 26). Cells were grown in Ham’s F-10 media (PAA, UK) [supplemented with 10% fetal bovine serum (FBS) (PAA, UK) and 1% penicillin/streptomycin].

XRCC1 deficient HeLa SilenciX® cells and control XRCC1 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.

MCF-7 breast cancer cells were grown in RPMI media (PAA, UK) [supplemented with 10% fetal bovine serum (FBS) (PAA,UK) and 1% penicillin/streptomycin].
XRCC1 knockdown using siRNAs: Two XRCC1 siRNA constructs (sequences listed in supplemental table S4), one negative scrambled control and siRNA for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (positive control) were used in these studies. The siRNA constructs were purchased from Ambion life technologies, UK. The transfection protocol was as described previously by Fan et.al (27). MCF-7 cells were plated in 6-well plates (2 ml medium/well without antibiotics). At 50% confluence, transfection was achieved using Lipofectamine TM 2000 (Invitrogen) according to the manufacturer’s protocol. Briefly, siRNA (100 pmol) and Lipofectamine (5µl) were each separately mixed with 250 µl Opti- MEM1 (GIBCO/Invitrogen) without FBS. After 5 minute incubation at room temperature, the siRNA and Lipofectamine solutions were combined and incubated for another 20 min at room temperature. This mixture was then added to plated cells, cultured at 37°C overnight and the medium was later replaced with fresh medium plus penicillin/streptomycin (1%). When the cells attained 100% confluence, they were trypsinized and subsequently transferred into 75 cm² flasks for continued growth and/or treatment. XRCC1 Knockdown was evaluated by western blotting at various time points after transfection (days 3, 5 and 7).

Clonogenic survival assay: Two hundred cells per well were seeded in six-well plates. Cells were allowed to adhere for 4 hours. Compounds (KU55933 or NU7441) were added at the indicated concentrations. The plates were left in the incubator for 10 days for Chinese hamster cells and 14 days for SilenciX cells. For siRNA transfected MCF-7 cells, two hundred cells per well were seeded three days after transfection, treated with compound and left in the incubator for 14 days. After
incubation, the media was discarded, fixed (with methanol and acetic acid mixture) stained with crystal violet and counted.

Surviving Fraction = \[
\frac{\text{No. of colonies formed}}{\text{(No. of cells seeded x Plating efficiency)}}\] \times 100. All clonogenic assays were done in triplicate.

**Neutral COMET assay:** This assay was performed as described previously (28). Briefly, sub-confluent cells were exposed to DSB inhibitors [KU55933 (5μM)] or [NU7441 (1.5 μM)]. At various time points after exposure (pre-treatment, 24 hours and 48 hours) cells were extracted and comet assays were performed. A total of 200 comet images were evaluated for olive tail moment for each time point (pre-treatment, 24 hours and 48 hours).

**γH2AX immunocytochemistry:** This assay was performed as described previously (28). Briefly, cells were incubated in medium containing ATM inhibitor [KU55933 (5μM)] or DNA-PKCS inhibitor [NU7441 (1.5 μM)] for 24 hours. The frequencies of cells containing γH2AX foci were determined in 100 cells per slide in three separate experiments. Nuclei containing more than six γH2AX foci were considered positive.

**Flow cytometric analyses (FACS) for cell cycle progression:** Cells grown to sub-confluence were treated with ATM inhibitor [KU55933 (5μM)] or DNA-PKCS inhibitor [NU7441 (1.5 μM)] for 24 hours and collected by trypsinization and centrifugation (1000 rpm for 5 minutes). FACS assay was performed as described previously (28).
Apoptosis detection by FITC-annexin V flow cytometric analysis: Cells were treated for 48 hours with ATM inhibitor [KU55933 (5μM)] or DNA-PKCS inhibitor [NU7441 (1.5 μM)]. Cells were later collected by trypsinization and centrifugation (1000 rpm for 5 minutes) and were washed twice with cold PBS and then re-suspended cells in 1X Binding buffer at a concentration of 1X10^6 cells/ml. Then 100μl of the solution (1X10^5 cells) was transferred to a 5 ml culture tube and 5μl of FITC Annexin V and 5μl PI were added. The cells were then gently vortex and incubated for 15 min at RT (25°C) in the dark. After the incubation, 400μl of binding buffer was added to each tube and was analyzed by flow cytometry within 1 hour. Data was analysed using FlowJo7.6.1 software.

Western blot analysis: This assay was performed as described previously (28). Primary antibodies used were: mouse anti -XRCC1 (Thermo Fisher Scientific, Waltham, MA, USA); rabbit anti- ATM (Cell Signalling Tech., Danvers, MA, USA), and rabbit anti-DNA-PKcs (Novus Biologicals, Southpark way, Littleton CO,USA).
RESULTS

Clinicopathological significance of XRCC1 expression: A total of 1297 tumours in the primary cohort were suitable for analysis of XRCC1 expression. 1093/1297 (84%) of the tumours were positive for XRCC1 expression, and 204/1297 (16%) tumours were negative for XRCC1 expression (Figure 1A). Normal breast terminal duct lobular units (TDLUs) showed moderate to strong nuclear XRCC1 expression throughout (Figure 1A). In invasive BC, loss of XRCC1 expression was highly significantly associated with aggressive clinicopathological features (Table 1) including high histological grade (p<0.0001), pleomorphism (p<0.0001), glandular de-differentiation (p=0.006), absence of hormonal receptors (ER-/PgR-/AR-) (p<0.0001), presence of basal like phenotypes (p=0.001) and triple negative phenotypes (p<0.0001) (Table 1).

