OXFORD

Carcinogenesis, 2016, Vol. 37, No. 1, 63–71

doi:10.1093/carcin/bgv163
Advance Access publication November 19, 2015
Original Manuscript

ORIGINAL MANUSCRIPT

Clinicopathological and prognostic significance of RECQL5 helicase expression in breast cancers

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Abstract

RECQL5 is a member of the RecQ family of DNA helicases and has key roles in homologous recombination, base excision repair, replication and transcription. The clinicopathological significance of RECQL5 expression in breast cancer is unknown. In this study, we have evaluated *RECQL5* mRNA expression in 1977 breast cancers, and RECQL5 protein level in 1902 breast cancers [Nottingham Tenovus series (n = 1650) and ER– cohort (n = 252)]. Expression levels were correlated to aggressive phenotypes and survival outcomes. High *RECQL5* mRNA expression was significantly associated with high histological grade (P = 0.007), HER2 overexpression (P = 0.032), ER+/HER2–/high proliferation genefu subtype (P < 0.0001), integrative molecular clusters (intClust 1and 9) (P < 0.0001) and poor survival (P < 0.0001). In subgroup analysis, high *RECQL5* mRNA level remains significantly associated with poor BCSS in ER+ cohort (P < 0.0001) but not in ER– cohort (P = 0.116). At the protein level, in tumours with low RAD51, high RECQL5 level was significantly associated with high histological grade (P < 0.0001), higher mitotic index (P = 0.008), dedifferentiation (P = 0.025), pleomorphism (P = 0.027) and poor survival (P = 0.003). In subgroup analysis, high RECQL5/low RAD51 remains significantly associated with poor BCSS in ER+ cohort (P = 0.010), but not in ER– cohort (P = 0.628). In multivariate analysis, high *RECQL5* mRNA and high RECQL5/low RAD51 nuclear protein coexpression independently influenced survival (P = 0.022) in whole cohort and in the ER+ subgroup. Preclinically, we show that exogenous expression of RECQL5 in MCF10A cells can drive proliferation supporting an oncogenic function for RECQL5 in breast cancer. We conclude that RECQL5 is a promising biomarker in breast cancer.

Introduction

RecQ helicases are a highly conserved family of proteins with critical roles in the maintenance of genomic stability (1–4). RECQL5 is a key member of the mammalian RecQ helicase family (1). It is a 3′–5′ helicase with weak Holiday junction unwinding

Received: July 23, 2015; **Revised:** October 18, 2015; **Accepted:** October 30, 2015

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Abbreviations

DM	distant metastases
HR	homologous recombination
IHC	immunohistochemistry
TMA	tissue microarray

activity (1). RECQL5 preferentially unwinds the lagging strand of DNA at replication fork structures and can promote strand exchange *in vitro* (5). Three isoforms of RECQL5 have been identified; RECQL5α (410aa), RECQL5β (435aa) and RECQL5γ (991aa) (6). Whereas RECQL5α and RECQL5γ are cytoplasmic, RECQL5β isoform has nuclear localization (6). RECQL5β (here in referred to as RECQL5) interacts with multiple DNA repair and metabolizing proteins including RAD51, RAD50, PARP1, FEN1, RNA polymerase II, WRN, BLM and Mre11, NBS1, PCNA, TopIIa and Top IIIa/b (1). RECQL5 has important roles in homologous recombination (HR), base excision repair (BER), DNA replication and transcription (1). Evidence for a role in HR includes findings that RECQL5-deficient cells have an increased rate of sister chromatid exchange (7), and that depleted cells accumulate γH2Ax and RAD51 foci (8). Mechanistically RECQL5's function in HR can be proposed from *in vitro* studies showing that RECQL5 physically interacts with RAD51, causing an ATPase-dependent disruption of RAD51 mediated presynaptic filament formation and hence has antirecombinase activity (9) similar to Bloom syndrome helicase (BLM) (7). Further RECQL5 associates with the MRN complex and inhibits MRE11 exonuclease activity (10), which may also contribute to the HR function of RECQL5. Interestingly while the MRN complex is required to bring RECQL5 to sites of DSBs, it's recruitment is independent of the helicase activity (11). RECQL5 may also have a role in BER. It's precise function in this pathway is not clear but RECQL5 interacts with the long patch BER proteins PCNA and FEN1, it's interaction stimulating FEN1 activity and RECQL5 colocalises with FEN1 after oxidative damage of DNA (12). In addition, the short patch BER protein XRCC1 is retained at sites of oxidative damage in the absence of RECQL5 and expression of PARP1 and XRCC1 maybe regulated by RECQL5 (13). A role in protecting cells from DNA replication stress has also been shown for RECQL5. RECQL5 foci increase following replication stress and overexpressing RECQL5 can overcome thymidine-induced replication stress (14). Likewise RECQL5 can prevent spontaneous replication fork collapse and RECQL5-depleted cells are hypersensitive the DNA replication inhibitor camptothecin (15,16). RECQL5 has also been implicated in regulation of transcription elongation and can suppress genomic instability associated with transcriptional stress (2,17,18).

Germline mutation of three of the RecQ helicases (BLM, WRN and RECQL4) leads to cancer predisposition syndromes namely Bloom syndrome, Werner syndrome and Rothmund–Thomson syndrome (3,4). Although RECQL5 helicase has not been associated with any disease phenotype, in preclinical studies, RECQL5-deficient mice cells show increased levels of spontaneous double strand breaks, are susceptible to gross chromosomal rearrangements and are prone to develop lymphomas and various solid tumours including breast cancer (8,19).

