Untangling the ATR-Chk1 network for prognostication, prediction and therapeutic target validation in breast cancer

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ABSTRACT

Background: ATR-Chk1 signalling network is critical for genomic stability. ATR-Chk1 may be deregulated in breast cancer and have prognostic, predictive and therapeutic significance.

Patients and methods: We investigated ATR and phosphorylated CHK1\textsuperscript{Ser345} protein (pChk1) expression in 1712 breast cancers (Nottingham Tenovus series). ATR and Chk1 mRNA were evaluated in 1950 breast cancers (METABRIC cohort). Pre-clinically, biological consequences of ATR gene knockdown or ATR inhibition by small molecule inhibitor (VE-821) were investigated in MCF-7 and MDA-MB-231 breast cancer cell lines and in non-tumorigenic breast epithelial cells (MCF10A).

Results: High ATR and high cytoplasmic pChk1 expression was significantly associated with higher tumour stage, higher mitotic index, pleomorphism and lymphovascular invasion. In univariate analysis, high ATR and high cytoplasmic pChk1 protein expression was associated with shorter breast cancer specific survival (BCSS). In multivariate analysis, high ATR remains an independent predictor of adverse outcome. At the mRNA level, high Chk1 remains associated with aggressive phenotypes including lymph node positivity, high grade, Her-2 overexpression, triple-negative phenotype and molecular classes associated with aggressive behaviour and shorter survival. Pre-clinically, Chk1 phosphorylation at serine 345 following replication stress (induced by gemcitabine or hydroxyurea treatment) was impaired in ATR knockdown and in VE-821 treated breast cancer cells. Doxycycline inducible knockdown of ATR suppressed growth, which was restored when ATR was re-expressed. Similarly, VE-821 treatment resulted in a dose dependent suppression of cancer cell growth and survival (MCF7 and MDA-MB-231) but had no effect on non-tumorigenic breast epithelial cells (MCF10A).
**Conclusions:** We provide evidence that ATR and Chk1 are promising biomarkers and rational drug target for personalized therapy in breast cancer.
INTRODUCTION

Ataxia telangiectasia mutated and Rad3 related protein (ATR), a serine threonine kinase belonging to the PIKK family (phosphoinositide 3-kinase-like family of protein kinase), is a key regulator of genomic integrity. ATR is activated by single stranded (ss)-double stranded (ds) DNA junctions generated at stalled replication forks that frequently occur at sites of DNA damage. Activated ATR in turn phosphorylates Chk1 at Ser\textsuperscript{345} and Ser\textsuperscript{317}, as well as several other target proteins involved in homologous recombination repair and DNA cross-link repair. Phosphorylation of Chk1 at Ser\textsuperscript{345} (pChk1) leads to its activation which not only promotes further autophosphorylation at Ser\textsuperscript{296}, but also results in phosphorylation and inactivation of Cdc25A and Cdc25c. Whereas Cdc25A activates S-phase progression, Cdc25C through CDK1 (Cdc2) regulates mitotic entry. In addition, Chk1 also targets many other proteins involved in cell cycle regulation and DNA repair. As a consequence of the considerable cross talk, ATR-Chk1 activation ultimately results in arrest of cell cycle progression allowing sufficient time for DNA repair to be completed for the maintenance of genomic integrity.

\textit{ATR} gene mutation is associated with Seckel syndrome which is characterised by growth retardation and microcephaly. However the role of ATR gene mutations or polymorphisms in cancer pathogenesis is less well defined. In breast cancer, no significant associations between \textit{ATR} or Chk1 polymorphism, cancer risk or survival outcomes have been demonstrated. The clinical significance of ATR or Chk1 mRNA or protein expression is also not fully known in human cancer. Despite a paucity of clinical studies, ATR and Chk1 have emerged as promising anti-cancer drug targets. In cell line models, dominant negative inhibition of ATR function resulted in hypersensitivity to multiple chemotherapeutic agents and radiation.
Similarly, ATR or Chk1 knockdown by siRNA lead to chemotherapy and radiotherapy potentiation. Pharmacological inhibition of ATR or Chk1 by small molecule inhibitors results in chemotherapy and radiotherapy potentiation in cancer cell lines as well as in xenograft models. Extensive preclinical data therefore provides compelling evidence to investigate ATR and/or Chk1 blockade as a promising therapeutic target in patients.

Our hypothesis is that altered ATR and pChk1 expression may influence breast cancer biology and behaviour, adversely impact clinical outcomes and could be suitable for rational therapeutic targeting in breast cancer. In the current study we have investigated ATR and Chk1 in large cohorts of primary breast cancers at the protein and mRNA level and have demonstrate for the first time that ATR and pChk1 overexpression is linked to aggressive phenotype and poor outcomes in breast cancer. Pre-clinically, in breast cancer cell lines we show that phosphorylation and activation of Chk1 at Ser\textsuperscript{345} is ATR dependent. ATR gene knockdown or ATR inhibition by small molecule inhibition impairs breast cancer cell growth. We conclude that ATR-pChk1 is a rational target for therapeutic application in breast cancer.
METHODS