Association between XRCC1 and biomarkers of DNA repair, cell cycle progression and apoptosis: As shown in table 1, loss of XRCC1 expression was significantly associated with loss of expression of other DNA repair proteins such as BRCA1 (p<0.0001) and TOP2A (p<0.0001). In addition, abnormal expression of tumour suppressor proteins such as p53 (p=0.03), p16 (p<0.001) and FHIT (p<0.0001) was more common in BCs with loss of XRCC1 expression compared to XRCC1 positive tumours, reflecting a higher level of genomic instability in XRCC1 negative tumours. Notably, loss of XRCC1 expression was also significantly associated with low expression of p53 downstream genes that regulate cell cycle progression and apoptosis such as p21 (p<0.001), MDM2 (p<0.001), MDM4 (p<0.001), Bcl2 (p<0.001) and Bax (p<0.001).
Survival analyses: Loss of XRCC1 expression in tumours showed an adverse outcome at 10 years with a 2-fold increase in the risk of death (p<0.0001) [Figure 1B], recurrence (p<0.0001) [Supplemental Figure 1A1] and distant metastasis (DM) (p<0.0001) [Supplemental Figure 1A2] compared with those tumours with positive XRCC1 expression. Investigating the clinical outcome of 678 patients with early stage lymph node negative tumours revealed that tumours with loss of XRCC1 expression displayed a worse prognosis than cases with positive XRCC1 expression [Figure 1C and supplemental Figure 1 (B1, B2,)]. Adverse clinical outcomes associated with loss of XRCC1 expression was also confirmed in 466 patients with locally advanced lymph node positive breast cancer [Figure 1D and supplemental Figure 1 (C1, C2)].

Prognostic significance of XRCC1 expression in low risk BC patients [Nottingham Prognostic Index (NPI) <3.4] who did not receive any adjuvant therapy: Low risk BC patients (NPI<3.4) were treated with surgery followed by radiotherapy only (n=368). At 10 years, BC with loss of XRCC1 expression showed a 2.5-4.0 fold increase in the risk of death (p<0.0001) [Figure 1E], recurrence (p<0.0001) [Supplemental Figure 2A2] and DM (p=0.0001) [Supplemental Figure 2A3] compared with those with positive XRCC1 expression.

Prognostic significance of XRCC1 expression in different adjuvant systemic therapy settings: Patients whose tumours were high risk (NPI ≥3.4), ER-α positive, received endocrine therapy, and XRCC1 negative had a 2-fold increase in risk of death (p<0.0001) [Figure 1F], recurrence (p<0.0001) [Supplemental Figure 2B2] and DM (p<0.0001) [Supplemental Figure 2B3] at 10 years compared with patients whose tumours were positive for XRCC1.
The poor outcome of BC with loss of XRCC1 expression was further confirmed in high risk BC receiving adjuvant CMF chemotherapy. At 10 years, patients with XRCC1 negative tumours showed a 2 fold increase in the risk of death (p=0.002) [Figure 1G], recurrence (p=0.001) [Supplemental Figure 2C2] and DM (p=0.02) [Supplemental Figure 2C3] compared with those whose tumours were XRCC1 positive.

**Multivariate Cox regression analysis:** In multivariate Cox regression analysis including other validated prognostic factors, such as lymph node stage, histological grade and tumour size (NPI components), XRCC1 expression was a powerful independent predictor for clinical outcome (p<0.0001) (Table 2).

**ER-α negative invasive breast cancer (validation cohort):** To confirm the above findings in the context of modern adjuvant anthracycline based chemotherapy, we validated XRCC1 expression in an independent cohort of 281 ER-α negative breast cancers. The characteristics of this cohort are summarised in supplementary Table S2. A total of 252 tumours were suitable for analyses of XRCC1 expression. 212/252 (84%) of the tumours were positive for XRCC1 expression with 40/252 (16%) tumours negative for XRCC1 expression. Patients with XRCC1 negative tumours showed 2 fold increase in the risk of death (HR: 2.1, 95% CI: 1.2-3.7, p=0.008) [Figure 2A], recurrence (HR: 2.1, 95% CI: 1.2-3.6, p=0.007) [Supplemental Figure 3A2] and DM (HR: 2.2, 95% CI: 1.2-3.8, p=0.006) [Supplemental Figure 3A3] compared with those whose tumours were XRCC1 positive.

**Prognostic significance of XRCC1 expression in triple negative breast tumours**
Sub-group analysis in all triple negative breast cancer patients was performed (n=455). Loss of XRCC1 expression was significantly associated with poor survival
either after receiving CMF or anthracycline (HR: 2.3, 95% CI: 1.4-3.7, p=0.0005) [Figure 2B], recurrence (HR: 2.5, 95% CI: 1.6-3.8, p=0.00006) [Supplemental Figure 3B2] and DM (HR: 2.3, 95% CI: 1.4-3.7, p=0.0005) [ Supplemental Figure 3B3] compared with those whose tumours were XRCC1 positive. Prognostic significance was also confirmed when analysed for the group receiving anthracycline chemotherapy only (HR: 2.9, 95% CI: 1.4-5.7, p=0.003) [Figure 2C] or CMF only (HR: 1.9, 95% CI: 0.99-3.8, p=0.05) [Figure 2D].