We hypothesized that RECQL5 may influence breast cancer pathogenesis. In this study, we have evaluated *RECQL5* mRNA expression in 1950 breast cancers, and RECQL5 protein level in 1902 breast cancers. We provide the first clinical evidence that RECQL5 may influence the development of aggressive breast cancer and have prognostic significance particularly in ER+ breast cancers. In addition, we show that exogenous expression of RECQL5 in MCF10A cells can drive proliferation supporting a oncogenic function for RECQL5 in breast cancer.

Materials and methods

RECQL5 and mRNA expression

RECQL5 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 1980 breast cancer samples is described by Curtis *et al.* (20). Patient demographics are summarized in Supplementary Table 1, available at *Carcinogenesis* Online. ER positive and/or lymph node negative patients did not receive adjuvant chemotherapy. ER negative 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 (20). RECQL5 expression was evaluated in this data set (RECQL5 probe ID: ILMN_1697682). 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 (20). Real time RT-qPCR was performed on the ABI Prism 7900HT sequence detection system (Applied Biosystems) using SYBR1 Green reporter. All the samples were analyzed as triplicates. 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. X-tile (Version 3.6.1) was used to identify a cut-off in gene expression values such that the resulting subgroups have significantly different survival courses. In addition, a corrected P value was produced for each analysis by using Monte-Carlo Cross Validation (MCCV) simulations (50 random populations) in X-tile to avoid the problem of over fitting and finding aberrantly low P value due to the analysis of multiple cut-points (21). The 2-fold cross validation was used to randomly split the data in to two halves and find the optimal cut-point of one half, and then divide the other half according to this cut-point. Then, the optimal cut-point of the second half was found and the first halve was similarly divided. Finally, a survival analysis of the entire dataset was performed based on the average of the optimal cut-points obtained from the MCCV simulations (21).

RECQL5 protein expression in breast cancer

The study was performed in a consecutive series of 1650 patients with primary invasive breast carcinomas who were diagnosed between 1986 and 1999 and entered into the Nottingham Tenovus Primary Breast Carcinoma series. Patient demographics are summarized in Supplementary Table 2, available at *Carcinogenesis* Online. This is a well-characterized series of patients with long-term follow-up that have been investigated in a wide range of biomarker studies (22–25). 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 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 premenopausal 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 1–233 months). Survival data, including overall survival, disease-free survival and development of locoregional and distant metastases (DM), was maintained on a prospective basis. Disease-free survival 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 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-α negative invasive BCs diagnosed and managed at the Nottingham University Hospitals between 1999 and 2007. All patients were primarily treated with surgery, followed by Radiotherapy and anthracycline chemotherapy. The characteristics of this cohort are summarized in Supplementary Table 3, available at *Carcinogenesis* Online.

Tumor Marker Prognostic Studies (REMARK) criteria, recommended by McShane *et al.* (26), were followed throughout this study. Ethical approval was obtained from the Nottingham Research Ethics Committee (C202313).

Tissue microarrays and immunohistochemistry (IHC)

Tumours were arrayed in tissue microarrays (TMAs) constructed with two replicate 0.6 mm cores from the centre and periphery of the tumours. The TMAs were immunohistochemically profiled for RECQL5 and other biological antibodies (Supplementary Table 4, available at *Carcinogenesis* Online) as described previously (22–25). 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). Leica Autostainer XL machine was used to dewax and rehydrate the slides. Pre-treatment antigen retrieval was performed on the TMA sections using sodium citrate buffer (pH 6.0) and heated for 20 min at 95°C in a microwave (Whirpool JT359 Jet Chef 1000W). A set of slides were incubated for 60 min with the primary anti-RECQL5 antibody (HPA029971, Sigma-Aldrich, UK) and anti-RAD51 antibody (clone Ab88572, Abcam Ltd., Cambridge, UK), at a dilution of 1:100 and 1:70, 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

The tumour cores were evaluated by two scorers (AA and TAF) and the concordance between the two scorer was excellent ($k = 0.79$). 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. RECQL5, RAD51 and TOPO2A expression was categorized based on the frequency histogram distributions. A median H score of ≥10 was taken as the cut-off for high RECQL5 and a median H score of ≥8 was taken as the cut-off for high RAD51 nuclear expression. For TOPO2A >25% staining cells were taken as high TOPO2A expression. Not all cores within the TMA were suitable for IHC analysis as some cores were missing or lacked tumour (<15% tumour).

Statistical analysis

Data analysis was performed using SPSS (SPSS, version 17 Chicago, IL). Where appropriate, Pearson's Chi-square, Fisher's exact, Student's *t* and ANOVA one-way tests were used. Cumulative survival probabilities were estimated using the Kaplan–Meier method, and differences between survival rates were tested for significance using the log-rank test. Multivariate analysis for survival was performed using the Cox proportional hazard model. The proportional hazards assumption was tested using standard log–log plots. Hazard ratios (HR) and 95% confidence intervals (95% CI) were estimated for each variable. All tests were two-sided with a 95% CI and a $P < 0.05$ considered significant. For multiple comparisons, *P* values were adjusted according to Holm–Bonferroni correction method (27).

