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Clinicopathological and Functional Significance of RECQL1 Helicase in Sporadic Breast Cancers

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Abstract

RECQL1, a key member of the RecQ family of DNA helicases, is required for DNA replication and DNA repair. Two recent studies have shown that germline RECQL1 mutations are associated with increased breast cancer susceptibility. Whether altered RECQL1 expression has clinicopathologic significance in sporadic breast cancers is unknown. We evaluated RECQL1 at the transcriptomic level (METABRIC cohort, $n = 1{,}977$) and at the protein level [cohort 1, $n = 897$; cohort 2, $n = 252$; cohort 3 (BRCA germline deficient), $n = 74$]. In RECQL1-depleted breast cancer cells, we investigated anthracycline sensitivity. High RECQL1 mRNA was associated with intClust.3 ($P = 0.026$), which is characterized by low genomic instability. On the other hand, low RECQL1 mRNA was linked to intClust.8 [luminal A estrogen receptor–positive (ER^+) subgroup; $P = 0.0455$] and intClust.9 (luminal B ER^+ subgroup; $P = 0.0346$) molecular phenotypes. Low *RECQL1* expression was associated with shorter breast cancer–specific survival ($P = 0.001$). At the protein level, low nuclear RECQL1 level was associated with larger tumor size, lymph node positivity, high tumor grade, high mitotic index, pleomorphism, dedifferentiation, ER negativity, and HER-2 overexpression ($P < 0.05$). In ER^+ tumors that received endocrine therapy, low RECQL1 was associated with poor survival ($P = 0.008$). However, in ER^- tumors that received anthracycline-based chemotherapy, high RECQL1 was associated with poor survival ($P = 0.048$). In RECQL1-depleted breast cancer cell lines, we confirmed doxorubicin sensitivity, which was associated with DNA double-strand breaks accumulation, S-phase cell-cycle arrest, and apoptosis. We conclude that RECQL1 has prognostic and predictive significance in breast cancers. *Mol Cancer Ther; 16(1); 239–50. ©2016 AACR.*

Introduction

DNA helicases unwind DNA, a process essential during replication and DNA repair. Human RecQ family of DNA helicases includes RECQL1, RECQL4, RECQL5, BLM, and WRN (1, 2). RECQL1 (also known as RECQL or RECQ1) is localized to chromosome 12p12 and encodes a 649 amino acid protein (3–6). RECQL1 is the smallest and the most abundant of human RecQ helicases. RECQL1 is an integral component of the replication complex and is required for the maintenance of replication fork progression (7–9). RECQL1 is also essential for the maintenance of genomic stability through roles in DNA repair. RECQL1, besides a DNA 3′-5′ helicase activity, can promote branch migration of Holliday junctions and also has strand annealing activity (10). Moreover, to accomplish its various biological functions, RECQL1 is known to interact with various proteins involved in DNA repair, including PARP1, RPA, RAD51, Top3α, EXO1, MSH2/6, MLH1-PMS2, and Ku70/80 (3–6). The essential role played by RECQL1 in DNA repair is underpinned by the fact that RECQL1 depletion in cells results in increased frequency of spontaneous sister chromatid exchanges, chromosomal instability, DNA damage accumulation, and increased sensitivity to cytotoxic chemotherapy (11).

Emerging data suggest a role for RECQL1 in breast cancer pathogenesis. Importantly, two recent studies have shown that germline RECQL1 mutations are associated with increased breast cancer susceptibility (12–14). Sun and colleagues have identified pathogenic mutations in *RECQL1* gene in 9 of 448 Chinese patients with BRCA-negative familial breast cancers (12). Similarly, Cybulski and colleagues identified deleterious mutations in 7 of 1,013 and 30 of 13,136 Polish breast cancer patients (13). Although germline mutations in RECQL1 are rare, the data provide evidence that RECQL1 is a tumor suppressor. However whether RECQL1 also influences sporadic breast cancer pathogenesis and prognosis is currently unknown.

In the current study, we have comprehensively investigated RECQL1 in large cohorts of sporadic breast cancer and have provided the first clinical evidence that altered RECQL1 expression is associated with aggressive breast cancers and poor

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prognosis. Preclinically, RECQL1 depletion in breast cancer cells increased anthracycline chemosensitivity. We conclude that RECQL1 expression has prognostic and predictive significance in sporadic breast cancers.

Materials and Methods

RECQL1 mRNA expression in breast cancer. *RECQL1* mRNA expression was investigated in METABRIC (Molecular Taxonomy of Breast Cancer International Consortium) cohort. The METABRIC study protocol, detailing the molecular profiling methodology in a cohort of 1,977 breast cancer samples, is described by Curtis and colleagues (15). Patient demographics are summarized in Supplementary Table S1. Estrogen receptor–positive (ER^+) and/or lymph node–negative patients did not receive adjuvant chemotherapy. ER^- and/or lymph node–positive patients received adjuvant chemotherapy. For this cohort, the mRNA expression was hybridized to Illumina HT-12 v3 platform (Bead Arrays), and the data were preprocessed and normalized as described previously. RECQL1 expression was evaluated in this dataset (RECQL1 probe ID: ILMN_1692705). The probe was a perfect match and quality for its target, having a GC content of 58%, 0 SNPs, and it does not possess a polyG tail at the end. Samples were classified into the intrinsic subtypes based on the PAM50 gene list. A description of the normalization, segmentation, and statistical analyses was described previously (15). Real-time qRT-PCR was performed on the ABI Prism 7900HT sequence detection system (Applied Biosystems) using SYBR1 Green reporter. All the samples were analyzed as triplicates. The χ^2 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. Xtile (Version 3.6.1) was used to identify a cutoff in gene expression values such that the resulting subgroups had significantly different survival courses (16).

RECQL1 protein expression in breast cancer. The study was performed in a consecutive series of 1,650 patients with primary invasive breast carcinomas who were diagnosed between 1986 and 1999 and entered into the Nottingham Tenovus Primary Breast Carcinoma series. Patient demographics are summarized in Supplementary Table S2. This is a well-characterized series of patients with long-term follow-up that have been investigated in a wide range of biomarker studies (17–23). All patients were treated in a uniform way in a single institution with standard surgery (mastectomy or wide local excision), followed by radiotherapy. Prior to 1989, patients did not receive systemic adjuvant treatment (AT). After 1989, AT was scheduled on the basis of prognostic and predictive factor status, including Nottingham Prognostic Index (NPI), ERα status, and menopausal status. Patients with NPI scores of <3.4 (low risk) did not receive AT. In premenopausal patients with NPI scores of ≥3.4 (high risk), classical cyclophosphamide, methotrexate, and 5-flurouracil (CMF) chemotherapy was given; patients with $ER\alpha^+$ tumors were also offered endocrine therapy. Postmenopausal patients with NPI scores of ≥3.4 and ERα positivity were offered endocrine therapy, whereas $ER\alpha^-$ patients received classical CMF chemotherapy. Median follow-up was 111 months (range, 1–233 months). Survival data, including breast cancer–specific survival (BCSS), disease-free survival (DFS), and development of locoregional and distant metastases (DM), were maintained on a prospective basis. DFS was defined as the number of months from diagnosis to the occurrence of local recurrence, local lymph node relapse or DM relapse. BCSS was defined as the number of months from diagnosis to the occurrence of breast cancer–related death. Local recurrence-free survival was defined as 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.