PRECLINICAL SYNTHETIC LETHALITY EVALUATION

The clinical data confirms that XRCC1 loss is an independent poor prognostic marker. As XRCC1 deficient cells are BER/SSBR deficient and reliant on DSB repair pathways for maintaining genomic stability, we hypothesized that XRCC1 deficient cells are likely to be hypersensitive to DSB repair inhibitors and could be exploited for synthetic lethality applications. Ataxia telangectasia mutated (ATM) is critical for DNA DSB sensing and signal transduction (29). DNA-dependent protein kinase catalytic subunit (DNA-PKcs) is an essential component of non-homologous end-joining (NHEJ) (30). We therefore investigated highly specific DSB repair small molecule inhibitors for synthetic lethality. KU55933 [2-(4-Morpholinyl)-6-(1-thianthrenyl)-4H-pyran-4-one] is a potent, selective and competitive ATM kinase inhibitor (Kᵢ = 2.2 nM, IC₅₀ values are 13, 2500, 9300, 16600, > 100000 and > 100000 nM at ATM, DNA-PKcs, mTOR, PI 3-kinase, PI 4-K and ATR respectively) (31). NU7441 [8-(4-Dibenzothienyl)-2-(4-morpholinyl)-4H-1-benzopyran-4-one] is a potent and selective DNA-dependent protein kinase (DNA-PKcs) inhibitor (IC₅₀ values are 14, 1700, 5000, >100000 and >100000 nM for DNA-PKcs, mTOR, PI 3-K,
ATM and ATR respectively (32). NU7026 [2-(4-Morpholinyl)-4H-naphthol[1,2-b]pyran-4-one] is an ATP-competitive inhibitor of DNA-dependent protein kinase (DNA-PKcs). IC\textsubscript{50} values are 0.23, 13.0, > 100 and > 100 µM for DNA-PKcs, PI3K, ATM and ATR respectively (33). ATM and DNA-PKcs inhibitors were investigated in a panel of XRCC1 deficient and proficient Chinese hamster and human cancer cells.

**Chinese Hamster (CH) cells:** We first confirmed XRCC1 deficiency in EM-C11 and EM-C12 cells by Western blot analysis (Figure 3A). EM-C11, EM-C12 as well as CHO9 are proficient in the expression ATM and DNA-PKcs (Figure 3A). KU55933 as well as NU7441 induce reduced survival in EM-C11 and EM-C12 cells compared to CHO9 cells (Figure 3B and Figure 3C). Similar reduced survival was also seen with cells treated with NU7026 (another DNA-PKcs inhibitor) (Supplemental Figure 4A). The neutral COMET assay specifically detects DSBs in DNA. Figure 3D summarizes the results for EM-C11, EM-C12 and CHO9 cells treated with 5 µM of KU55933. Compared to pre-treatment samples, after 24 hours of exposure to KU55933, XRCC1 deficient cells accumulate significantly higher DSBs. Similar results were also seen in XRCC1 deficient cells treated with 1.5 µM of NU7441 (Figure 3E). DSBs induce phosphorylation of H2AX at serine 139 (γH2AX), and accumulation of γH2AX foci in the nucleus is a marker of DSBs. Nuclei containing more than six γH2AX foci were considered positive for DSB accumulation. Figure 3F and 3G show that EM-C11 and EM-C12 cells accumulate significantly more γH2AX foci in their nucleus compared to control cells after treatment with 5 µM of KU55933. Similar results were also seen in XRCC1 deficient cells treated with 1.5 µM with NU7441 (Figure 3H). Accumulation of DSBs may delay cell cycle progression. In EM-C11, EM-C12 and CHO9 cells exposed to DSB repair inhibitor for 24 hours cell cycle
progression was evaluated. Figures 4A and 4B summarise the data for cells treated with 5 μM of KU55933. At 24 hours, EM-C11 and EM-C12 were shown to be significantly arrested in G2/M phase of the cell cycle compared to CHO9 cells. Similar results were observed in XRCC1 deficient cells treated with 1.5 μM with NU7441 (Figure 4C). Increased early apoptosis at 48 hours is demonstrated in EM-C11 and EM-C12 cells compared to CHO9 cells treated with 5 μM of KU55933 or 1.5 μM of NU7441 (supplemental figure 4B).

**Human HeLa SilenciX cells:** Figure 4D confirms that XRCC1 SilenciX cells are XRCC1 deficient compared to control SilenciX cells. Both cell lines are proficient for ATM and DNA-PKcs protein expression. Figure 4E demonstrates that KU55933 (5 μM) is selectively toxic to XRCC1 deficient SilenciX cells in comparison to control XRCC1 proficient SilenciX cells. Similar results were also demonstrated in XRCC1 deficient cells treated with 1.5 μM with NU7441 (Figure 4F). Neutral comet assays in cells treated with 5 μM of KU55933 (Figure 4G) show that the mean tail moment was significantly higher in XRCC1 deficient SilenciX cells at 24 hours (p= 0.04) and at 48 hours (p= 0.009) post-treatment in comparison to XRCC1 proficient control SilenciX cells. Similar results were also demonstrated in XRCC1 deficient cells treated with 1.5 μM with NU7441 (Figure 4H). The mean tail moment was higher in XRCC1 deficient SilenciX cells at 24 hours (p= 0.03) and at 48 hours (p=0.01) post-treatment in comparison to XRCC1 proficient control SilenciX cells. Figure 4I shows that XRCC1 deficient SilenciX cells accumulate significantly more γH2AX foci in their nucleus compared to control cells after treatment with 5 μM of KU55933. Similar results were also seen in XRCC1 deficient cells treated with 1.5 μM with NU7441
(Figure 4J). Figure 4K and 4L demonstrates that SilenciX cells were arrested at the G2/M phase of the cell cycle after treatment with 5 μM of KU55933 or 1.5 μM of NU7441 respectively. Figure 4M confirms substantial apoptosis at 48 hours in SilenciX cells treated with 5 μM of KU55933 or 1.5 μM of NU7441 compared to control cells.