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) authenticated cell lines were purchased from ATCC and were grown in RPMI (MCF-7, MDA-MB-231) or DMEM (MDA-MB-468 and MDA-MB-436) medium with the addition of 10% fetal bovine serum and 1% penicillin/streptomycin. Cell lysates were prepared and Western blot analysis performed. Primary anti-RECQL5 antibody (HPA029971, Sigma-Aldrich, UK) was incubated over night at room temperature at a dilution of 1:2000. Primary anti-β actin antibody [1:10 000 dilution (Abcam)] was used as a loading control. Infrared dye-labeled secondary antibodies (Li-Cor) [IRDye 800CW Mouse Anti-Rabbit IgG and IRDye 680CW Rabbit Anti-Mouse IgG] were incubated at a dilution of 1:10 000 for 1 h. Membranes were scanned with a Li-Cor Odyssey machine (700 and 800nm) to determine protein expression.

RECQL5 knockdown breast cancer cells using siRNAs

MCF7 cells were transfected with 50 nM RECQL5 siRNA (Dharmacon, Lafayette, CO) using *DharmaFECT* 1 reagent (Dharmacon, Lafayette, CO) and left for 48 h. The cell lysates were prepared and western blotted for RECQL5 as above but immunoreactive protein was visualized using ECL reagents (Amersham Pharmacia) following manufacturer's instructions. The sequences used for Q5β-1 and Q5β-3 siRNA constructs were 5′-UGAAGAAGGUGGCCGAUAU-3′ and 5′-CUGCAAAUGUUGUGGUCAA-3′, respectively.

RECQL5 overexpression in MCF10A cells and proliferation assay

About 10 000 MCF10A cells were plated into replica wells of 6 well plate and transfected with pcDNA-RECQL5β (a generous gift from Dr Pavel Janscak - Institute of Molecular Cancer Research, University of Zurich) or empty vector control. Following trypsinisation to remove cells from the plate live cells counted every 24 h post-transfection using trypan blue exclusion. In parallel, cell lysates were prepared and western blotted for RECQL5 as above but immunoreactive protein was visualized using ECL reagents (Amersham Pharmacia) following manufacturer's instructions.

Quantitative real-time PCR

Total RNA was extracted from MCF-7, MDA-MB-231, MDA-MB-468 and MDA-MB-436 cells using RNeasy Mini kit (QIAGEN, UK). The quantification of the extracted RNA was done using a NanoDrop 2000c Spectrophotometer (Thermo Scientific, UK). The cDNA was synthesized from 0.5 μg of total RNA using RT^2 first strand kit (QIAGEN, UK). qPCR was performed using SYBR Green PCR Master mix (Applied Biosystems, Warrington, UK) with primer set (RECQL5 QuantiTect Prier Assay, Cat. No. QT00084973, QIAGEN) targeting RECQL5 gene. The glyceraldehyde-3-phosphate dehydrogenase housekeeper gene was used as an internal control (GAPDH QuantiTect Prier Assay, Cat. 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.

Results

High RECQL5 mRNA levels associate with aggressive sporadic breast cancer

We initially profiled a panel of breast cancer cell lines. MDA-MB-436, MDA-MB-468 MCF-7 and MDA-MB 231 have robust expression of *RECQL5* mRNA (Figure 1A). We then proceeded to investigate *RECQL5* mRNA expression in the METABRIC cohort. 1306/1977 (66%) of breast tumours had low *RECQL5* mRNA expression and 34% (671/1977) of breast tumours had high *RECQL5* mRNA expression. High *RECQL5* mRNA expression was significantly associated with aggressive clinicopathological features including high histological grade, HER2 over expression, and triple negative phenotypes (ps < 0.05) (Table 1). High *RECQL4* mRNA expression was also found to be significantly correlated as described previously breast cancer molecular phenotypes; PAM50.Her2 ($P < 0.0001$) and Genufu subtype (ER+/Her2-/High proliferation) ($P < 0.01$) breast tumours (Table 1). On the other hand, PAM50.LumA and Genufu subtypes (ER+/Her2−/low proliferation) were associated with low levels of *RECQL5* mRNA (ps