We also evaluated an independent series of 252 $ER\alpha^-$ invasive breast cancers diagnosed and managed at the Nottingham University Hospitals (Nottingham, United Kingdom) between 1999 and 2007. All patients were primarily treated with surgery, followed by radiotherapy and anthracycline chemotherapy. The characteristics of this cohort are summarized in Supplementary Table S3. In addition, we also explored RECQL1 expression in a cohort of BRCA germline-deficient tumors. Patient demographics in this cohort are summarized in Supplementary Table S4.

Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) criteria, recommended by McShane and colleagues (24), were followed throughout this study. Ethical approval was obtained from the Nottingham Research Ethics Committee (C202313).

Tissue microarrays and IHC. Tumors were arrayed in tissue microarrays (TMA) constructed with 0.6-mm cores sampled from the periphery of the tumors. The TMAs were immunohistochemically profiled for RECQL1 and other biological antibodies (Supplementary Table S5) as described previously (18, 19, 21, 23). 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: 1,250 tests), and the Leica Bond Primary Antibody Diluent (AR9352), each used according to the manufacturer's instructions (Leica Microsystems). Leica Autostainer XL machine was used to dewax and rehydrate the slides. Pretreatment antigen retrieval was performed on the TMA sections using sodium citrate buffer (pH 6.0) and heated for 20 minutes at 95°C in a microwave (Whirlpool JT359 Jet Chef 1000W). A set of slides was incubated for 60 minutes with the primary anti-RECQL1 antibody (Bethyl Laboratories, catalog no. A300-450A) at a dilution of 1:1,000, respectively. Negative and positive (by omission of the primary antibody and IgG-matched serum) controls were included in each run. The negative control ensured that all the staining was produced from the specific interaction between antibody and antigen.

Evaluation of immune staining. 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, and 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. RECQL1 expression was categorized on the basis of the frequency histogram distributions. The tumor cores were evaluated by two scorers (A. Arora and M.A. Aleskandarany), and the concordance between the two scorers was excellent ($k = 0.79$). Xtile (Version 3.6.1) was used to identify a cutoff in protein expression values such that the resulting subgroups had significantly different survival courses. An H-score of ≥215 was taken as the cutoff for high RECQL1 level. Not all cores within the TMA were suitable for

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IHC assessments, as some cores were missing or containing inadequate invasive cancer (<15% tumor).

Statistical analysis. Data analysis was performed using SPSS (SPSS, version 17). Where appropriate, Pearson χ^2, Fisher exact, Student *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 hazards model. The proportional hazards assumption was tested using standard log–log plots. HRs 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 Benjamini–Hochberg method (25).

Breast cancer cell lines and culture. MCF-7 ($ER^+/PR^+/HER2^-$, BRCA1 proficient), MDA-MB-231 ($ER^-/PR^-/HER2^-$, BRCA1 proficient), MDA-MB-468 ($ER^-/PR^-/HER2^-$, BRCA1 proficient), and MDA-MB-436 ($ER^-/PR^-/HER2^-$, BRCA1 deficient) were purchased from ATCC and were grown in RPMI (MCF-7) or DMEM (MDA-MB-231, MDA-MB-468, and MDA-MB-436) medium with the addition of 10% FBS and 1% penicillin/streptomycin. Cells in culture were routinely checked for mycoplasma contamination by PCR (Sigma, catalog no. MP0035).The characterization of the cells was performed by ATCC and passaged in the laboratory for fewer than 6 months.

RECQL1 depletion in breast cancer cells. On-Target plus SMARTpool siRNAs against RECQL1 (NM_032941) and nontargeting control (CTL) were purchased from Dharmacon (catalog nos. L-013597-00-0005 and D-001810-10-05, respectively). We have previously established the specificity of the siRNA pool (5). All siRNA transfections (in MCF-7, MDA-MB-231, and MDA-MB-468 breast cancer cells) were performed by reverse transfection at a final concentration of 20 nmol/L using Lipofectamine RNAiMAX (Invitrogen, catalog no. 13-778-075) as instructed by the manufacturer. Stable shRNA-mediated knockdown of RECQL1 in MDA-MB-231 cells was achieved using a lentiviral system (26). Briefly, lentivirus particles were produced by cotransfecting 293T cells with the pLKO.1 lentiviral shRNA expression vector containing the RECQL1 targeting sequence (5′-GAGCTTATGTTACCAGTTA-3′) or the gene encoding luciferase (5′-ACGCTGAGTACTTCGAAATGT-3′) with the packaging plasmids psPAX2 and pM2D.G and used to transduce MDA-MB-231 cells, followed by selection with puromycin (8 μg/mL). All cells were cultured in a humidified atmosphere containing 5% CO_2 at 37°C and routinely checked for mycoplasma contamination (Sigma, catalog no. MP0035). The level of RECQL1 depletion was verified by Western blotting.

Western blot analysis. Whole-cell lysates were prepared in RIPA buffer containing protease inhibitor cocktail (Sigma, catalog no. 11873580001), and protein was quantified using Bio-Rad DC Protein Assay Kit (Bio-Rad, catalog no. 5000111). Fifty micrograms of total protein per lane was used for immunoblotting. The following primary antibodies were used: RECQL1 (1:1,000; Bethyl Laboratories, catalog no. A300-450A), γH2AX (1:1,000; Cell Signaling Technology, catalog no. 2577), GAPDH (1:1,000; Cell Signaling Technology, catalog no. 5174), ERα (1:100, EP1 clone, Dako, catalog no. IS08430-2.), and β-actin (1:10,000; Abcam, catalog no. ab8226). Following incubation with infrared dye-labeled (Li-Cor; IRDye 800CW mouse anti-rabbit IgG and IRDye 680CW rabbit anti-mouse IgG; 1:10,000) or HRP-conjugated secondary antibodies (Vector Laboratories) for 1 hour, membranes were scanned with a Li-Cor Odyssey machine (700 and 800 nm) or GeneGnome XRQ Chemidoc System (Syngene) to determine protein expression, and signal intensities were quantified using ImageJ.

qRT-PCR. Total RNA was extracted from MCF-7, MDA-MB-231, MDA-MB-468, and MDA-MB-436 cells using RNeasy Mini Kit (QIAGEN). The quantification of the extracted RNA was done using a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific). The cDNA was synthesized from 0.5 μg of total RNA using RT^2 First Strand Kit (QIAGEN). The real-time qPCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) with primer set (RECQL1 QuantiTect Primer Assay, catalog no. QT00034503, QIAGEN) targeting RECQL1 gene. The GAPDH housekeeper gene was used as an internal control (GAPDH QuantiTect Prier Assay, catalog no. QT00079247, QIAGEN). The real-time PCR for each RNA sample was performed in triplicate. NTC (no template control) was used to rule out cross-contamination of reagents and surfaces. NTC included all the RT-PCR reagents except the RNA template. Minus reverse-transcriptase (− RT) control was used to rule out genomic DNA contamination.

Cytotoxicity and cell-cycle analysis. Cells, stably transduced or 48 hours after siRNA transfection, plated in quadruplicates into a 96-well plate (5×10^3 cells/well) were treated with increasing concentrations of doxorubicin, and cell viability was measured after 5 days by the WST-8–based colorimetric assay using Cell Counting Kit-8 (Dojindo Laboratories) according to the manufacturer's instructions. For cell-cycle analysis, cells were fixed in cold ethanol before being stained with propidium iodide (Sigma, 0.45 mg/mL). Resuspended cells were analyzed for DNA content by flow cytometry performed on a BD Accuri C6 flow cytometer equipped with BD Accuri C6 software (BD Biosciences). Means from two independent experiments were plotted with their respective SEMs. Statistically significant differences between cell populations was confirmed using a two-tailed *t* test, assuming equal variances, and are presented in the figures as *, $P \leq 0.05$; **, $P \leq 0.005$.