**MCF-7 breast cancer cells:** We first confirmed XRCC1 knockdown using siRNA in MCF-7 cells. After transfection, cell lysates were sampled on days 3, 5 and 7 for XRCC1 knock down by western blot analysis. On day 3, there was robust XRCC1 knock down (more than 80%) in cells transfected with construct 1 or construct 2 (Figure 5A) compared to scrambled negative control. We used GAPDH as a positive control and also demonstrated efficient knockdown of GAPDH in cells (Figure 5A). KU55933 treatment reduced survival in XRCC1 deficient cells compared to proficient cells (Figure 5B). Figure 5D summarizes the results for neutral comet assay with KU55933. Significant DSBs accumulation was observed in XRCC1 deficient cells at 24 hours \( p=0.001 \) (construct 1), \( P=0.003 \) (construct 2) and 48 hours \( p=0.002 \) (construct 1), \( p=0.05 \) (construct 2) compared to XRCC1 proficient cells. This was associated with significant number of γH2AX foci [Figure 5E, \( p= 0.05 \) (construct 1), \( p= 0.02 \) (construct 2)] and accumulation of cells in G2/M phase of the cell cycle [Figure 5F, \( p= 0.05 \) (construct 1), \( p= 0.05 \) (construct 2)] in XRCC1 deficient cells compared to proficient cells. Similarly, NU7441 treatment was more toxic to XRCC1 deficient cells (Figure 5C) and associated with accumulation of DSBs (Figure 5G) at 24 hours \( p=0.002 \) (construct 1), \( P=0.01 \) (construct 2) and 48 hours \( p=0.01 \) (construct 1), \( p=0.005 \) (construct 2) and formation of more γH2AX foci [Figure 5H,
p = 0.01 (construct 1), p = 0.04 (construct 2) and G2/M cell cycle arrest [Figure 5I, p = 0.002 (construct 1), p = 0.007 (construct 2)].

The data presented in human cancer cells as well as in CH cells provide compelling evidence that in XRCC1 deficient cells DSB repair inhibitors induce synthetic lethality by inducing accumulation of DSB leading to cell cycle arrest and death.
DISCUSSION:

XRCC1 is a key scaffolding protein intimately involved in BER, SSBR and B-NHEJ. This is the first study to evaluate XRCC1 protein expression in breast cancer. Survival analyses confirmed that XRCC1 negativity is an independent predictor of poor clinical outcome. We validated our data in an independent cohort of ER negative tumours and confirmed the negative prognostic significance. In triple negative tumours, XRCC1 negativity remains significantly associated with poor clinical outcome compared to XRCC1 positive tumours. XRCC1 may be a useful tool in the stratification of BC patients for adjuvant therapy. Our data suggests that otherwise low risk (NPI < 3.4) XRCC1 negative tumours should be included within high risk groups and would benefit from adjuvant systemic therapy. Moreover, poor clinical outcome with loss of XRCC1 expression was also confirmed in high risk BC (NPI ≥ 3.4), suggesting that alternative aggressive systemic chemotherapy may be warranted in this group. However, one limitation of this study is that these historical patients received CMF chemotherapy, and whether XRCC1 expression can stratify patients who receive a more modern anthracycline/taxol based chemotherapy remains to be established in follow-on studies.

Our clinical data is in contrast to preclinical studies wherein XRCC1 deficient cell lines have been shown to be sensitive to cytotoxic therapy (3, 4). Mutator phenotype is a well-recognised phenomenon where DNA repair deficiency leads to genomic instability which further leads to increased accumulation of mutations and selection of aggressive malignant clones that may be resistant to therapy (2, 34). DNA polymerase beta, another key factor in DNA base excision repair, has been shown to exhibit the mutator phenotype phenomenon (2, 34). We speculate that loss of
XRCC1 leads to genomic instability and a mutator phenotype phenomenon may be operating in human cancer cells deficient in XRCC1 expression. In order to test this hypothesis in human tumours we conducted correlative studies with markers of DNA repair, cell cycle and apoptosis. XRCC1 negative tumours were biologically more aggressive as evidenced by high histological grade, pleomorphism, glandular de-differentiation, absence of hormonal receptors, and presence of basal like phenotypes, triple negative phenotypes and loss of CK18. XRCC1 negativity was highly associated with loss of other DNA repair factors such as BRCA1 and TOP2A implying intrinsic genomic instability in these tumours. Mutant p53 (that promotes genomic instability) and loss of down-stream markers (MDM2 and MDM4) was significantly associated with loss of XRCC1. p53 resistant tumours are known to be resistant to chemotherapy. In a previous study, we investigated the expression of inactive p53 in this cohort and demonstrated resistance to therapy (20). The association with loss of Topo II expression and XRCC1 deficiency provides additional evidence for therapy resistance seen in this cohort. Whether gene silencing or post transcriptional mechanisms operate to reduce XRCC1 protein expression remains unclear, although a recent preclinical study suggests that the PI3K-Akt/MAP-kinase pathway, which is frequently dysregulated in breast cancer (35, 36), may be involved in regulating XRCC1 expression in cells (37).