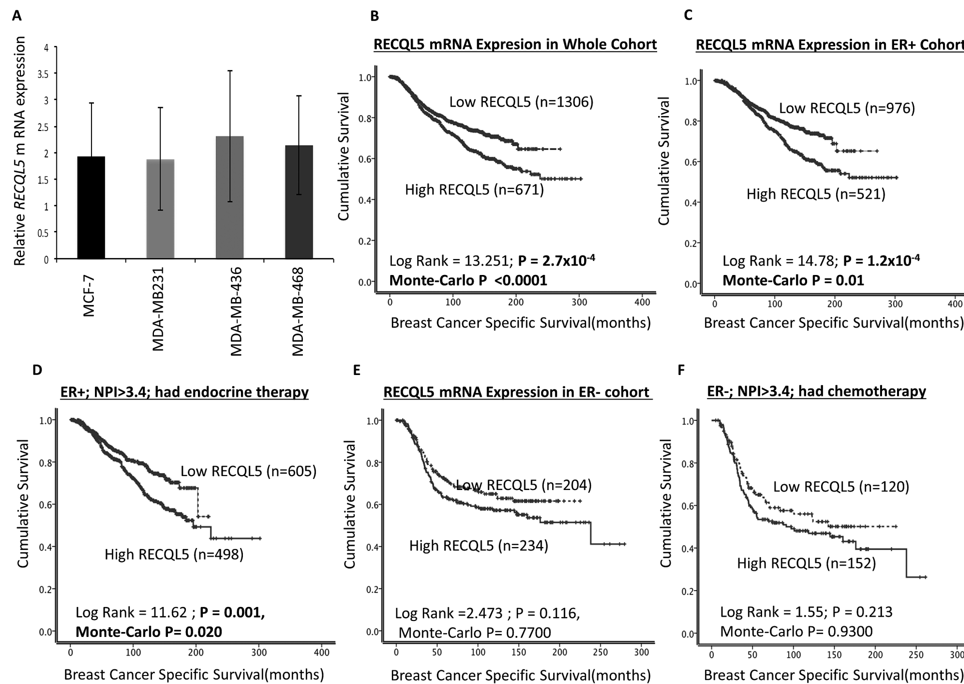

Figure 1. (**A**) *RECQL5* mRNA expression in breast cancer cell lines. (**B**) Kaplan–Meier curves showing BCSS based on *RECQL5* mRNA expression in the whole cohort. (**C**) Kaplan–Meier curves showing BCSS based on *RECQL5* mRNA expression in ER+ cohort; (**D**) Kaplan–Meier curves showing BCSS (Breast cancer specific survival) based on *RECQL5* mRNA expression in ER+ cohort that had endocrine therapy; (**E**) Kaplan–Meier curves showing BCSS based on *RECQL5* mRNA expression in ER– cohort. (**F**) Kaplan–Meier curves showing BCSS based on *RECQL5* mRNA expression in ER– cohort that had chemotherapy.

< 0.05) (Table 1). Interestingly, PAM50.Basal was also more common in tumours with low levels of *RECQL5* mRNA (ps < 0.05). Similarly, low *RECQL5* mRNA expression was associated with intClust.3 ($P < 0.00001$) and intClust.4 (ps < 0.001) molecular phenotypes that have good prognosis (28). However, high *RECQL5* mRNA expression was significantly associated with intClust.1 ($P < 0.00001$), and intClust.9 ($P < 0.00001$) that have intermediate to poor clinical outcome (20).

We then proceeded to survival analysis in METABRIC cohort. High *RECQL5* mRNA expression was associated with poor BCSS ($P < 0.0001$) in the whole cohort (Figure 1B). In the ER+ subgroup, high *RECQL5* mRNA expression was found to be associated with poor BCSS ($P < 0.0001$) (Figure 1C). In the ER+ subgroup that received adjuvant endocrine therapy, high *RECQL5* mRNA expression remained associated with poor BCSS ($P < 0.001$) (Figure 1D). However, in ER– subgroup, *RECQL5* mRNA expression did not significantly influence outcome in the ER– cohort, including ER– patients who received chemotherapy ($P = 0.116$ and $P = 0.213$ respectively) (Figure 1E and F).

In the multivariate Cox regression analysis, that included other validated prognostic factors, *RECQL5* mRNA expression was a powerful independent predictor for BCSS in the whole cohort ($P = 0.038$), ER+ subgroup ($P = 0.046$) but not in the ER– subgroup ($P = 0.615$) (Table 2).

Together the data provides evidence that *RECQL5* mRNA level has clinicopathological significance and influence prognosis, particularly in ER+ breast cancers. We proceeded to evaluate RECQL5 protein expression in breast cancers.

RECQL5 protein expression in sporadic breast cancer

We initially investigated RECQL5 protein level in breast cancer cell lines and in MCF10A breast epithelial cells. As shown in Figure 2A, MDA-MB-436, MDA-MB-468 MCF-7 and MDA-MB 231 have robust expression of RECQL5 protein. On the other hand, in MCF10A cells, RECQL5 expression was low compared to MCF7 cells (Figure 2B). When RECQL5 was overexpressed in MCF10A (Figure 2B) we observed increased proliferation in RECQL5 overexpressing MCF10A cells compared to control MCF10A cells (Figure 2C). Taken together, the data suggest that RECQL5 is a marker of proliferation and could have prognostic and/or predictive significance in human breast cancers. We proceeded to IHC investigation in human tumours. To provide evidence for the specificity of the anti-RECQL5 antibody used for IHC studies, we generated RECQL5 knockdown (KD) MCF7 cells using two siRNA constructs. As shown in Figure 2D, control cells have robust RECQL5 expression seen as a single band while RECQL5 KD cells show almost complete loss of this band—demonstrating the specificity of the RECQL5 antibody.

We observed nuclear-only localization of RECQL5 protein in breast cancers (Figure 2D). There was no cytoplasmic staining in breast tumours. 46.3% (556/1200) of tumours showed low nuclear RECQL5 expression and 53.7% (644/1200) of tumours revealed high nuclear RECQL5 expression. We also investigated RECQL5 expression in 30 normal breast tissues. There was no cytoplasmic staining in normal tissues. We observed that 30/30 (100%) normal tissues showed high nuclear RECQL5 expression. Taken together, the data suggest differential expression RECQL5 in normal tissues compared to tumours tissues. We initially investigated RECQL5 protein alone in breast cancer tissues. Tumours with low nuclear RECQL5 levels were significantly associated with high grade, high mitotic index, tubule formation, tumour type, HER2 overexpression, ER-, triple negative and basal type phenotype (ps < 0.05) (Supplementary Table 5, available at *Carcinogenesis* Online). In addition, low RECQL5 expression was found to be associated with low levels of other DNA repair proteins such as BRCA1, XRCC1, FEN1, SMUG1, APE1, Polβ, ATM, ATR, Chk1, Chk2, TOP2A, Rad51 and DNA-PKc (ps < 0.01) (Supplementary Table 5, available at *Carcinogenesis* Online). We