Immunofluorescence staining analysis. For γH2AX staining, control and RECQL1 knockdown cells were grown on coverslips in the medium containing 0.1 μmol/L doxorubicin for 4 hours and allowed to recover in drug-free medium for indicated time periods. Cells were fixed in 3.75% paraformaldehyde for 10 minutes at room temperature, permeabilized in 0.5% Triton X-100 in PBS for 10 minutes, and blocked with 1% BSA in PBS for 1 hour at room temperature, followed by incubation with mouse monoclonal anti-γH2AX (1:200; Upstate Biotechnology, JBW301) antibody for 1 hour at 37°C. After three washes in PBS for 5 minutes each, the cells were incubated with Alexa Fluor 488 goat anti-mouse IgG (1:400; Invitrogen) secondary antibody for 1 hour at 37°C, washed three times with PBS, and mounted in ProLong Gold containing DAPI (Invitrogen). Immunostained cells were imaged with a Nikon fluorescence microscope (Eclipse Ti) equipped with imaging capabilities and Elements imaging

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software. Scoring for each individual condition (siRNA or shRNA, cell line, drug treatment, etc.) within an experiment was carried out on at least 10 separate fields of view and about 50 to 100 cells in total. Means from two independent experiments were plotted with their respective SEMs. Statistically significant differences between cell populations were confirmed using a two-tailed *t* test, assuming equal variances, and are presented in the figures as *, $P \leq 0.05$; **, $P \leq 0.005$.

Results

RECQL1 mRNA expression in human breast cancer

We then evaluated *RECQL1* mRNA expression in the METABRIC cohort. A total of 31.7% (626/1,971) of breast tumors had low *RECQL1* mRNA expression and 68.3% (1,345/1,971) of breast tumors had high *RECQL1* mRNA expression. Clinicopathologic associations are summarized in Supplementary Table S6. The METABRIC study by joint clustering of copy number and gene expression data identified 10 novel biological subgroups [labeled integrative clusters (intClust) 1–10; ref. 15]. We investigated whether *RECQL1* mRNA expression would associate with these distinct biological subgroups (Supplementary Table S7). High *RECQL1* mRNA was associated with intClust.3 ($P = 0.026$), which is characterized by low genomic instability (15). On the other hand, low *RECQL1* mRNA was linked to intClust.8 ($P = 0.0455$) and intClust.9 ($P = 0.0346$) phenotypes. Of note, intClust.8 belongs to luminal A ER^+ subgroup, whereas intClust.9 belongs to luminal B ER^+ subgroup (15).

We then proceeded to survival analysis in the METABRIC cohort. Low *RECQL1* mRNA expression was associated with poor BCSS ($P = 0.001$) in the whole cohort (Fig. 1B). In ER^+ tumors, low *RECQL1* mRNA expression remained associated with poor BCSS ($P = 0.001$; Fig. 1C), including in patients who received adjuvant endocrine therapy ($P = 0.003$; Fig. 1D). However, in ER^- tumors, *RECQL1* mRNA expression, although borderline, did not significantly influence outcome in the ER^- cohort, including in patients who received adjuvant chemotherapy ($P = 0.071$ and $P = 0.071$, respectively; Fig. 2E and F).

Together, the data provide evidence that *RECQL1* mRNA level has clinicopathologic and prognostic significance in various subtypes of breast cancers. We then proceeded to evaluate RECQL1 protein level in breast cancers.

RECQL1 protein level in human breast cancer

A total of 897 early breast cancers were suitable for RECQL1 expression analysis. We observed only nuclear expression where 677 of 897 (75.5%) tumors had low RECQL1 level and 220 of 897 (25.5%) had high RECQL1 level (Fig. 2B). We also evaluated 15 normal breast tissues for RECQL1 expression where high nuclear staining in the terminal duct lobular units in the sections was observed (mean H-score = 226), suggesting differential expression of RECQL1 in breast cancer tissues compared with normal breast tissue. No cytoplasmic staining was observed in any normal breast or tumor tissue.

As shown in Table 1, in the whole cohort, low nuclear RECQL1 levels were significantly associated with larger tumor size, lymph node positivity, higher tumor stage, high tumor grade, high mitotic index, pleomorphism, dedifferentiation, and tumor type (*P*s < 0.05). ER^-, progesterone-negative (PR^-), and HER-2 overexpression was more common in tumors with low nuclear RECQL1 protein level ($P < 0.05$). High-risk NPI >3.4 was also more common in tumors with low RECQL1 level ($P = 0.0006$). Low PARP1, BRCA1 negative, low RAD51, low ATM, low nuclear pChk1, low nuclear Chk2, low XRCC1, low FEN1, low SMUG1, and low DNA-PKcs were significantly more likely in tumors with low nuclear RECQL1 protein level ($P < 0.05$). Moreover, low RECQL1 tumors were also significantly associated with low levels of other RecQ helicases, including RECQL4, BLM, and WRN (*P*s < 0.05). We then proceeded to analysis separately in ER^+ and ER^- cohort.

In ER^+ tumor (Supplementary Table S7), low nuclear RECQL1 level was significantly associated with higher mitotic index ($P = 0.033$). PR^- and high-risk NPI >3.4 was also more common in tumors with low RECQL1 level ($P < 0.05$). Low XRCC1 and low TOPO2A were also more likely in tumors with low nuclear RECQL1 protein level ($P < 0.05$). However, in ER^- tumors (Supplementary Table S8), no significant clinicopathologic associations were observed.

We then proceeded to survival analyses. In the whole cohort, patients whose tumors had low RECQL1 level were significantly more likely to have shorter BCSS compared with those with high RECQL1 level ($P = 0.001$) (Fig. 2C). In ER^+ tumors, similarly, low RECQL1 was associated with poor BCSS ($P = 0.008$; Fig. 2D), including in patients who received adjuvant endocrine therapy ($P = 0.021$; Fig. 2E). However, in patients who received no endocrine therapy, RECQL1 level did not influence survival ($P = 0.485$; Supplementary Fig. S1A). In ER^- tumors, RECQL1 did not influence survival, including in patients who received CMF chemotherapy (Supplementary Fig. S1B–S1D). However, in this historical cohort, patients received CMF chemotherapy, which is currently not the standard adjuvant treatment in breast cancer. We therefore investigated RECQL1 level and survival in a further cohort of 252 ER^- tumors that received more modern anthracycline-based adjuvant chemotherapies. The characteristics of this cohort are summarized in Supplementary Table S3. As the long-term follow-up data have not yet matured, we investigated the impact of RECQL1 expression on DFS at 5 years in patients who received adjuvant doxorubicin chemotherapy. At 5 years, 176 of 252 were alive, 73 of 252 were dead from breast cancer recurrence, and 3 of 252 died from other causes. Patients with high RECQL1 expression were more likely to suffer disease recurrence compared with patients with low RECQL1 expression ($P = 0.048$; Fig. 2F).

We also investigated RECQL1 expression in 49 BRCA1 germline-deficient and 25 BRCA2 germline-deficient breast tumors. No significant clinicopathologic associations were observed (data not shown). RECQL1 expression also did not influence survival outcomes of BRCA1/2 germline-deficient tumors (data not shown).

Taken together, the data suggest that RECQL1 overexpression may predict resistance to doxorubicin chemotherapy in sporadic ER^- breast cancers. To investigate this possibility further, we proceeded to preclinical studies in breast cancer cell lines.