Clinical study

Patients: The study was performed in a consecutive series of 1712 patients with primary invasive breast carcinoma who were diagnosed from 1986 to 1999 and entered into the Nottingham Tenovus Primary Breast Carcinoma series. Patient demographics are summarised in supplementary table S1. This is a well-characterized series of patients with long-term follow-up that have been investigated in a wide range of biomarker studies \{Ellis, 1992 \#32; Ellis, 1992 \#32; Elston, 1991 \#31\}. All patients were treated in a uniform way in a single institution with standard surgery (mastectomy or wide local excision) with radiotherapy. Prior to 1989, patients did not receive systemic adjuvant treatment (AT). After 1989, AT was scheduled based on prognostic and predictive factor status, including Nottingham Prognostic Index (NPI), oestrogen receptor-α (ER-α) status, and menopausal status. Patients with NPI scores of <3.4 (low risk) did not receive AT. In pre-menopausal patients with NPI scores of ≥3.4 (high risk), classical Cyclophosphamide, Methotrexate, and 5-Flurouracil (CMF) chemotherapy was given; patients with ER-α positive tumours were also offered endocrine therapy. Postmenopausal patients with NPI scores of ≥3.4 and ER-α positivity were offered endocrine therapy, while ER-α negative patients received classical CMF chemotherapy. Median follow up was 111 months (range 1 to 233 months). Survival data, including overall survival, disease-free survival (DFS), and development of loco-regional and distant metastases (DM), was maintained on a prospective basis. DFS was defined as the number of months from diagnosis to the occurrence of local recurrence, local lymph node (LN) relapse or DM relapse. Breast cancer specific survival (BCSS) was defined as the number of months from diagnosis to the occurrence of BC related-death. Local recurrence free survival (LRS) was defined the number of months from diagnosis to the occurrence of local recurrence. DM-free survival was defined as the number of months from
diagnosis to the occurrence of DM relapse. Survival was censored if the patient was still alive at the time of analysis, lost to follow-up, or died from other causes.

Tumor Marker Prognostic Studies (REMARK) criteria, recommended by McShane et al (McShane, 2005 #37), were followed throughout this study. Ethical approval was obtained from the Nottingham Research Ethics Committee (C202313).

**Construction of Tissue Microarray (TMA):** TMAs were constructed as previously described (refs). Four micron sections of the tissue array block were cut and placed on Superfrost Plus slides for immunohistochemical staining.

**Immunohistochemistry (IHC):** The TMAs were immunohistochemically profiled for ATR, phosphorylated Chk1 and other biomarkers included in this study (Supplementary Table S2) as previously described (Sultana, 2013 #4088). Immunohistochemical staining was performed using the Thermo Scientific Shandon Sequenza chamber system (REF: 72110017), in combination with the Novolink Max Polymer Detection System (RE7280-K: 1250 tests), and the Leica Bond Primary Antibody Diluent (AR9352), each used according to the manufacturer’s instructions (Leica Microsystems). The tissue slides were deparaffinised with xylene and then rehydrated through five decreasing concentrations of alcohol (100%, 90%, 70%, 50% and 30%) for two minutes each. Pre-treatment antigen retrieval was performed on the TMA sections using sodium citrate buffer (pH 6.0), heated for 20 minutes at 95°C in a microwave (Whirlpool JT359 Jet Chef 1000W). A set of slides were incubated for 18 hours at 4°C with the primary mouse monoclonal anti-ATR antibody, clone 1E9 (H00000545-M03, Novus Biologicals, Cambridge, UK), at a dilution of 1:20. A further set of slides were incubated for 60 minutes with the primary rabbit polyclonal anti-phosphorylated Chk1 antibody (Ab58567, Abcam, Cambridge, UK), at a dilution of 1:140.
To validate the use of TMAs for immunophenotyping, full-face sections of 40 cases were stained and protein expression levels of DNA-PKcs 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 immunohistochemical staining:** The tumour cores were evaluated by specialist pathologists and oncologists blinded to the clinico-pathological characteristics of patients. 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. H-score in a range of 0 – 300 was generated. The median H-score of 100 was taken as the cut-off and low ATR or pChk1 expression was classed as H-score of ≤ 100 and >100 was classed as high for ATR or pChk1 expression. Not all cores within the TMA were suitable for IHC analysis as some cores were missing or lacked tumour. HER2 expression was assessed according to the new ASCO/CAP guidelines using IHC and chromogenic in situ hybridisation (CISH) [Wolff, 2007 #36].

**ATR and Chk1 gene expression:** The mRNA expression of ATR and Chk1 was performed in the METABRIC (Molecular Taxonomy of Breast Cancer International Consortium) cohort. The METABRIC study protocol, detailing the molecular profiling methodology in a cohort of 1980 breast cancer samples is described by Curtis et al (Curtis et al.). Patient demographics are summarized in supplementary Table S3 of supporting information. ER positive and/or
lymph node negative patients did not receive adjuvant chemotherapy. ER negative and/or lymph node positive patients received adjuvant chemotherapy. RNA was extracted from fresh frozen tumours and subjected to transcriptional profiling on the Illumina HT-12 v3 platform. The data was pre-processed and normalized as described previously (Curtis et al.). ATR and Chk1 expression was investigated in this data set and correlated with clinicopathological features, molecular classes and outcome. X-tile (version 3.6.1, Yale University, USA) was used to identify a cut-off in gene expression values to divide the population into high/low subgroups prior to analysis. The Chi-square test was used for testing association between categorical variables and a multivariate Cox model was fitted to the data using as endpoint breast cancer specific death. Cumulative survival probabilities were estimated using the Kaplan–Meier method.