Table 1. Association between *RecQL5* mRNA expression and clinicopathologic variables in METABRIC cohort

	RECQL5 mRNA expression		*P* values	
	Low	High		
Variable	N (%)	N (%)	Unadjusted	Adjusted
Pathological parameters				
Lymph node stage				
Negative	684 (52.6%)	351 (52.5%)	0.990	25.74
Positive (1–3)	207 (15.9%)	107 (15.9%)		
Positive (>3)	409 (31.5%)	213 (31.7%)		
Grade				
G1	129 (10.3%)	40 (6.2%)	**0.007**	**0.023**
G2	512 (41.0%)	258 (40.2%)		
G3	607 (48.6%)	343 (53.5%)		
Tumour size (cm)				
T 1a+b(1.0)	61 (4.7%)	31 (4.7%)	0.097	0.157
T 1c(>1.0–2.0)	532 (41.0%)	234 (35.6%)		
T2 (>2.0–5)	639 (49.2%)	362 (55.0%)		
T3 (>5)	67 (68.4%)	31 (31.6%)		
NPI				
≤3.4	461 (35.3%)	219 (32.6%)	0.238	0.269
>3.4	137 (64.7%)	1160 (67.4%)		
Her2 overexpression (No)	1159 (88.7%)	573 (85.4%)	**0.032**	0.075
(Yes)	147 (11.3%)	98 (14.6%)		
Triple negative (No)	1079 (82.6)	581 (88.6)	**0.023**	0.066
(Yes)	227 (17.4)	90 (13.4)		
ER (negative)	322 (24.7%)	148 (22.1%)	0.199	0.246
(positive)	984 (75.3%)	523 (77.9%)		
PgR (negative)	603 (46.2%)	333 (49.6%)	0.145	0.188
(Positive)	703 (53.8%)	338 (50.4%)		
Genefu subtype				
ER-/Her-2 negative	110 (16.3%)	40 (12.6%)	0.129	0.186
ER+/Her-2 negative/high proliferation	229 (33.9%)	137 (43.1%)	**0.005**	**0.018**
ER+/Her-2 negative/low proliferation	265 (39.1%)	103 (32.4%)	**0.038**	**0.082**
Her-2 positive	72 (10.7%)	38 (11.9%)	0.543	0.564
PAM50 subtype				
PAM50.Her2	130 (11.2%)	108 (17.6%)	**1.5×10^{-4}**	**0.0008**
PAM50.Basal	233 (20.1%)	97 (15.8%)	**0.029**	0.075
PAM50.LumA	488 (42.1%)	227 (37.1%)	**0.042**	0.084
PAM50.LumB	309 (26.6%)	180 (29.4%)	0.214	0.252
IntClust subgroups				
intClust.1	49 (3.8%)	88 (13.1%)	**8.3×10^{-15}**	**0.00001**
intClust.2	55 (4.2%)	17 (2.5%)	0.059	0.102
intClust.3	221 (16.9%)	69 (10.3%)	**7.8×10^{-5}**	0.0005
intClust.4	267 (20.4%)	76 (11.3%)	**3.9×10^{-7}**	**0.00001**
intClust.5	120 (63.5%)	69 (36.5%)	0.433	0.469
intClust.6	50 (3.8%)	36 (5.4%)	0.113	0.172
intClust.7	113 (8.7%)	40.2 (11.3%)	0.056	0.104
intClust.8	187 (14.3)	113 (16.8%)	0.139	0.191
intClust.9	74 (5.7%)	72 (10.7%)	**4.6×10^{-5}**	**0.0004**
intClust.10	170 (13.0%)	55 (8.2%)	**0.001**	**0.0043**

Bold = Statistically significant; HER, human epidermal growth factor receptor 2; ER, oestrogen receptor; PgR, progesterone receptor; triple negative, ER–/PgR–/HER2–. Adjusted *P* values were calculated using Benjamini–Hochberg false discovery rate method to adjust for multiple testing.

then conducted the univariate survival analysis. At protein level, RECQL5 expression alone failed to show any statistically significant correlation with BCSS in whole cohort (Supplementary Figure 1A, available at *Carcinogenesis* Online). We then proceeded to subgroup analysis and again found no statistically significant association with survival in ER+ (Supplementary Figure 1B, available at *Carcinogenesis* Online) and ER– subgroups (Supplementary Figure 1C, available at *Carcinogenesis* Online). As RECQL5 alone, despite having clinicopathological associations with aggressive phenotype, did not have prognostic significance we hypothesized that RECQL5 may operate in the context of RAD51 or TOPO2A to influence clinical outcomes. We therefore proceeded to co-expression analysis.