RECQL1 depletion and doxorubicin chemosensitivity in breast cancer cell lines

RECQL1 deficiency leads to genomic instability and sensitivity to a range of genotoxins (3–6). However, the impact of RECQL1 depletion in breast cancer cells and anthracycline sensitivity has not been investigated.

We initially profiled MCF-7, MDA-MB-468, and MDA-MB 231 breast cancer cell lines. At the mRNA level, MCF-7 and

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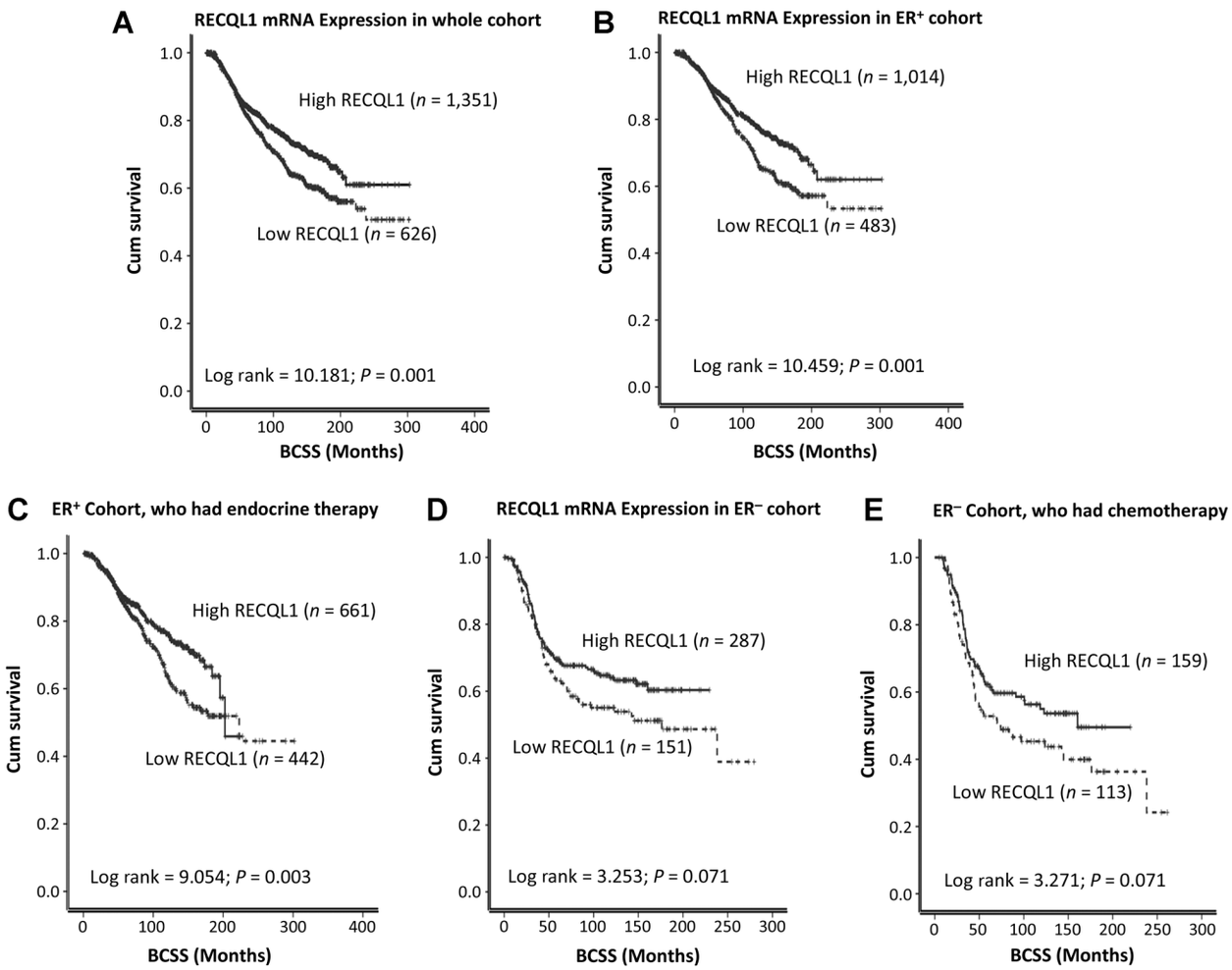

Figure 1.

RECQL1 mRNA expression and breast cancer survival. **A,** *RECQL1* mRNA expression and survival in the whole cohort. **B,** *RECQL1* mRNA expression and survival in ER^+ tumors. **C,** *RECQL1* mRNA expression and survival in patients with ER^+ tumors who received endocrine therapy. **D,** *RECQL1* mRNA expression and survival in ER^- tumors. **E,** *RECQL1* mRNA expression and survival in patients with ER^- tumors who received chemotherapy. Cum, cumulative.

MDA-MB-231 cells have high *RECQL1* mRNA expression compared with MDA-MB-468 cells (Fig. 3A). At the protein level, all three cell lines have robust RECQL1 protein expression (Fig. 3B). We then utilized siRNA to transiently deplete RECQL1 in MDA-MB-468, MDA-MB-231, and MCF-7 cells. We transfected cells with a control siRNA (siControl) or a pool of 4 siRNAs (SMARTpool, 20 nmol/L) targeting RECQL1 (siRECQL1; Fig. 3C1 and 3D1 and Supplementary Fig. S2A1). As compared with control cells, RECQL1-depleted cells displayed significantly reduced survival to doxorubicin treatment in MDA-MB-468, MDA-MB 231 (Fig. 3C2 and 3D2), and MCF-7 cells (Supplementary Fig. S2A2; $P < 0.05$ at all drug concentrations tested). To determine whether increased sensitivity to doxorubicin was also sustained in cells depleted of RECQL1 over a longer period of time, we transduced MDA-MB-231 cells with a RECQL1-specific shRNA (Fig. 3E1). As compared with control shRNA (shCTL)–transduced MDA-MB-231 cells, the RECQL1 shRNA (shRECQL1)–transduced cells displayed significantly reduced survival to doxorubicin treatment (Fig. 3E2).

To determine whether the cellular level of RECQL1 protein modulates overall DNA damage in breast cancer cell lines, we examined γH2AX as a surrogate of DNA double-strand breaks in control and RECQL1 knockdown cells exposed to doxorubicin. MDA-MB-231 cells stably transduced with control or RECQL1 shRNA were treated with 0.1 μmol/L doxorubicin for 4 hours, and the percentage of cells exhibiting ≥5 γH2AX foci at various time points following recovery from drug treatment was determined by immunofluorescence (Fig. 3F). Consistent with constitutively elevated DNA damage upon RECQL1 knockdown reported in other cell types (8, 27), RECQL1-depleted MDA-MB-231 cells displayed spontaneous γH2AX foci under untreated condition. Doxorubicin treatment induced comparable level of DNA double-strand breaks in both control and RECQL1 knockdown MDA-MB-231 cells. However, following 8-hour recovery from the doxorubicin treatment, significantly greater fraction of RECQL1-depleted cells was scored positive for γH2AX foci. After 24 hours in drug-free medium, γH2AX foci were persistent in about 25% RECQL1-depleted cells as compared with 5% control