Statistical analyses: Data analysis was performed using SPSS (SPSS, version 17 Chicago, IL). Where appropriate, Pearson’s Chi-square, Fisher’s exact, $\chi^2$ for trend, Student’s t and ANOVAs one way tests were performed using SPSS software (SPSS, version16 Chicago, IL). Cumulative survival probabilities were estimated using the Kaplan–Meier method. Differences between survival rates were tested for significance using the log-rank test. Multivariate analysis for survival was performed using the Cox hazard model. The proportional hazards assumption was tested using standard log-log plots. Each variable was assessed in univariate analysis as a continuous and categorical variable and the two models were compared using an appropriate likelihood ratio test. Hazard ratios (HR) and 95% confidence intervals (95% CI) were estimated for each variable. All tests were two-sided with a 95% CI. P values for each test were adjusted with Benjamini and Hochberg multiple P-value adjustment and an adjusted p value of <0.05 was considered significant.

Pre-clinical study
**Chemicals and reagents:** All chemicals and reagents were obtained from Sigma (St Louis, MO, USA) unless otherwise stated. VE-821 was from Vertex Pharmaceuticals (Abingdon, UK).

**Cells and cell lines:** MCF7 (p53 wild type, ER +ve human breast adenocarcinoma) and MDA-MB-231 (p53 mutant, triple negative human breast adenocarcinoma) cells were grown in RPMI-1641 media containing 10% FBS, 100 units/ml penicillin and 0.1 mg/ml streptomycin. MCF10A (p53 wild type, immortalised human non-tumorigenic breast epithelium) cells from ATCC (Manassus, VA, USA) were maintained in DMEM/Ham’s F12 Nutrient mixture with 5% horse serum (Gibco, Life Technologies, Paisley, UK), 0.5 µg/ml hydrocortisone, 20 ng/ml epidermal growth factor, 10 µg/ml insulin and 100 ng/ml cholera toxin. Cells were maintained at 37°C and 5% CO₂ and were authenticated (LGC Standards, Teddington, UK) and confirmed to be mycoplasma free (Mycoalert, Lonza, Basel, Switzerland).

**siRNA knockdown of ATR:** MCF7 cells were added to either scrambled siRNA (5’ – UUCUCCGAACGUGUCACGUdtdt) or ATR specific siRNA (5’-CAUCUUAUCCCAUGCGUGUdtdt) diluted in OptiMEM (Gibco, Life Technologies, Paisley, UK) and Lipofectamine RNAiMax (Life Technologies, Paisley, UK) to a final siRNA concentration of 10 nM. Cells were allowed to adhere for 48 hours before being treated for 1 hour with 1 µM gemcitabine or 10 mM hydroxyurea.

**shRNA knockdown of ATR:** Lentivirus particles containing ATR-specific shRNA were formed by transfecting HEK293T cells with pCMVΔ8.91 packaging vector, pMD2.G envelope vector and pTRIPZ doxycycline-inducible lentiviral vector containing ATR shRNA (Thermo, Northumberland, UK). Virus particles released into the media were collected and purified via ultracentrifugation using Lenti-X concentrator (Clontech, Mountain View, USA). MCF7 cells were transduced with ATR shRNA lentivirus and stably expressing clones identified and grown by selection using 1 µg/ml puromycin. MCF7
shATR cells were maintained in full media supplemented with 1 µg/ml puromycin. shATR expression was induced by supplementing media with 1 µg/ml doxycycline. Cells grown in the absence of doxycycline were used as a control.

**Gel electrophoresis and western blotting:** Cells were prepared for gel electrophoresis by lysis using Phosphosafe extraction reagent (Merck, Darmstadt, Germany) and sonication. Protein concentration was determined using a Pierce BCA protein assay (Thermo, Northumberland, UK). Lysates were diluted in 4 x XT sample buffer (Bio-Rad, Hemel Hempstead, UK) and subjected to gel electrophoresis using 4-15% Tris-Glycine cells (Bio-Rad, Hemel Hempstead, UK). Proteins were transferred onto Hybond C-Extra nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK) using western blotting. Proteins were detected using primary antibodies incubated overnight at 4°C: goat anti-ATR (1:300 N-19 – Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-pChk1\(^{\text{Ser345}}\) (1:300 133D3 – Cell Signalling Technologies, Danvers, MA, USA), mouse anti-Chk1 (1:300 G-4 – Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-actin (1:1000 AC40 – Sigma, Poole, UK), mouse anti-β-actin (1:10000 2D1D10 – Genscript, Piscataway, NJ, USA). Secondary antibodies were anti-goat-HRP (1:2000, Santa Cruz Technologies, Santa Cruz, CA, USA), anti-mouse-HRP (1:2000 – Dako UK Ltd, Ely, UK) and anti-rabbit-HRP (1:1000 – Dako UK Ltd, Ely, UK). Chemiluminescence from ECL Prime detection reagent (GE Healthcare, Buckinghamshire, UK) was detected using a G-box (Syngene, Cambridge, UK) and band intensities were measured by densitometry using Genetools software (Syngene, Cambridge, UK).