RECQL5 coexpression with RAD51 or TOPO2A in sporadic breast cancer

RECQL5 physically interacts with RAD51 and disrupts RAD51 mediated presynaptic filament formation (1,9). Tumours with

Table 2. Multivariate analysis of RECQL5 mRNA expression in breast cancer

	P Value	Risk ratio	95% CI for risk ratio	
			Lower	Upper
BCSS (whole cohort)				
Grade	**0.000004**	1.447429	1.237421	1.693079
Size (cm)	**0.000001**	1.121003	1.076755	1.167069
LN status	**0.000001**	1.875371	1.666800	2.110040
RECQL5 mRNA expression	**0.023530**	1.223718	1.027549	1.457337
BCSS (ER± cohort)				
Grade	**0.0002**	1.411263	1.176981	1.692178
Size (cm)	**0.000001**	1.180525	1.123389	1.240566
LN status	**0.000001**	1.822326	1.578493	2.103825
RECQL5 mRNA expression	**0.045**	1.242008	1.004707	1.535359
BCSS (ER− cohort)				
Grade	0.968564	1.008054	0.676448	1.502217
Size (cm)	0.239188	1.046124	0.970452	1.127695
LN status	**0.000001**	1.908038	1.539252	2.365181
RECQL5 mRNA expression	0.635591	1.078023	0.790092	1.470886

Bold: statistically significant; CI, confidence interval; HR, hazard ratio; LN, lymph node.

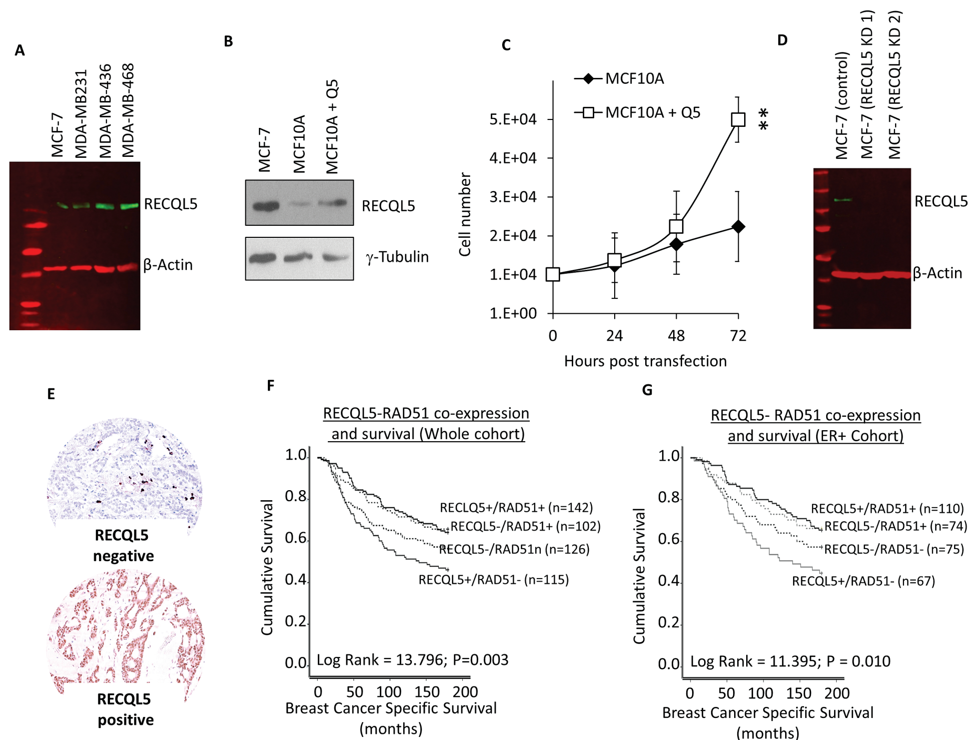

Figure 2. (**A1**) Western blot of RECQL5 expression in breast cancer cell lines. (**A2**) MCF10A breast cells were transfected with RECQL5 or empty vector and. Extracts were made 48h post transfection and western blotted for RECQL5. RECQL5 level in MCF7 cells is shown for comparison. (**B**) MCF10A breast cells were transfected with RECQL5 or empty vector and cell number counted every 24h. Mean and standard deviation are shown for three independent repeats. **Indicates a statistical significance of $P < 0.01$ at 72h using Student's *t* test. (**C**) Western blot of RECQL5 expression in MCF-7 (RECQL5 wild-type) and MCF-7 RECQL5 knock down cell line. (**D**) Microphotograph of RECQL5 protein expression in breast tumours. (**E**) Kaplan–Meier curves showing BCSS based on RECQL5-RAD51 coexpression in the whole cohort. (**F**) Kaplan–Meier curves showing BCSS based on RECQL5-RAD51 coexpression in ER+ cohort.

high RECQL5-low RAD51 nuclear protein levels were significantly associated with high grade, high mitosis and pleomorphism (all $P < 0.05$) (Table 3). Tumours with low RECQL5-low RAD51 nuclear protein levels were significantly associated with tubule formation, NPI > 3.4 and ER/PR negativity (all $P < 0.05$) (Table 3). In univariate analysis, tumours with high RECQL5-low RAD51 nuclear protein levels were associated with poor BCSS in the whole cohort ($P = 0.003$) (Figure 2E). In subgroup analysis, ER+ tumours with high RECQL5-low RAD51 nuclear protein levels were associated with poor BCSS ($P = 0.010$) (Figure 2F). In ER− cohort, RECQL5-RAD51 co-expression did not influence survival ($P = 0.628$) (Supplementary Figure 1D, available at *Carcinogenesis* Online). We have shown previously a direct interaction between RECQL5 and Topoisomerase IIα (TOP2A) where RECQL5 specifically stimulated the decatenation activity of TOP2A (29). Tumours with low RECQL5-high TOPO2A were