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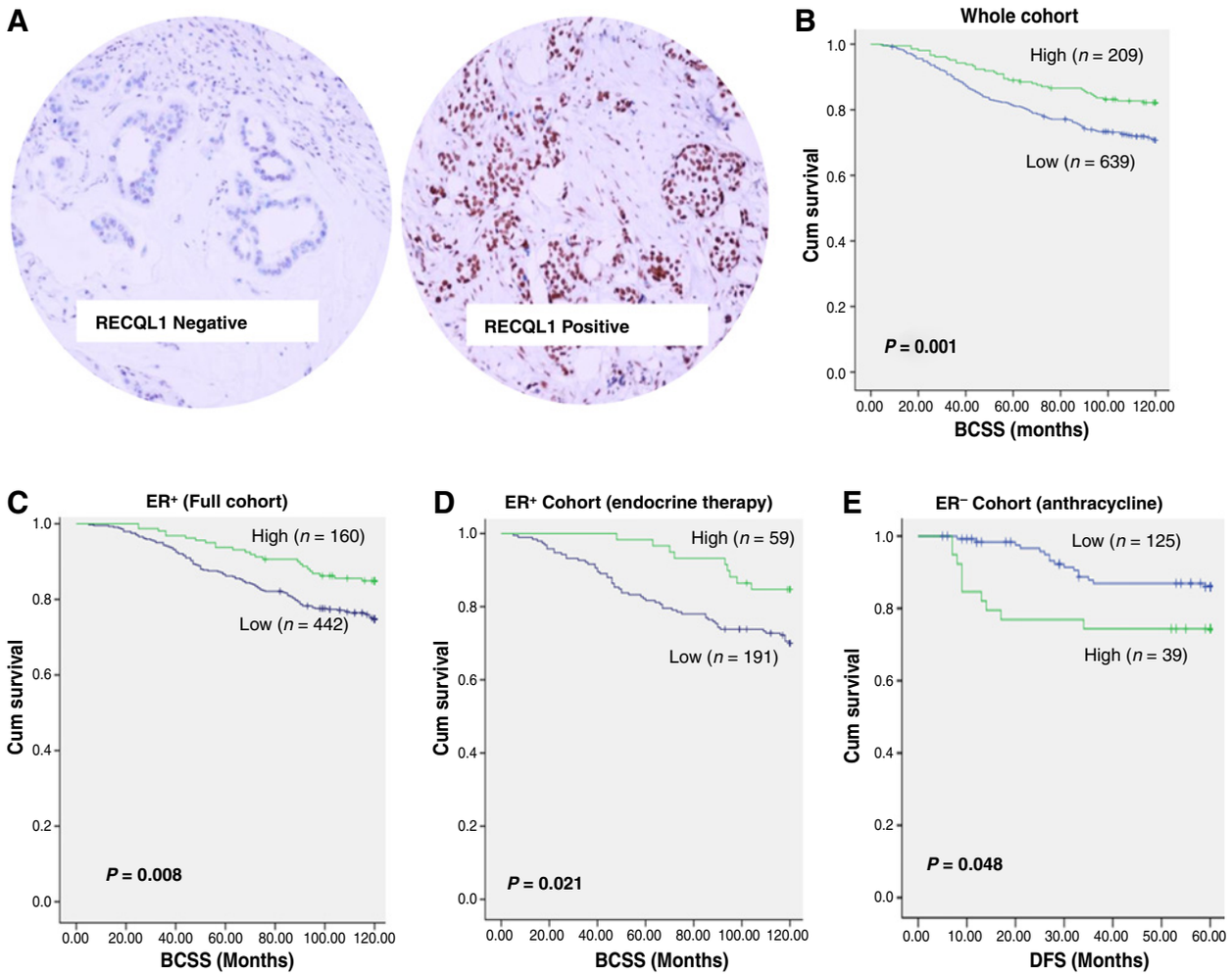

Figure 2.
RECQL1 protein level and breast cancer survival. **A,** Photomicrographs of RECQL1 protein expression in breast cancers. **B,** RECQL1 protein level and survival in the whole cohort. **C,** RECQL1 protein level and survival in ER$^+$ tumors. **D,** RECQL1 protein level and survival in patients with ER$^+$ tumors who received endocrine therapy. **E,** RECQL1 protein level and survival in patients with ER$^-$ tumors who received anthracycline chemotherapy. Cum, cumulative.

MDA-MB-231 cells (Fig. 3G). We note that the initial numbers of γH2AX-positive cells induced spontaneously in control versus RECQL1-depleted cells are different; however, the difference between control and RECQL1 knockdown cells for the percentage of γH2AX-positive cells during recovery (8 and 24 hours) from doxorubicin treatment is statistically significant ($P \leq 0.05$). These results suggest that RECQL1 promotes repair of doxorubicin-induced DNA damage. In MCF-7 cells, similarly, RECQL1-depleted cells retain statistically significant proportion of γH2AX-positive cells at 8 and 24 hours following recovery in drug-free medium (Supplementary Fig. S2B).

We next analyzed cell-cycle progression in these cells using FACS analysis (Fig. 4). Stable knockdown of RECQL1 in MDA-MB-231 cells resulted in predominant accumulation in G_0–G_1 phase of the cell cycle. Cell-cycle distribution of control and RECQL1 knockdown MDA-MB-231 cells was largely unaltered after doxorubicin treatment (0.1 μmol/L, 4 hours). MDA-MB-231 cells, with or without knockdown of RECQL1 expression, accumulated in the S-phase following 8 hours after recovery from treatment, and in G_2–M following 24-hour recovery in drug-free medium; however, a significantly greater proportion of RECQL1-depleted cells remained in the S-phase at 8 ($P \leq 0.05$) and 24 hours ($P \leq 0.05$) of recovery (Fig. 4A and B). Doxorubicin-induced S-phase arrest is consistent with the formation of DNA adducts that prevent replication fork progression and formation of double-strand breaks downstream of topoisomerase II inhibition. Relative accumulation in the S-phase, together with the increased double-strand breaks and sensitivity to doxorubicin observed in RECQL1 knockdown MDA-MB-231 cells, is consistent with a role of RECQL1 in resolving stalled or broken replication forks and DNA repair. Doxorubicin-induced DNA strand breaks can ultimately result in apoptosis. To determine the extent of doxorubicin-induced cell death in RECQL1 knockdown cells, we analyzed the percentage of MDA-MB-231 cells stably transduced with control or RECQL1 shRNA having sub-G_1 DNA content (Fig. 4C and D). Following treatment with doxorubicin (0.1 μmol/L) for 24 hours, 9 ± 1.73% control and 15 ± 2.61% RECQL1 knockdown cells were in the sub-G_1

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Table 1. RECQL1 protein levels and breast cancer