**Growth assay:** MCF7, MCF7 shATR or MDA-MB-231 cells were seeded at a density of 1000 cells/well into 96-well tissue culture plates. To assess the effect of ATR knockdown on cell growth MCF7 shATR cells were treated with 1 µg/ml doxycycline for 3 days prior to seeding then growth was measured over the next 11 days in fresh medium. For growth inhibition assays cells were treated for 24 hours with VE-821 then allowed to grow in fresh
media for 5 days. Cells were fixed using Carnoy’s fixative and allowed to dry overnight. Cell growth was measured by staining cells with 10 µg/ml DAPI in 0.001% Triton-X-100. Cells were solubilised using 10 mM EDTA (pH 2.2) and the fluorescence measured at 460 nm using a MFX Microtiter Plate Fluorimeter (Dynex Technologies, Chantilly, VA, USA).

**Clonogenic survival assay:** MCF7, MDA-MB-231 or MCF10A cells seeded at a density of 1 x 10^5 cells/ml. Following treatment with VE-821 for 24 hours cells were counted and re-seeded at low density for colony formation. Colonies were allowed to grow for 2 weeks in fresh media before being fixed using Carnoy’s fixative and stained using 1% crystal violet. Colonies were counted and the % plating efficiency calculated.
RESULTS

ATR and pChk1 protein expression correlate to aggressive breast cancer phenotypes

**ATR:** ATR was detected in the nuclei of the tumour cells with no cytoplasmic or membranous expression. High ATR expression was seen in 409/1298 (31.5%) tumours compared to 889/1298 (68.5%) tumours that had low/negative ATR expression (Figure 1A) (Table 1). High ATR expression was associated with higher stage (p=0.036), higher tumour grade (p<0.001) with higher mitotic index (p<0.001) and cellular pleomorphism (p<0.001) and lymphovascular invasion (p=0.009) (Table 1). High ATR expression was associated with absent/reduced expression of DNA damage response (DDR) proteins including BRCA1, XRCC1, SMUG1 and DNA-PKcs (p<0.05). High MIB1, TOPO2A and CDK1 and Low Bax and MDM2 were more frequently seen in high ATR expressing tumours (p<0.05).

**pChk1:** High cytoplasmic pChk1 expression was seen in 672/1712 (39.3%) tumours compared to 1040/1712 (60.7%) tumours that had low ATR expression (Figure 1A) (Table 2). High cytoplasmic pChk1 protein expression was significantly associated with higher grade, higher mitotic index, nuclear pleomorphism, histological tumour type and lymphovascular invasion (p<0.05). Triple-negative and basal-like phenotypes were more likely in high cytoplasmic pChk1 protein expressing tumours (p<0.05). Absence/reduced expression of DDR proteins including BRCA1, XRCC1, APE1, polβ, but increased expression of ATR and DNA-PKcs were also more likely associated with high cytoplasmic pChk1 protein expressing tumours (p<0.05). High MIB1, high TOPO2A, high CDK1 and low MDM2 was likely in high cytoplasmic pChk1 protein expressing tumours (p<0.05).

High nuclear pChk1 expression was seen in 264/1712 (15.4%) tumours compared to 1448/1712 (84.6%) tumours that had low nuclear pChk1 expression (Figure 1A) (Supplementary Table S4). In contrast to cytoplasmic expression, low nuclear pChk1 protein expression was significantly associated with features of aggressive behaviour including
higher grade, higher mitotic index, de-differentiation and nuclear pleomorphism (p<0.05). ER-, PR-, AR-, Her-2 positive tumours were more likely in low nuclear pChk1 protein expressing tumours (p<0.05). Absence of BRCA1, low XRCC1, low SMUG1, low APE1, low SMUG1, low polβ and low DNA-PKcs were also more likely associated with low nuclear pChk1 protein expressing tumours (p<0.05). High MIB1, low TOPO2A and low MDM2 was likely in low nuclear pChk1 protein expressing tumours (p<0.05).

**ATR-pChk1 combined analysis:** The data presented above suggests that differential ATR/pChk1 expression may influence breast cancer phenotypes. To evaluate further, we performed ATR/pChk1 combined analysis. As shown in supplementary table S5, tumours that had high ATR expression/high cytoplasmic pChk1 expression/low nuclear pChk1 expression had larger size, higher stage, higher grade, higher mitotic index, de-differentiation, pleomorphism, HER-2 overexpression, ER-/PR-/AR-, high MIB1, low TOP2A, low Bax and high CDK1 phenotypes (p<0.05). In addition, such tumours also exhibited a genomic instability phenotype characterised by absence of BRCA1, low XRCC1, low SMUG1, low polβ and low DNA-PKcs expression (p<0.01). Interestingly, although there was no association with p53 mutation, low MDM2 was more likely in tumours with high ATR expression/high cytoplasmic pChk1 expression/low nuclear pChk1 expressing tumours.