Pre-clinically, we then investigated if XRCC1 deficiency could be exploited for stratified treatment strategy in breast cancer. The ability of PARP inhibitors to induce synthetic lethality in BRCA deficient breast cancers (38) suggests that additional factors within BER/SSBR may be suitable for personalized approaches. XRCC1 is a key factor in BER and SSBR. As XRCC1 deficient cells may be reliant on DSB repair
pathways to maintain cellular survival, we hypothesised that XRCC1 deficiency in somatic tumours could be exploited for synthetic lethality application by blocking the DSB repair pathway in cells. Ataxia telengectasia mutated (ATM) and DNA-dependent protein kinase catalytic subunit (DNA-PKcs) are members of the phosphatidylinositol-3-kinase-like protein kinase (PIKK) family. ATM is a key sensor and transducer of DNA damage signalling during homologous recombination (HR) in response to DSBs generated during replication (29). DNA-PKcs is an essential component of non-homologous end-joining (NHEJ) (30). Whereas NHEJ operates throughout the cell cycle, HR is limited to S and G2. However, the mechanisms by which cells choose between NHEJ and HR remains to be established. In addition, the molecular complexities of interactions between DNA-PKcs, ATM, NHEJ and HR are only beginning to emerge (39). For example, it appears that there may be more than 40 phosphorylation sites in DNA-PKcs and how post translational modifications of DNA-PKcs impact HR is unknown (39). Moreover, novel interactions between XRCC1, ATM and DNA-PKcs have also been recently reported (40, 41). ATM-Chk2-dependent phosphorylation of XRCC1 was been shown to promote BER by Chou et al. (40). In another study, DNA-PKcs was shown to phosphorylate BRCT1 domain of XRCC1 at serine 371 and cause XRCC1 dimer dissociation. In addition, XRCC1 was also shown to stimulate phosphorylation of p53-Ser 15 by DNA-PKcs in that study (41). XRCC1 is also a key component of the backup NHEJ pathway (42, 43). Therefore given the interesting links, we investigated whether a synthetic lethality relationship exists between XRCC1, ATM and DNA-PKcs. Detailed in vitro studies in XRCC1 deficient and proficient cells treated with ATM inhibitor (31) or DNA-PKcs (32) inhibitors were conducted.
We demonstrate herein that highly specific and potent ATM and DNA-PKcs inhibitors are synthetically lethal in XRCC1 deficient cells. We have concluded synthetic lethality for the following reasons. Firstly, in Chinese Hamster (CH) cells deficient in XRCC1 we observed increased sensitivity to DSB repair inhibitors and confirmed this observation in XRCC1 deficient human cancer cells. We have recently demonstrated a similar phenomenon in CH cells as well as in human cancer cells deficient in APE1, another key factor in BER (28). Secondly, functional analyses in XRCC1 deficient CH and human cells confirmed that DSB repair inhibitors led to an accumulation of DSBs, arrest of G2/M cell cycle progression and induction of apoptosis. We present a working model for DSB repair inhibition as a synthetic lethality strategy in XRCC1 deficient cells. In brief, XRCC1 deficient cells have elevated SSBs that are eventually converted to toxic DSBs. In cells deficient in DSB repair (such as by pharmacological inhibition of ATM or DNA-PKcs), DSBs would persist and lead to the observed synthetic lethality. In cells that are proficient in DSB repair, DSBs would be repaired and cells would survive.

In conclusion, we provide the first clinical evidence that XRCC1 deficiency is an independent poor prognostic biomarker in breast cancer patients. Pre-clinically we have demonstrated that XRCC1 deficiency can be exploited for synthetic lethality application in breast cancer by blocking DSB repair using ATM or DNA-PKcs inhibitors. Accelerated pharmaceutical development of DSB repair inhibitors is urgently warranted to expand synthetic lethality strategies in breast cancer.
References:

Table 1: Association between XRCC1 expression and clinico-pathologic variables

<table>
<thead>
<tr>
<th>Variable</th>
<th>XRCC1 Expression</th>
<th>(\chi^2)</th>
<th>Adjusted p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Loss (N=204) n (%)</td>
<td>Positive (N=1093) n (%)</td>
<td></td>
</tr>
<tr>
<td><strong>A) Pathological Parameters</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumour Size</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 a + b (≤1.0)</td>
<td>16 (9)</td>
<td>85 (9)</td>
<td></td>
</tr>
<tr>
<td>T1 c (&gt;1.0-2.0)</td>
<td>86 (46)</td>
<td>525 (55)</td>
<td></td>
</tr>
<tr>
<td>T2 (&gt;2.0-5)</td>
<td>80 (43)</td>
<td>344 (35)</td>
<td></td>
</tr>
<tr>
<td>T3 (&gt;5)</td>
<td>3 (2)</td>
<td>9 (1)</td>
<td></td>
</tr>
<tr>
<td>Lymph node stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>113 (61)</td>
<td>594 (62)</td>
<td></td>
</tr>
<tr>
<td>Positive (1-3 nodes)</td>
<td>59 (32)</td>
<td>309 (32)</td>
<td></td>
</tr>
<tr>
<td>Positive (&gt;3 nodes)</td>
<td>14 (7)</td>
<td>62 (6)</td>
<td></td>
</tr>
<tr>
<td><strong>Grade</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>23 (12)</td>
<td>173 (18)</td>
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</tr>
<tr>
<td>G2</td>
<td>31 (17)</td>
<td>345 (36)</td>
<td></td>
</tr>
<tr>
<td>G3</td>
<td>131 (71)</td>
<td>445 (46)</td>
<td></td>
</tr>
<tr>
<td><strong>Tumour Types</strong></td>
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<td></td>
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<tr>
<td>IDC-NST</td>
<td>122 (66)</td>
<td>548 (57)</td>
<td>5.2x10^{-3}</td>
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<tr>
<td>Tubular</td>
<td>29 (16)</td>
<td>195 (20)</td>
<td></td>
</tr>
<tr>
<td>Medullary</td>
<td>5 (3)</td>
<td>101 (11)</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>12 (6)</td>
<td>19 (2)</td>
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</tr>
<tr>
<td><strong>Mitotic Index</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>M1 (low; mitoses &lt; 10)</td>
<td>39 (21)</td>
<td>375 (39)</td>
<td>1.1x10^{-3}</td>
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<tr>
<td>M2 (medium; mitoses 10-18)</td>
<td>27 (15)</td>
<td>182 (19)</td>
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</tr>
<tr>
<td>M3 (high; mitosis &gt;18)</td>
<td>119 (64)</td>
<td>406 (42)</td>
<td></td>
</tr>
<tr>
<td><strong>Pleomorphism</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (small-regular uniform)</td>
<td>3 (2)</td>
<td>25 (3)</td>
<td>1.2x10^{-3}</td>
</tr>
<tr>
<td>2 (Moderate variation)</td>
<td>45 (24)</td>
<td>408 (42)</td>
<td></td>
</tr>
<tr>
<td>3 (Marked variation)</td>
<td>137 (74)</td>
<td>532 (55)</td>
<td></td>
</tr>
<tr>
<td><strong>Tubule formation</strong></td>
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<td></td>
</tr>
<tr>
<td>1 (&gt;75% of definite tubule)</td>
<td>7 (4)</td>
<td>53 (5)</td>
<td>0.006*</td>
</tr>
<tr>
<td>2 (10%-75% definite tubule)</td>
<td>47 (25)</td>
<td>346 (36)</td>
<td></td>
</tr>
<tr>
<td>3 (&lt;10% definite tubule)</td>
<td>131 (71)</td>
<td>564 (59)</td>
<td></td>
</tr>
<tr>
<td><strong>B) Aggressive phenotype</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Her2 overexpression (No)</td>
<td>168 (84)</td>
<td>934 (90)</td>
<td></td>
</tr>
<tr>
<td>(Yes)</td>
<td>32 (16)</td>
<td>138 (10)</td>
<td></td>
</tr>
<tr>
<td>Triple negative (No) (Yes)</td>
<td>120 (67)</td>
<td>807 (86)</td>
<td>1.5x10^{-3}</td>
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<tr>
<td>Basal like (No) (Yes)</td>
<td>135 (79)</td>
<td>815 (86)</td>
<td>0.001*</td>
</tr>
<tr>
<td>p63 (Negative) (Positive)</td>
<td>168 (95)</td>
<td>908 (99)</td>
<td>0.001*</td>
</tr>
<tr>
<td>Vimentine (Negative) (Positive)</td>
<td>95 (63)</td>
<td>582 (60)</td>
<td>0.038</td>
</tr>
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<td>FHIT (Negative) (Positive)</td>
<td>56 (35)</td>
<td>149 (18)</td>
<td>1.6x10^{-3}</td>
</tr>
<tr>
<td>CK18 (Negative) (Positive)</td>
<td>104 (65)</td>
<td>672 (62)</td>
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</tr>
<tr>
<td><strong>C) Hormonal receptors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ER (Negative) (Positive)</td>
<td>78 (47)</td>
<td>212 (24)</td>
<td>1.1x10^{-6}</td>
</tr>
<tr>
<td>PGR (Negative) (Positive)</td>
<td>89 (53)</td>
<td>679 (76)</td>
<td></td>
</tr>
<tr>
<td>AR (Negative) (Positive)</td>
<td>114 (64)</td>
<td>373 (41)</td>
<td>6.4x10^{-2}</td>
</tr>
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<td><strong>D) DNA Repair</strong></td>
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<td></td>
<td></td>
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<tr>
<td>BRCA1 (Negative) (Positive)</td>
<td>54 (35)</td>
<td>124 (16)</td>
<td>2.6x10^{-2}</td>
</tr>
<tr>
<td>TOP2A (Negative) (Positive)</td>
<td>99 (65)</td>
<td>654 (64)</td>
<td></td>
</tr>
<tr>
<td><strong>E) Cell cycle/apoptosis regulators</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>p53 (Wild) (Mutant)</td>
<td>127 (74)</td>
<td>722 (81)</td>
<td>0.03*</td>
</tr>
<tr>
<td>p21 (Negative) (Positive)</td>
<td>110 (71)</td>
<td>446 (55)</td>
<td>2.3x10^{-3}</td>
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<tr>
<td>MDM4 (Negative) (Positive)</td>
<td>131 (87)</td>
<td>573 (61)</td>
<td>5.2x10^{-2}</td>
</tr>
<tr>
<td>MDM2 (Negative) (Positive)</td>
<td>134 (88)</td>
<td>584 (74)</td>
<td>1.4x10^{-2}</td>
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<tr>
<td>Bax (Negative) (Positive)</td>
<td>101 (63)</td>
<td>457 (67)</td>
<td>0.001*</td>
</tr>
<tr>
<td>Bcl2 (Negative) (Positive)</td>
<td>102 (54)</td>
<td>303 (35)</td>
<td>6.5x10^{-5}</td>
</tr>
<tr>
<td>p16 (Negative) (Positive)</td>
<td>121 (76)</td>
<td>693 (68)</td>
<td>0.001*</td>
</tr>
</tbody>
</table>

* Statistically significant; **: grade as defined by NGS; BRCA1: Breast cancer 1, early onset; HER2: human epidermal growth factor receptor 2; ER: oestrogen receptor; PGR: progesterone receptor; CK: cytokeratin; Basal-like: ER-, HER2 and positive expression of either CK5/6, CK14 or EGFR; Triple negative: ER-/PGR-/HER2-.