	RECQL1 protein level		*P*	
Variable	**Low *n* (%)**	**High *n* (%)**	**Unadjusted**	**Adjusted**
(A) Pathological parameters				
Tumor size				
<1 cm	53 (7.8)	31 (14.1)	**0.031**	**0.0472**
>1–2 cm	338 (49.9)	108 (49.1)		
>2–5 cm	268 (39.6)	78 (35.5)		
>5 cm	18 (2.7)	3 (1.4)		
Lymph node status				
Negative	351 (58.5)	143 (68.8)	**0.029**	**0.0483**
Positive (1–3)	199 (33.2)	54 (26.0)		
Positive (>3)	50 (8.3)	11 (5.3)		
Tumor stage				
1	410 (60.5)	157 (70.7)	**0.017**	**0.0313**
2	210 (29.6)	52 (23.4)		
3	67 (9.9)	13 (5.9)		
Tumor grade				
G1	88 (13.0)	46 (20.9)	**0.0001**	**0.0018**
G2	230 (33.9)	89 (40.5)		
G3	360 (53.1)	85 (38.6)		
Mitotic index				
M1 (low; mitoses <10)	212 (32.2)	105 (48.2)	**0.0001**	**0.0012**
M2 (medium; mitoses 10–18)	131 (19.9)	34 (15.6)		
M3 (high; mitoses >18)	315 (47.9)	79 (36.2)		
Tubule formation				
1 (>75% of definite tubule)	25 (3.8)	19 (8.7)	**0.006**	**0.0162**
2 (10%–75% definite tubule)	213 (32.4)	78 (35.8)		
3 (<10% definite tubule)	420 (63.8)	121 (55.5)		
Pleomorphism				
1 (small-regular uniform)	13 (2.0)	7 (3.2)	**0.001**	**0.0044**
2 (moderate variation)	218 (33.2)	101 (46.5)		
3 (marked variation)	425 (64.8)	109 (50.2)		
Tumor type				
IDC-NST	421 (63.3)	107 (49.5)	**0.017**	**0.0298**
Tubular carcinoma	122 (18.3)	59 (27.3)		
Medullary carcinoma	17 (2.6)	6 (2.8)		
ILC	63 (9.5)	27 (12.5)		
Others	8 (1.2)	4 (1.9)		
Mixed NST/lobular/special type	34 (5.1)	13 (6.0)		
ER				
Negative	187 (28.1)	42 (19.7)	**0.015**	**0.0309**
Positive	478 (71.9)	171 (80.3)		
PR				
Negative	310 (48.0)	67 (31.5)	**0.0001**	**0.0007**
Positive	336 (52.0)	146 (68.5)		
Her2 overexpression				
No	559 (84.1)	197 (90.0)	**0.032**	**0.0431**
Yes	106 (15.9)	22 (10.0)		
Triple-negative phenotype				
No	570 (83.8)	183 (81.7)	0.459	0.4725
Yes	110 (16.2)	41 (18.3)		
NPI				
≤3.4	117 (27.7)	88 (41.5)	**0.0001**	**0.0006**
>3.4	462 (72.3)	124 (58.5)		
(B) DNA repair				
XRCC1 (nuclear)				
Low	98 (18.6)	9 (5.6)	**0.0001**	**0.0005**
High	430 (81.4)	151 (94.4)		
BRCA1 (nuclear)				
Low	104 (18.9)	18 (10.7)	**0.014**	**0.0306**
High	447 (81.1)	150 (89.3)		
SMUG1 (nuclear)				
Low	288 (58.2)	64 (45.1)	**0.002**	**0.007**
High	207 (41.8)	78 (54.9)		
FEN1 (nuclear)				
Low	389 (75.4)	103 (65.2)	**0.012**	**0.0280**
High	127 (24.6)	55 (34.8)		

(Continued on the following page)

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Table 1. RECQL1 protein levels and breast cancer (Cont'd)

	RECQL1 protein level		*P*	
Variable	**Low *n* (%)**	**High *n* (%)**	**Unadjusted**	**Adjusted**
FEN1 (cytoplasmic)				
Low	285 (55.2)	70 (44.3)	**0.016**	**0.0311**
High	231 (44.8)	88 (55.7)		
PARP1				
Low	270 (49.0)	67 (39.6)	**0.033**	**0.0481**
High	281 (51.0)	102 (60.4)		
TOPO2				
Low	237 (47.0)	64 (38.6)	0.057	0.0739
High	267 (53.0)	102 (61.4)		
KU 70/80				
Low	60 (13.0)	22 (17.7)	0.181	0.2112
High	400 (87.0)	102 (82.3)		
DNA-PKcs				
Low	87 (18.0)	7 (5.6)	**0.001**	**0.0044**
High	397 (82.0)	117 (94.4)		
ATR				
Low	110 (35.4)	38 (37.6)	0.682	23.87
High	201 (64.6)	63 (62.4)		
Chk1 (nuclear)				
Low	605 (89.1)	164 (73.2)	**0.0001**	**0.0005**
High	74 (10.9)	60 (26.8)		
Chk1 (cytoplasmic)				
Low	211 (31.1)	75 (33.5)	0.451	0.478
High	468 (68.9)	149 (66.5)		
ATM				
Low	238 (56.9)	54 (41.5)	**0.002**	**0.007**
High	180 (43.1)	76 (58.5)		
CHK2				
Low	112 (25.2)	19 (15.7)	**0.029**	0.327
High	333 (74.8)	102 (84.3)		
RAD51 (nuclear)				
Low	234 (55.1)	43 (39.1)	**0.003**	**0.0095**
High	191 (44.9)	67 (60.9)		
(C) Other RecQ helicases				
RECQL5 (nuclear)				
Low	273 (46.5)	68 (40.0)	0.133	0.1605
High	314 (53.5)	102 (60.0)		
RECQL4 (nuclear)				
Low	245 (65.9)	173 (50.6)	**0.00004**	**0.0014**
High	127 (34.1)	169 (49.4)		
RECQL4 (cytoplasmic)				
Low	297 (53.6)	73 (46.2)	0.100	0.125
High	257 (46.4)	85 (53.8)		
BLM (nuclear)				
Low	163 (27.7)	28 (16.9)	**0.005**	**0.0146**
High	426 (72.3)	138 (83.1)		
BLM (cytoplasmic)				
Low	438 (74.5)	114 (70.3)	0.292	0.3194
High	150 (25.5)	48 (29.6)		
WRN (nuclear)				
Low	197 (48.8)	53 (42.1)	0.188	0.214
High	207 (51.2)	73 (57.9)		
WRN (cytoplasmic)				
Low	221 (54.7)	52 (41.3)	**0.008**	**0.02**
High	183 (45.3)	74 (58.7)		

NOTE: Bold = statistically significant; triple negative: $ER^-/PR^-/HER2^-$.
Abbreviations: IDC-NST, invasive ductal carcinoma-non-specific type; ILC, invasive lobular carcinoma.

population; treatment for 48 hours resulted in 13 ± 2.04% control and 19.2 ± 2.01% RECQL1 knockdown cells in the sub-G_1 population (Fig. 4C and D). In RECQL1-depleted MCF-7 cells, although S-phase accumulation was not evident (Supplementary Fig. S2C), there was a significant accumulation of sub-G_1 cells upon 24 and 48 hours of doxorubicin treatment (Supplementary Fig. S2D).

RECQL1 depletion and ERα levels

Given the recent evidence that RECQL1 may modulate gene expression (26, 28), we conducted preliminary studies to explore whether RECQL1 may impact upon ERα expression in breast cancer cell lines. In control cells, as expected, ERα expression was not detectable in MDA-MB-468 cells and MCF-7 cells had proficient ERα expression. We detected ERα expression in MDA-MB-231.