**ATR and pChk1 protein expression associate with poor survival outcomes in patients**

**Univariate analysis:** High ATR expression was associated with worse breast cancer specific survival in patients (p=0.001) (Figure 1B1). High cytoplasmic pChk1 expression was associated with shorter BCSS (p=0.005) (Figure 1B2). In contrast, low nuclear pChk1 expression was associated with worse BCSS (p=0.007) (Supplementary Figure 1A). Investigating nuclear and cytoplasmic pChk1 together we found that patients whose tumours have low nuclear and high cytoplasmic Chk1 have the worst survival compared to tumours that have high nuclear and low cytoplasmic Chk1 (p<0.001) (Figure 1B3). We then combined ATR and pChk1 in the analysis. As shown in figure 1B3, high ATR/low nuclear Chk1/high
cytoplasmic Chk1 tumours have the worst survival (p<0.001). As p53 may be involved in the activation of ATR-Chk1 pathway we also conducted an exploratory analysis in p53 mutant and proficient tumours. As shown in Figures 2A and 2B, high ATR/p53 mutants, high cytoplasmic pChk1/p53 mutants have the worst survival compared to tumours that are p53 wild-type. Similarly, high nuclear pChk1/p53 mutants have the worst survival compared to tumours that are p53 wild-type (Supplementary Figure 1B).

We then proceeded to various sub-group analysis in ER+ and ER- breast cancer cohorts (Supplementary Figures 2 and 3). In patients with ER-negative tumours that received no chemotherapy, high ATR remains associated with poor survival (Supplementary Figures 2A1) but this association was not observed in the ER-negative tumours that received adjuvant chemotherapy. On the other hand, in ER- negative tumours that received adjuvant chemotherapy (anthracycline or CMF chemotherapy), cytoplasmic pChk1 was associated with poor survival. Interestingly, whereas, high cytoplasmic pChk1 was associated with poor survival after CMF chemotherapy (Supplementary Figures 2B3), low cytoplasmic pChk1 was associated with poor survival after anthracycline chemotherapy (Supplementary Figures 2B2). Nuclear pChk1 did not influence survival in various sub-groups (Supplementary Figures 2C). In ER+ tumours, no significance was evident except for nuclear pChk1, where low expression was associated with poor survival to adjuvant endocrine therapy (Supplementary Figures 3).

**Multivariate analysis:** High ATR was an independent predictor of worse BCSS in multivariate analysis (p=0.006) (Supplementary table S6). Stage, grade, endocrine therapy, **bcl-2** expression status were other independent markers of poor survival in patients. The expression of cytoplasmic or nuclear Chk1 did not independently influence survival.

**ATR and Chk1 mRNA expression in breast cancer**
The data presented above provides evidence that high ATR and high Chk1 protein expression have prognostic significance in breast cancer. To investigate whether RNA of these genes are also associated with outcome and to assess the role of RNA-protein translation on the clinical significance of genes, we explored ATR and Chk1 mRNA expression in the METABRIC cohort that comprises 1950 breast tumours (nature reference).

Interestingly, high ATR mRNA was not significantly associated with any clinicopathological features or survival in the METABRIC cohort (Figure 2B, Supplementary Table S7, supplementary Figure 3A and supplementary Figure 3C). On the other hand, as shown in supplementary table S8, high Chk1 mRNA was significantly associated with high tumour T-stage, high grade, lymph node positivity, high risk NPI score (>3.4), HER-2 over expression, ER negative, triple negative, and molecular classes associated with aggressive behaviour and poor outcome including Genefu subtypes (ER-/Her-2 negative, ER+/Her-2 negative/high proliferation), PAM50.Her-2, PAM50. Basal, integrative molecular cluster (intClust).1, intClust.5, intClust.9 and intClust.10 phenotypes (p<0.01). Patients with tumours that had high Chk1 mRNA had significantly shorter BCSS compared to tumours that had low Chk1 mRNA expression (p<00001) (Figure 2C). In ER+ tumours that received adjuvant endocrine therapy, high Chk1 mRNA was significantly associated with worse BCSS (p<0.001) (Supplementary Figure 3B). Conversely, in ER- tumours that received adjuvant chemotherapy, high Chk1 mRNA was not associated with worse BCSS (Supplementary Figure 3D).

Taken together, protein as well as mRNA expression data suggests that for ATR, protein over expression is likely to be due to a post-transcriptional/translational mechanism. However for Chk1, protein over expression is due to transcriptional up-regulation.
Replication stress induced phosphorylation of Chk1 at serine\textsuperscript{345} is ATR dependent in breast cancer cells