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Table 2: Multivariate analysis using Cox regression analysis confirms that XRCC1 protein expression is independent prognostic factor

<table>
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<th>Variable</th>
<th>BCSS</th>
<th>DFS</th>
<th>DM-FS</th>
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<tbody>
<tr>
<td></td>
<td>HR (CI 95%)</td>
<td>HR (CI 95%)</td>
<td>HR (CI 95%)</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>p</td>
<td>p</td>
</tr>
<tr>
<td>XRCC1 (negative)</td>
<td>1.9 (1.5-2.5)</td>
<td>1.8 (1.5-2.3)</td>
<td>1.8 (1.4-2.2)</td>
</tr>
<tr>
<td></td>
<td>5.0x10^{-7}**</td>
<td>1.3x10^{-7}**</td>
<td>5.3x10^{-9}**</td>
</tr>
<tr>
<td>Tumour size</td>
<td>1.3 (1.2-1.5)</td>
<td>1.2 (1.1-1.4)</td>
<td>1.3 (1.2-1.5)</td>
</tr>
<tr>
<td></td>
<td>5.2x10^{-8}**</td>
<td>5.8x10^{-7}**</td>
<td>7.7x10^{-7}**</td>
</tr>
<tr>
<td>Grade</td>
<td></td>
<td>1.1x10^{-7}**</td>
<td>0.007**</td>
</tr>
<tr>
<td>G1</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>G2</td>
<td>2.0 (1.2-3.2)</td>
<td>1.3 (0.9-1.8)</td>
<td>1.7 (1.2-2.6)</td>
</tr>
<tr>
<td>G3</td>
<td>3.5 (2.2-5.5)</td>
<td>1.2 (1.1-1.4)</td>
<td>2.5 (1.7-3.8)</td>
</tr>
<tr>
<td>Lymph node</td>
<td></td>
<td>1.6x10^{-7}**</td>
<td>5.6x10^{-7}**</td>
</tr>
<tr>
<td></td>
<td>1.6x10^{-7}**</td>
<td>5.6x10^{-7}**</td>
<td>3.0x10^{-7}**</td>
</tr>
<tr>
<td></td>
<td>1.6 (1.3-2.1)</td>
<td>1.4 (1.2-1.8)</td>
<td>1.6 (1.3-2.0)</td>
</tr>
<tr>
<td></td>
<td>(1.3-2.1)</td>
<td>(1.2-1.8)</td>
<td>(1.3-2.0)</td>
</tr>
<tr>
<td></td>
<td>4.4 (3.2-6.1)</td>
<td>3.8 (2.8-5.1)</td>
<td>4.5 (3.2-6.1)</td>
</tr>
</tbody>
</table>

** Statistically significant

BCSS; Breast cancer specific survival, DFS; disease free survival, DM-FS: distant metastases, HR; hazard ratio, CI; confident interval
Figure Legends

**Figure 1**: A. Microphotographs of XRCC1 expression in normal (1) and breast cancer tissue (2-5) (magnification x 200). Kaplan Meier curves showing breast cancer specific survival (BCSS) in the whole cohort (B), lymph node negative sub-group (C) and in lymph node positive sub-group (D), low risk BC (NPI<3.4) received no adjuvant therapy (E), high risk BC (NPI ≥3.4) ER + who received endocrine therapy (F), high risk BC (NPI ≥3.4) who received chemotherapy (G). See text for details.

**Figure 2**: Kaplan Meier curves showing breast cancer specific survival (BCSS) in the validation cohort. ER- high risk BC received anthracycline (A), Triple negative high risk that received chemotherapy (anthracycline or CMF) (B), Triple negative high risk that received anthracycline chemotherapy only (C), Triple negative high risk that received CMF chemotherapy only (D). See text for details.

**Figure 3**: A. Western blot analysis in chinese hamster (CH) cells (CHO9, EM-C11, EM-C12). Clonogenic survival assays for CH cells treated with KU55933 (B) and NU7441 (C) at indicated concentrations (see methods for details). (D) Neutral COMET assays for CH cells treated with KU55933. Compared to pre-treatment samples, after 24 hours of exposure to ATM inhibitor, the mean tail moment was significantly higher in EM-C11 and EM-C12 cells at 24 hours [(p=0.01)(p=0.05) respectively] and at 48 hours [(p=0.05)(p=0.04) respectively] in comparison to CHO9 cells) (E) Neutral COMET assays for CH cells treated with NU7441. The mean tail moment was significantly higher in EM-C11 and EM-C12 cells at 24 hours [(p=0.05)(p=0.04) respectively] and at 48 hours [(p= p=0.006)(p=0.01) respectively]). (F). Microphotographs of γH2AX immunocytochemistry in CH cells showing >6 γH2AX foci in EM-C11 and EM-C12 cells compared to CHO9 cells after 24 hours of treatment with KU55933. (G) In CHO9 cells, the mean γH2AX positive cells was 7 in pre-treatment cells and increased to 15 after the 24 hour treatment.
with KU55933. In EM-C11 cells, the mean γH2AX positive cells was 6 in pre-treatment cells and increased to 36 after the 24 hour treatment (p=0.01). (H) γH2AX immunocytochemistry data for NU7441 is shown here. Cells were exposed to 1.5 μM DNA-PKcs inhibitor for 24 hours and compared to control samples prior to compound treatment. In CHO9 cells, the mean γH2AX positive cells was 8 in pre-treatment cells and increased to 12 after the 24 hour treatment. In EM-C11 cells, the mean γH2AX positive cells was 10 in pre-treatment cells and increased to 26 after the 24 hour treatment (p=0.06).
Figure 4: A. A typical FACS read out in CH cells treated with 24 hours of KU55933 is shown here. Quantification of various phases of the cell cycle is shown for CH cell treated with KU55933 (B) and NU7441 (C). D. Western blot analysis in human cancer cells (control silenciX and XRCC1 deficient silenciX cells). Clonogenic survival assays for human cells treated with KU55933 (E) and NU7441 (F) at indicated concentrations is shown here (see methods for details). Neutral COMET assays for human cells treated with KU55933 (G) and NU7441 (H). (I) γH2AX immunocytochemistry in human cells showing >6 γH2AX foci in XRCC1 deficient silenciX cells compared to control silenciX after 24 hours of treatment with KU55933. In XRCC1 proficient control SilenciX cells, the mean γH2AX positive cells was 7 in pre-treatment cells and increased to 10 after a 24 hour treatment with KU55933. In XRCC1 deficient SilenciX cells, the mean γH2AX positive cells was 8 in pre-treatment cells and increased to 16 after a 24 hour treatment (p=0.04). (J). γH2AX immunocytochemistry in cells treated with NU7441. In XRCC1 proficient control SilenciX cells, the mean γH2AX positive cells was 7 in pre-treatment cells and increased to 8 after a 24 hour treatment with NU7441. In XRCC1 deficient SilenciX cells, the mean γH2AX positive cells was 8 in pre-treatment cells and increased to 15 after a 24 hour treatment (p=0.001) (see methods and results section for details). (K). Quantification of various phases of the cell cycle in human cells treated with 24 hours of KU55933. (L). Quantification of various phases of the cell cycle in human cells treated with 24 hours of NU7441. (M). Quantification of human cells (control and XRCC1 deficient SilenciX) undergoing apoptosis after 48 hour treatment with KU55933 (5 μM) and NU7441 (1.5 μM) [number in black colour bold=early phase apoptosis cells), number in red colour bold = late apoptosis].