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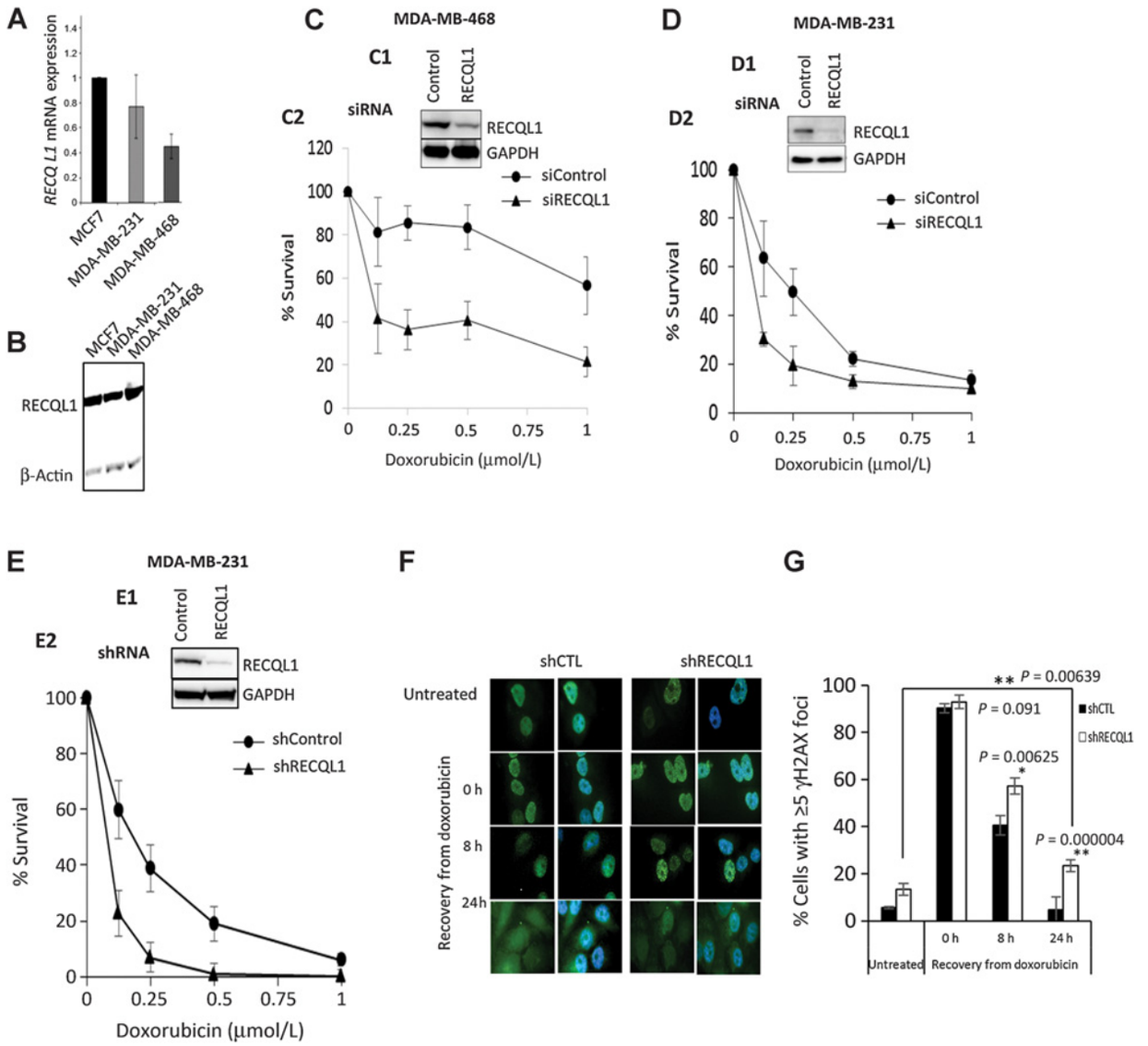

Figure 3.

RECQL1 depletion and doxorubicin sensitivity in breast cancer cell lines. **A,** *RECQL1* mRNA expression in MCF-7, MDA-MB-231, and MDA-MB-468 breast cancer cell lines. **B,** RECQL1 protein level in MCF-7, MDA-MB-231, and MDA-MB-468 breast cancer cell lines. **C,** Transient RECQL1 depletion by siRNA (**C1**) and doxorubicin sensitivity in MDA-MB-468 (**C2**; the graph shows the cellular surviving fractions measured at different doses of doxorubicin treatment in control and RECQL1-depleted cells. Surviving fraction values are the mean ± SEM from three independent experiments). **D,** Transient RECQL1 depletion by siRNA (**D1**) and doxorubicin sensitivity in MDA-MB-231 (**D2**; the graph shows the cellular surviving fractions measured at different doses of doxorubicin treatment in control and RECQL1-depleted cells. Surviving fraction values are the mean ± SEM from three independent experiments). **E,** Stable RECQL1 depletion by shRNA (**E1**) and doxorubicin sensitivity in MDA-MB-231 (**E2**; the graph shows the cellular surviving fractions measured at different doses of doxorubicin treatment in control and RECQL1-depleted cells. Surviving fraction values are the mean ± SEM from three independent experiments). **F,** Representative immunofluorescence staining of γH2AX foci (green) and its "Merge" with nuclear DNA stain DAPI (blue) in control and RECQL1-depleted MDA-MB-231 cells is shown here. **G,** Analysis of γH2AX foci in MDA-MB-231 cells stably transduced with control or RECQL1 shRNA. The percentage of cells exhibiting ≥5 γH2AX foci at indicated time points following recovery from doxorubicin treatment (0.1 μmol/L for 4 hours) was determined by immunofluorescence. Quantitative data shown represent the average from two independent experiments with associated SEMs.

Although unexpected, previous studies have reported ERα expression in MDA-MB-231 cells (29, 30). As shown in Supplementary Fig. S3, 48 hours after RECQL1 siRNA transfection, we observed significant depletion of ERα levels in MCF-7 and MDA-MB-231 cells. The data suggest that either RECQL1 depletion impairs ERα expression or promotes ERα degradation. Detailed mechanistic studies are currently under way to explore these possibilities.

Taken together, preclinical and clinical data provide evidence that RECQL1 has prognostic and predictive significance in breast cancers.

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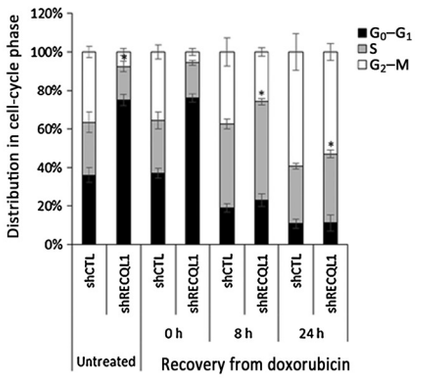
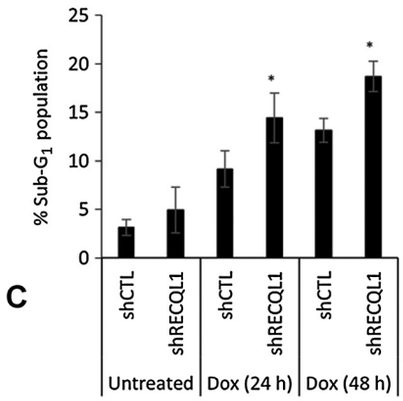
A



B

P	Untreated	DOX		
		0 h	8 h	24 h
	shCTL	shCTL	shCTL	shCTL
G_0–G_1	0.000148003	3.61E-05	0.353524	0.245683
S-phase	0.032920469	0.058841	0.004212	0.002189
G_2–M	0.000962009	0.001217	0.024416	0.055906

C



D

			Dox (24 h)		Dox (48 h)	
		0 h	24 h		48 h	
	shCTL	shRECQL1	shCTL	shRECQL1	shCTL	shRECQL1
Sub-G_1	3.117	4.926	91.463	14.898	12.975	19.195
SEM	0.830	2.348	1.891	2.561	2.04	2.13
P	0.022		4.98E-05		1.74E-05	

Figure 4.
RECQL1 depletion and cell-cycle progression. **A,** Cell-cycle distributions of MDA-MB-231 cells stably transduced with either control or RECQL1 shRNA at the indicated times following recovery from doxorubicin (Dox) treatment (0.1 μmol/L for 4 hours). **B,** Data shown represent the average from two independent experiments with associated SEMs. Individual *P* values are summarized as a table here. **C,** Sub-G_1 population in control and RECQL1-depleted MDA-MB-231 cells after doxorubicin treatment (0.1 μmol/L) for indicated time. **D,** Data shown represent the average from two independent experiments with associated SEMs. Individual *P* values are summarized as a table here.