Table 3. RECQL5–RAD51 protein coexpression and breast cancer

Variable	RECQL5 and RAD51 protein coexpression					
	RQ5n−/RAD51n− N (%)	RQ5n+/RAD51n− N (%)	RQ5n−/RAD51n+ N (%)	RQ5n+/RAD51n+ N (%)	P value (unadjusted)	P value (adjusted)
Pathological parameters						
Tumour size						
≤1 cm	12 (6.8)	9 (5.8)	10 (7.6)	16 (8.6)	**0.031**	**0.045**
>1–2 cm	77 (43.8)	65 (41.9)	73 (55.7)	107 (57.5)		
>2–5 cm	84 (47.7)	78 (50.3)	45 (34.4)	62 (33.3)		
>5 cm	3 (1.7)	3 (1.9)	3 (2.3)	1 (0.5)		
Tumour stage						
1	96 (54.5)	83 (53.2)	79 (60.3)	112 (60.2)	0.370	0.401
2	64 (36.4)	57 (36.5)	44 (33.6)	52 (28.0)		
3	16 (9.1)	16 (10.3)	8 (6.1)	22 (11.8)		
Tumour grade						
G1	13 (7.4)	18 (11.5)	14 (10.7)	37 (19.9)	**4.1×10^{-5}**	**0.0005**
G2	59 (33.5)	38 (24.4)	53 (40.5)	73 (39.2)		
G3	104 (59.1)	100 (64.1)	64 (48.9)	76 (40.9)		
Mitotic index						
M1 (low; mitoses < 10)	45 (26.0)	34 (22.5)	41 (31.5)	72 (40.0)	**0.008**	**0.017**
M2 (medium; mitoses 10–18)	38 (22.0)	28 (18.5)	24 (18.5)	38 (21.1)		
M3 (high; mitosis >18)	90 (52.0)	89 (58.9)	65 (50.0)	70 (38.9)		
Tubule formation						
1 (>75% definite tubule)	5 (2.9)	2 (1.3)	7 (5.4)	7 (3.9)	**0.025**	**0.046**
2 (10–75% definite tubule)	45 (26.0)	49 (32.5)	43 (33.1)	75 (41.7)		
3 (<10% definite tubule)	123 (71.1)	100 (66.2)	80 (61.5)	98 (54.4)		
Pleomorphism						
1 (small-regular uniform)	0 (0.0)	1 (0.7)	2 (1.6)	2 (1.1)	**0.027**	**0.043**
2 (Moderate variation)	61 (35.5)	42 (27.8)	48 (37.2)	82 (45.6)		
3 (Marked variation)	111 (64.5)	108 (71.5)	79 (61.2)	96 (53.3)		
Tumour type						
IDC-NST	121 (69.9)	113 (72.4)	75 (58.1)	102 (55.1)	**0.002**	**0.005**
Tubular carcinoma	21 (12.1)	22 (14.1)	27 (20.9)	50 (27.0)		
Medullary carcinoma	7 (4.0)	2 (1.3)	3 (2.3)	0 (0.0)		
ILC	14 (8.1)	8 (5.1)	13 (10.1)	20 (10.8)		
Others	3 (1.7)	1 (0.6)	0 (0.0)	1 (0.5)		
Lymph node status						
Negative	81 (54.4)	69 (51.1)	72 (60.0)	97 (58.4)	0.353	0.417
Positive (1–3)	56 (37.6)	54 (40.0)	44 (36.7)	53 (31.9)		
Positive (>3)	12 (8.1)	12 (8.9)	4 (3.3)	16 (9.6)		
Aggressive phenotype						
Her2 overexpression						
No	153 (87.4)	123 (80.4)	105 (81.4)	159 (88.3)	0.105	0.136
Yes	22 (12.6)	30 (19.6)	24 (18.6)	21 (11.7)		
Triple negative phenotype						
No	149 (84.7)	130 (83.3)	115 (87.8)	151 (81.2)	0.457	5.94
Yes	27 (15.3)	26 (16.7)	16 (12.2)	35 (18.8)		
NPI						
≤3.4	31 (18.3)	31 (21.2)	34 (27.4)	68 (38.4)	**1.1×10^{-4}**	**0.0005**
>3.4	138 (81.7)	115 (78.8)	90 (72.6)	109 (61.6)		
Hormone receptors						
ER						
Negative	60 (35.1)	53 (34.9)	32 (25.4)	28 (15.7)	**7.6×10^{-5}**	**0.0005**
Positive	111 (64.9)	99 (65.1)	94 (74.6)	150 (84.3)		
PgR						
Negative	91 (54.5)	77 (52.0)	49 (39.5)	63 (35.6)	**0.001**	**0.003**
Positive	76 (45.5)	71 (48.0)	75 (60.5)	114 (64.4)		

Adjusted *P* values were calculated using Benjamini–Hochberg false discovery rate method to adjust for multple testing.
Bold = statistically significant; ER, oestrogen receptor; HER2, human epidermal growth factor receptor 2; PgR, progesterone receptor; Triple negative, ER−/PgR−/HER2−.

significantly associated with high grade, high mitotic index, dedifferentiation tumour type, high risk NPI and PR negativity (Supplementary Table 6, available at *Carcinogenesis* Online). In univariate analysis, RECQL5-TOPO2A co-expression did not influence survival (Supplementary Figures 1E–G, available at *Carcinogenesis* Online).