Multivariate analysis in human tumours provides evidence that ATR independently influences survival in patients and is key biomarker. To provide further preclinical evidence we proceeded to mechanistic studies in breast cancer cell lines. We first generated ATR knockdown (KD) breast cancer cells using an ATR specific siRNA construct. As shown in Figure 3A1 and 3A2, we achieved more than 90% KD of ATR in MCF7 cells. To induce replication stress, ATR wild type or ATR KD MCF7 cells were treated with 1\(\mu\)M of gemcitabine or 10 mM of hydroxyurea (HU). Phosphorylation of Chk1 at serine\textsuperscript{345} was impaired, upon gemcitabine or HU treatment, in ATR KD cells but not in MCF7 cells treated with scrambled control (Figure 3A1 and 3A2). The data shows that Chk1 phosphorylation is ATR dependent in breast cancer cells. To provide further evidence, we treated MCF7 with VE-821, a potent and selective ATP competitive inhibitor of ATR (\(K_i\) and \(IC_{50}\) of 13 nM and 26 nM respectively). Whereas gemcitabine treatment in the absence of VE-821 induced robust phosphorylation of Chk1 at serine\textsuperscript{345}, there was a dose dependent inhibition of Chk1 phosphorylation by VE-821 in Gemcitabine treated cells (Figures 3B1-3) \([IC_{50} (\mu M): MCF7 = 3.62 \pm 1.94, MDA-MB-231 = 0.57 \pm 0.30]\). In MDA-MD-231 and in non-tumorigenic breast epithelium cells (MC710A) we also observed a similar impairment of Chk1 phosphorylation by VE-821 (Figures 3B2 and 3B3) \([IC_{50} (\mu M) = 1.16 \pm 1.32]\). Taken together the data confirms that Chk1 activation is ATR dependent in MCF7, MDA-MB-231 and MCF10A cells.

ATR is required for breast cancer cell growth

To investigate whether ATR depletion has biological consequences in cells, we generated stable ATR KD MCF7 cells using doxycycline inducible shRNA. Following 3 days of treatment with doxycycline, robust KD of ATR was evident (Figure 4A1). Doxycycline was then removed and cell were monitored for growth and ATR protein expression over 11 days.
As shown in Figure 4A1 and 4A2, ATR deficiency arrested cell growth. However upon re-expression of ATR, cell growth was restored. Doxycycline treatment alone in un-transfected MCF7 cells had no effect (supplementary Figure 4).

We then investigated the effect of VE-821 in breast cancer cells. As shown in Figure 4B, a dose dependent suppression of cell growth was evident in MCF7 (GI₅₀ = 0.25 μM) and in MDA-MB-231 cells (GI₅₀ = 1.70 μM). There was also a significant direct correlation between growth inhibition and ATR inhibition in MCF7 and MDA-MB-231 cells (Figure 4C). To investigate whether VE-821 has selective toxicity to cancer cells, we performed clonogenic cell survival assays in MCF7, MDA-MB-231 and MCF10A cells. VE-821 exhibited selective toxicity in breast cancer cells (MCF7 and MDA-MB-23) compared to non-tumorigenic breast epithelium cells (MCF10A) (Figure 4D).

Taken together the preclinical study provides evidence that ATR is essential for breast cancer growth. In addition, the data also concurs with clinical study that demonstrated ATR overexpression as an independent prognostic factor in breast cancer.
DISCUSSION

ATR, a key factor in the maintenance of genomic integrity, is activated by single stranded (ss)-double stranded (ds) DNA junctions generated at stalled replication forks either during replication stress or during genotoxic therapy. Activated ATR in turn phosphorylates Chk1 at Ser 345 (pChk1) resulting in regulation of cell cycle progression and DNA repair. This is the first study to comprehensively investigate ATR and pChk1 in large cohorts of breast cancers. The data presented here provides compelling evidence that ATR and pChk1 have prognostic and predictive significance in breast cancer.

Chk1 is a key mediator of checkpoint regulation, genomic stability and cellular survival. Emerging evidence suggests that Chk1 undergoes alterations in sub-cellular localisation in response to DNA damage. Under normal conditions, Chk1 is localized to the nucleus. Upon DNA damage, ATR induced phosphorylation of Chk1 results in rapid localization to the cytoplasm. Nuclear Chk1 is known to activate RAD-51 dependent DNA repair whereas cytoplasmic Chk1 may be involved in cytoplasmic downstream checkpoint events. Chk1 phosphorylated at serine 345 is known to accumulate in the cytoplasm (Naidi et al JCB). To address whether cytoplasmic or nuclear pChk1 has clinicopathological significance in breast cancer, we evaluated pChk1 expression in the nucleus as well as in the cytoplasm of breast tumour cells. The data presented here provides evidence that over expression of cytoplasmic pChk1 is associated with aggressive features such as high grade, higher mitotic index including association with high MIB1, pleomorphism, triple negative, basal-like phenotype and poor survival. The data would concur with preclinical observation that suggest a genomic instability phenotype for cells that sequester Chk1 in the cytoplasm thereby driving a mutator phenotype characterised by aggressive pathology and clinical behaviour. Interestingly, nuclear pChk1 overexpression appears to be associated with lower grade, lower mitotic index, better differentiated tumours and improved survival in our study. Moreover, nuclear pChk1 overexpressing tumours are likely to be ER positive and associate with high
expression of other DNA repair proteins such as BRCA1, XRCC1, FEN1, SMUG1, APE1, polymerase beta and DNA-PKcs. The data would concur with preclinical observations suggesting a genomic stability role for nuclear Chk1. Combined analysis also showed that tumours with high cytoplasmic/low nuclear pChk1 tumours have the worst survival compared with low cytoplasmic/high nuclear tumours implying that altered sub-cellular localisation of Chk1 has clinicopathological significance. A striking observation in the current study was that Chkl mRNA overexpression was very highly significantly associated with aggressive phenotypes (such as lymph node positivity, high grade, high risk NPI score, ER+ luminal phenotype, Her-2 overexpression, triple negative phenotype) and poor survival. Chkl mRNA levels were also linked to biologically distinct integrative clusters reported in the METABRIC study. High Chkl mRNA level was frequent in intClust 10 subgroup which is the most highly genomically instable sub group with basal-like features. Whereas low Chkl mRNA level was seen in intClust 3 subgroup that is characterised by low genomic instability. In addition, high Chkl mRNA level is also frequently seen in intClust 5 (HER-2 enriched with worst survival), intClust 9 (8q cis-acting/20qamplified mixed subgroup), and intClust 1 (17q23/20q cis-acting luminal B subgroup) subgroups that also manifest an aggressive phenotype. On the other hand, low Chkl mRNA level is linked to intClust 4 (includes both ER-positive and ER-negative cases with a flat copy number landscape and termed the ‘CNA-devoid’ subgroup with extensive lymphocytic infiltration), intClust 7 (16p gain/16q loss with higher frequencies of 8q amplification luminal A subgroup) and intClust 8 subgroups (classical 1q gain/16q loss luminal A subgroup) (18). High Chkl mRNA was associated with poor survival in the METABRIC cohort. Interestingly, intClust 10, intClust 9, intClust 5 and intClust 1 sub-groups that are associated with high Chkl levels were also associated with poor prognosis in METABRIC study (18).In contrast, intClust 3, intClust 4, intClust 7 and intClust 8 that are associated with low Chkl expression, are associated with good to intermediate prognosis. In a study in triple negative breast cancer, Chkl was found to be overexpressed by transcriptional up-regulation through E2F1 Transcription factor. Similarly,
in lung cancer and colorectal cancer high levels of Chk1 may be associated with poor differentiation and worse survival. Together the data provides further evidence that Chk1 is a key biomarker and a rational drug target in breast cancer.