Figure 5: A. Western blot analysis confirming XRCC1 knockdown in MCF-7 cells transfected with siRNA constructs 1 and 2. Scrambled negative control as well as GAPDH positive control is also shown here. B. Clonogenic survival assay. XRCC1 deficient cells are hypersensitive to KU55933. C. Clonogenic survival assay. NU7441 treatment was more toxic to XRCC1 deficient
cells compared to proficient cells. D. Neutral COMET assays. XRCC1 knockdown MCF-7 cells accumulate more DSBs after treatment with KU55933. E. After KU55933 treatment, XRCC1 knockdown cells accumulate significant number of γH2AX foci compared to proficient cells (see text for details). F. XRCC1 knockdown cells accumulate in G2/M phase of the cell cycle after treatment with KU55933. G. Neutral COMET assays. XRCC1 knockdown cells MCF-7 cells accumulate more DSBs after treatment with NU7441. H. After NU7441 treatment, XRCC1 knockdown cells accumulate significant number of γH2AX foci compared to proficient cells (see text for details). I. XRCC1 knockdown cells accumulate in G2/M phase of the cell cycle after treatment with NU7441.
1. Normal breast tissue
2. Negative
3. Low
4. Moderate
5. Strong

B. Whole patients cohort (n=1163)
Positive XRCC1 (n=977)
Negative XRCC1 (n=186)
Log rank = 41.9
HR (95% CI) = 2.2 (1.7-2.8), p = 9.8x10^{-11}

C. Lymph node negative (n=678)
Positive XRCC1 (n=571)
Negative XRCC1 (n=107)
Log rank = 23.7
HR (95% CI) = 2.4 (1.7-3.5), p = 1.1x10^{-6}

D. Lymph node positive (n=466)
Positive XRCC1 (n=391)
Negative XRCC1 (n=75)
Log rank = 18.5
HR (95% CI) = 2.1 (1.5-2.9), p = 1.7x10^{-5}

E. Low risk BC (NPI < 3.4) received no adjuvant therapy (n=368)
Positive XRCC1 (n=339)
Negative XRCC1 (n=37)
Log rank = 20.0
HR (95% CI) = 3.8 (2.0-7.2), p = 0.000003

F. High risk BC (NPI ≥ 3.4)/ER+ who received endocrine therapy (n=504)
Positive XRCC1 (n=438)
Negative XRCC1 (n=66)
Log rank = 14.4
HR (95% CI) = 2.1 (1.4-3.0), p = 0.0002

G. High risk BC (NPI ≥ 3.4) who received CMF chemotherapy (n=229)
Positive XRCC1 (n=183)
Negative XRCC1 (n=46)
Log rank = 9.2
HR (95% CI) = 2.0 (1.3-3.3), p = 0.002

Figure 1
A. ERα- high risk BC received Anthracycline

B. Triple negative high risk received chemotherapy (CMF or Anthracycline)

C. Triple negative high risk received Anthracycline

D. Triple negative high risk received CMF

Figure 2
**Figure 3**

A. Western blot analysis showing the expression levels of β-Actin, XRCC1, ATM, and DNA-PKcs in CHO9, EM-C11, and EM-C12 cell lines.

B. Graph illustrating the log surviving fraction against concentration (M) for CHO9, EM-C11, and EM-C12 cell lines.

C. Graph showing the log surviving fraction against concentration (M) for CHO9, EM-C11, and EM-C12 cell lines.

D. Bar graphs depicting the mean tail moment of CHO9, EM-C11, and EM-C12 cell lines treated with KU55933.

E. Bar graphs showing the mean tail moment of CHO9, EM-C11, and EM-C12 cell lines treated with NU7441.

F. Immunofluorescence images of CHO9, EM-C11, and EM-C12 cell lines untreated and treated with KU55933.

G. Bar graph illustrating the percentage of cells with >5 γH2AX foci in CHO9, EM-C11, and EM-C12 cell lines treated with KU55933.

H. Bar graph showing the percentage of cells with >5 γH2AX foci in CHO9, EM-C11, and EM-C12 cell lines treated with NU7441.
Figure 5
Targeting XRCC1 deficiency in breast cancer for personalized therapy

Rebeka Sultana, Tarek Abdel-Fatah, Rachel Abbotts, et al.

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