Discussion

RECQL1 is a key member of the RecQ family of DNA helicases. RECQL1 has important roles in the maintenance of replication fork progression, DNA repair, and gene expression mechanisms (3–6). Recently, RECQL1 germline mutations were discovered in non-BRCA hereditary breast cancer patients (12, 13), implying a critical tumor-suppressive function for RECQL1. However, the role of RECQL1 in cancer pathogenesis appears to be complex. In normal cells, RECQL1 may function as a "caretaker of the genome" (3–6). On the other hand, established tumors may be dependent on RECQL1 to tolerate replication-induced DNA damage, a feature seen in proliferating cancer cells. In fact, RECQL1 has been shown to be overexpressed in glioblastoma (31), hepatocellular carcinoma (32), ovarian cancers (33), melanoma (34), and head and neck cancer models (35). Whether RECQL1 also impacts sporadic breast cancer pathogenesis is currently unknown. We have conducted comprehensive analysis and demonstrated prognostic and predictive significance of RECQL1 in sporadic breast cancers.

Genomic analyses have revealed that breast cancer represents a heterogeneous group of diseases with distinct prognostic outcomes (15). In addition to ER, PR, and HER-2 expression status, markers of proliferation and genomic stability appear to influence biological and clinical behavior of breast cancers (15, 36, 37). Given the role of RECQL1 in DNA replication and repair, we anticipated differential roles of RECQL1 in various molecular subtypes of breast cancers. As expected, high *RECQL1* mRNA was associated with intClust.3, which is characterized by low genomic instability (15). On the other hand, low *RECQL1* mRNA was linked to intClust.8 luminal A ER^+ subgroup (low proliferating) phenotype (15). Interestingly, low *RECQL1* mRNA was also observed in intClust.9 phenotype, which belongs to luminal B subgroup, implying a more complex role for RECQL1 in this subgroup. In the METABRIC cohort, low *RECQL1* mRNA was associated with poor survival. At the protein level, similarly, low RECQL1 was associated with aggressive phenotypes and poor survival, including in ER^+ tumors. However, a limitation to the current study is that mRNA expression and protein expression studies were conducted in two independent cohorts. Although, low levels of RECQL1 appear to be prevalent in the breast cancers, the mechanism for such downregulation is currently unknown. As epigenetic silencing of the BRCA1 promoter has been reported in up to 11% to 14% of breast tumors (37), it is likely that similar mechanisms may be operating for RECQL1 in sporadic breast cancers. An interesting observation was that we did not observe any cytoplasmic staining for RECQL1. This is in contrast to the cytoplasmic staining observed for BLM, RECQL4, and WRN in breast cancers (16, 38, 39). The data suggest differential regulation of localization for different RecQ helicases. In preclinical studies, RECQL1 deficiency has been shown to promote genomic instability resulting in increased frequency of spontaneous sister chromatid exchanges, chromosomal instability, DNA damage accumulation, and mutagenesis (3–6). A "mutator phenotype" (38) due to RECQL1 deficiency may therefore promote aggressive phenotypes in ER^+ breast cancers. In the current study, we observed that low PARP1, BRCA1 negative, low RAD51, low ATM, low nuclear pChk1, low nuclear Chk2, low XRCC1, low FEN1, low SMUG1, and low DNA-PKcs were significantly more likely in tumors with low nuclear RECQL1 protein level. The data suggest that RECQL1 loss may increase genomic instability, which may in turn lead to dysregulation of other DNA repair factors, thereby promoting a "mutator phenotype." A novel observation in the

current study is that low RECQL1 also influenced survival in ER^+ cohorts that received endocrine therapy, implying that RECQL1 could also have predictive significance. Given the recent evidence that RECQL1 may modulate gene expression (26, 28), we speculate that ER and/or ER-mediated gene expression could be influenced by low RECQL1 in tumors. To explore this hypothesis, we investigated ERα protein levels in control and RECQL1-depleted breast cancer cells. We observed significant depletion of ERα levels in RECQL1-depleted MCF-7 and MDA-MB-231 cells. The preliminary data would suggest that either RECQL1 depletion impairs ERα expression or promotes ERα degradation. Therefore, detailed mechanistic studies are required to explore these possibilities in detail.

In ER^- subgroup, RECQL1 did not appear to influence survival either in patient who received no chemotherapy or who received historical CMF chemotherapy. Interestingly, in ER^- tumors that received the more modern anthracycline chemotherapy, we observed that overexpression of RECQL1 was associated with poor DFS. The data suggest that ER^- tumors may be dependent on RECQL1 to tolerate replication-induced DNA damage, such as those induced by doxorubicin chemotherapy. To support this hypothesis, we depleted RECQL1 in breast cancer cells. We not only demonstrated doxorubicin sensitivity in RECQL1-depleted cells but also showed that the observed sensitivity was associated with DNA double-strand breaks accumulation, S-phase cell-cycle arrest, and apoptosis.

RecQ family of DNA helicases includes RECQL1, RECQL4, RECQL5, BLM, and WRN (1, 2). We have recently investigated the expression of RECQL4, RECQL5, BLM, and WRN in breast cancers (17, 39–41). Whereas high RECQL4, high RECQL5, and high BLM expressions were associated with aggressive breast cancers (17, 39, 40), low WRN expression was linked to poor outcomes (41). Interestingly, *RecQ* helicase mRNA levels are linked to biologically distinct integrative clusters reported in the METABRIC study (15). For example, intClust 3 subgroup that is characterized by low genomic instability was consistently seen with tumors with low *BLM*, low *RECQL4*, and low *RECQL5* mRNA levels. On the other hand, high *RECQL1* or high *WRN* mRNA levels correlated to intClust 3 subgroup. Similarly, intClust 9 (8q cis-acting/20qamplified mixed subgroup with aggressive phenotype) was more common in tumors with high *BLM*, high *RECQL4*, high *RECQL5*, low *RECQL1*, or low *WRN* mRNA levels. Taken together, the mRNA and protein expression data would suggest that differential helicase expressions lead to distinct molecular phenotypes. We speculate that proliferative functions (of BLM, RECQL4, and RECQL5 helicases) and genomic stability functions (of RECQL1 and WRN) may influence breast cancer pathogenesis. Moreover, the data presented here would also suggest that RecQ helicase–deficient sporadic tumors may be suitable for a synthetic lethality approach, an exciting new personalized treatment strategy recently demonstrated for PARP inhibitors in BRCA-deficient cancers (42). Moreover, given the recent development of helicase inhibitors (1), such as those targeting BLM (43, 44), our data would indicate potential application for these new helicase inhibitors for personalization of breast cancer therapy.

In conclusion, we have shown that RECQL1 has prognostic and predictive significance in breast cancer.

Disclosure of Potential Conflicts of Interest

G.R. Ball is the chief scientific officer at CompanDX Ltd. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Arora, S. Parvathaneni, M.A. Aleskandarany, A.R. Green, E.A. Rakha, S. Sharma
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Arora, S. Parvathaneni, M.A. Aleskandarany, D. Agarwal, R. Ali, T. Abdel-Fatah, A.R. Green, G.R. Ball, I.O. Ellis, S. Sharma, S. Madhusudan
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Study supervision: S. Sharma, S. Madhusudan

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