A surprising observation in the METABRIC cohort was that \textit{ATR} mRNA expression was not associated with any adverse clinicopathological features or survival implying that ATR protein overexpression is likely due to post-transcriptional/translational mechanisms. ATR protein overexpression was associated with aggressive tumours (such as high grade, size, higher mitotic index, pleomorphism) and poor survival. As proficient ATR and pChk1 indicate a functional ATR-Chk1 signalling pathway in cells, we performed combined analysis and demonstrated that tumours that are high ATR/high cytoplasmic pChk1/low nuclear Chk1 have the most aggressive phenotype and the worst survival.

Preclinical studies provide a link between p53 and ATR-Chk1 network. Loss of p53 may lead to increased replication stress and influence ATR/Chk1 expression. Interestingly, p53 deficient tumours appear to be sensitive to blockade by ATR or Chk1 inhibitors. In the current study, although there was no association with p53 mutation, low MDM2 was more likely in tumours with high ATR expression/ high cytoplasmic pChk1 expression /low nuclear pChk1 expressing tumours. We also demonstrate that ATR high/p53 mutants and Chk1 high/p53 mutants have the worst survival compared to that ATR low/p53 wild type and Chk1 high/p53 wild type. The data suggests that such breast cancer would be particularly suitable for personalized therapy.

An unexpected finding in the current study was a lack of clear evidence for predictive significance of ATR or Chk1 in breast cancer patients receiving adjuvant chemotherapy. At the mRNA level \textit{ATR} or \textit{Chk1} , did not influence survival in ER- negative tumours receiving chemotherapy. At the protein level, only cytoplasmic pChk1 overexpression appears to be associated with worse survival in patients treated with CMF (cyclophosphamide, methotrexate and 5-fluorouracil) chemotherapy. Surprisingly, low cytoplasmic pChk1 was
associated with poor survival in patients receiving anthracycline chemotherapy. This is in contrast to previous pre-clinical observation suggesting anthracycline sensitivity in Chk1 deficient cancer cell lines. Therefore further clinical studies are required to confirm these observations.

In the multivariate model, high ATR expression was independently associated with worse BCSS implying that ATR is an important prognostic biomarker in breast cancer. To provide additional evidence, we investigated in breast cancer cell lines. Firstly, we showed that ATR was directly involved in phosphorylation of Chk1 at serine 345 in breast cancer cells. Secondly, ATR knock down reduced proliferation and survival. Thirdly, VE-821, a specific and potent small molecule inhibitor of ATR not only blocked ATR induced Chk1 phosphorylation but also reduced breast cancer cell survival in a dose dependent manner. Taken together, the clinical and pre-clinical data provides compelling evidence that ATR is a promising target for anti-cancer therapy.

In conclusion, we provide confirmatory evidence that ATR-Chk1 influences breast cancer pathogenesis and clinical outcomes. Our data would support accelerated evaluation of ATR and Chk1 inhibitors currently under clinical development for personalized therapy in breast cancer patients.
### Table 1. ATR protein expression in breast cancer.

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<th>ATR PROTEIN EXPRESSION</th>
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<td>High N (%)</td>
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<tr>
<td>&gt;1-2cm</td>
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<tr>
<td>&gt;2-5cm</td>
<td>296 (33.3)</td>
<td>187 (45.7)</td>
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<tr>
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<td>12 (2.9)</td>
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<tr>
<td>2</td>
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<td>133 (32.4)</td>
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<tr>
<td>3</td>
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<td>42 (10.2)</td>
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<td>2 (10%-75% definite tubule)</td>
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**B) Aggressive phenotype**
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<td>56 (20.4)</td>
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Bold = statistically significant; 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-
Table 2. Cytoplasmic pChk1 expression in breast cancer.

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<th>VARIABLE</th>
<th>pCHK1 (Cyto) PROTEIN EXPRESSION</th>
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<td>Low N (%)</td>
<td>High N (%)</td>
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<td>A) Pathological Parameters</td>
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<tr>
<td>Tumour Size</td>
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<tr>
<td>&lt;1cm</td>
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<td>107 (10.3)</td>
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<td>&gt;1-2cm</td>
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<td>&gt;5cm</td>
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<td>Tumour Stage</td>
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<tr>
<td>1</td>
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<td>634 (60.8)</td>
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<tr>
<td>2</td>
<td>183 (27.2)</td>
<td>304 (29.1)</td>
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<tr>
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<td>45 (6.7)</td>
<td>105 (10.1)</td>
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<td>Tumour Grade</td>
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<td>G1</td>
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<td>166 (16.0)</td>
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<td>G2</td>
<td>226 (33.6)</td>
<td>339 (32.6)</td>
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<td>G3</td>
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<td>535 (51.4)</td>
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<td>M2 (medium; mitoses 10-18)</td>
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<td>1 (&gt;75% of definite tubule)</td>
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<td>60 (5.8)</td>
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<tr>
<td>2 (10%-75% definite tubule)</td>
<td>213 (32.1)</td>
<td>346 (33.4)</td>
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<tr>
<td>3 (&lt;10% definite tubule)</td>
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E) Cell cycle/apoptosis regulators
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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-
FIGURE LEGENDS

Figure 1. ATR and pChk1 protein expression in breast cancer. A. Microphotograph of ATR and pChk1 negative positive breast cancer tissue. B. Kaplan Meier curves showing breast cancer specific survival (BCSS) and ATR expression (B1). Kaplan Meier curves showing breast cancer specific survival (BCSS) and cytoplasmic pChk1 expression (B2). Kaplan Meier curves showing breast cancer specific survival (BCSS) and nuclear pChk1 expression (B3). Kaplan Meier curves showing breast cancer specific survival (BCSS) and combined nuclear/cytoplasmic pChk1 expression (B4). Kaplan Meier curves showing breast cancer specific survival (BCSS) and combined ATR/ pChk1 expression (B5).

Figure 2. A. Kaplan Meier curves showing breast cancer specific survival (BCSS) and combined p53/ATR expression (A1). Kaplan Meier curves showing breast cancer specific survival (BCSS) and combined p53/cytoplasmic pChk1 expression expression (A2). Kaplan Meier curves showing breast cancer specific survival (BCSS) and combined p53/nuclear pChk1 expression (A2). B. Kaplan Meier curves showing breast cancer specific survival (BCSS) and ATR mRNA expression. C. Kaplan Meier curves showing breast cancer specific survival (BCSS) and Chk1 mRNA expression.

Figure 3. A. ATR is responsible for Chk1 phosphorylation at serine 345 following replication stress. A1. MCF7 cells were subjected to 10 nM siRNA for 48 hours before being treated with 1 µM gemcitabine (Gem) or 10 mM hydroxyurea (HU) for 1 hour. Cells were harvested, lysed and the proteins separated using gel electrophoresis. ATR, pChk1$^{\text{Ser345}}$ and β-actin were detected using western blotting. Bands were quantified using densitometry (A2 and A3). See text for details. B. VE-821 inhibits gemcitabine-induced ATR activity as measured by pChk1$^{\text{Ser345}}$. MCF7, MDA-MB-231 or MCF10A cells were treated with 1 µM gemcitabine ± VE-821 for one hour before being harvested and lysed. Proteins were separated and detected using western blotting. Blot shown is in MDA-MB-231 cells and is representative of all experiments (B1). Concentration-response curve (B2) data shown is the
mean ± standard deviation of three individual experiments in each cell line. IC50 values from the 3 independent experiments are shown in B3.

**Figure 4.** ATR is required for cell growth in MCF7 cells. Cells with doxycyclin (Dox) – inducible shATR were incubated with or without Dox for 3 days. Then Dox was removed and cells were cultured for further indicated days. Cell growth was analysed using DAPI fluorescence (A1). ATR expression was monitored by western blotting (A2). Knockdown of ATR following Dox induction suppressed growth, which was restored when ATR was re-expressed. **B.** ATR inhibitor VE-821 reduces breast cancer cell growth. MCF7 or MDA-MB-231 were treated for 24 hours with a dose range of VE-821. Cells were then allowed to grow for 5 days in fresh media. Cell growth was measured by DAPI fluorescence. **C.** There was a direct correlation between growth inhibition and ATR inhibition in MCF7 and MDA-MB-231 cells. **D.** VE-821 is selective against breast cancer cells compared to non-cancer cells. MCF7, MDA-MB-231 and MCF10A cells were seeded into 6-well tissue culture plates and allowed to adhere for 24 hours. Cells were treated with VE-821 for 24 hours before being counted and re-seeded for colony formation. Cells were then allowed to grow for 14 days. Colonies were then fixed, stained and counted.