Table 4. Multivariate analysis of RECQL5-RAD51 protein coexpression in breast cancer

	B	P value	Exp (B)	95.0% CI for Exp (B)	
				Lower	Upper
BCSS in the whole cohort					
RECQL5-RAD51 coexpression	−0.069	**0.022**	0.934	0.880	0.990
NPI	0.769	**0.001**	2.158	1.378	3.379
er status	0.293	0.097	1.340	0.949	1.893
HER2 status	0.728	**0.0001**	2.071	1.432	2.994
BCSS in the ER± cohort					
RECQL5-RAD51 coexpression	−0.063	0.073	0.939	0.876	1.006
NPI	0.784	**0.001**	2.190	1.376	3.484
HER2 status	0.848	**0.001**	2.335	1.448	3.767
BCSS in the ER− cohort					
RECQL5-RAD51 coexpression	−0.081	0.172	0.923	0.822	1.036
NPI	0.651	0.519	1.917	0.265	13.882
HER2 status	0.551	0.057	1.735	0.984	3.062

Bold = statistically significant.

In multivariate analysis (Table 4), RECQL5-RAD51 coexpression remained statistically significant independent marker of prognosis ($P = 0.022$) in the whole cohort. NPI and HER-2 expression were other factors independently associated with BCSS (P values 0.001 and 0.0001, respectively). In the ER+ subgroup, RECQL5-RAD51 coexpression was of borderline significance ($P = 0.07$), but not in the ER− subgroup ($P = 0.172$).

Discussion

RECQL5 is a key member of the RecQ family of DNA helicases (1–4). RECQL5 is a multifunctional protein with roles in DNA replication, chromosomal segregation, DNA repair (including HR, single strand break repair and base excision repair) and transcription. Deficiency of DNA repair factors such as RECQL5 could predispose an individual to cancer through increased genomic instability. On the other hand, in established tumours high levels of RECQL5 may promote survival of cancer cells that are constantly under DNA damaging oxidative stress. RECQL5 deficient mice are predisposed to lymphomas and solid tumours including breast tumours (1–4). In humans, polymorphisms within the *RECQL5* gene may predispose an individual to cancer including: osteosarcomas, laryngeal carcinomas and breast cancers (30–33). In a recent study in colorectal cancers, low RECQL5 expression was observed at the mRNA and protein levels (19). The authors concluded that RECQL5 deficiency may predispose to colorectal cancer. However, the study had several limitations, including a small cohort and lack of clear evidence of clinicopathological associations or survival outcomes (19).

The clinicopathological significance of RECQL5 in sporadic breast cancer is unknown. In the current study we have comprehensively evaluated RECQL5, and unravelled its complex role in breast cancers. At the mRNA level, we observed high expression in 34% of tumours. High *RECQL5* mRNA expression was significantly associated with aggressive phenotypes and adverse survival. The prognostic significance was particularly pronounced in ER+ subgroup, supporting the observation that high *RECQL5* mRNA expression was more likely in ER+/HER2−/high proliferation Geneufu subtype tumours. At the protein level, we observed high RECQL5 levels in 53.7% of breast cancers. However, it should be pointed out that RECQL5 mRNA expression and RECQL5 protein expression were investigated in two independent cohorts. Therefore, a limitation to the current study is that we were unable to perform direct correlations between mRNA and protein expression. In contrast to mRNA expression data, although low RECQL5 expression was associated with aggressive phenotype, RECQL5 protein alone did not influence survival outcomes in patients. The data suggest that RECQL5 may be subjected to complex post-transcriptional regulation. Alternatively, there may be a threshold effect for expression level and this threshold is more accurately measured by the analysis of mRNA levels. Another possibility is that the complex role of RECQL5 may operate in the context of RAD51. RECQL5 directly interacts with RAD51 (9) and the anti-recombinogenic role of RECQL5 may be active through disruption of RAD51 mediated presynaptic filament formation during HR (34). Therefore, we conducted RECQL5-RAD51 co-expression studies and observed that tumours with high RECQL5/low RAD51 not only manifest aggressive phenotypes but were also associated with poor survival. In subgroup studies, similar to mRNA data, the prognostic significance was more pronounced in ER+ breast cancer but not in ER− tumours. Consistent with high RECQL5 being associated with aggressive phenotypes, over expression of RECQL5 in the normal breast cell line MCF10A increased proliferation. Interestingly RAD51 is reported as having relatively low expression in MCF10A cells (35) and the functional relationship between RECQL5, RAD51, proliferation, recombination and tumourigenesis will be the subject of future investigations. Taken together, the data provides the first clinical evidence that RECQL5 may influence breast cancer pathogenesis.

RECQL5 interacts with and is a general transcription elongation factor for RNA Pol II (2,17,18). Loss of RECQL5 leads to a genome-wide increase in the average rate of gene transcription, transcriptional stress and recombination (17) suggesting RECQL5 has a function in resolving such stress. RNA polymerases generate positive supercoiling ahead of the transcription apparatus, which in turn feeds back to reduce the processional rate of RNA polymerase. Topoisomerases are ubiquitous enzymes that remove supercoiling and therefore are required for normal transcription elongation. Specifically, type II topoisomerases generate transient protein-concealed double strand breaks while removing torsional stress from the DNA and collisions with the transcription apparatus can convert these protein–DNA complexes into permanent DNA strand breaks (36). Previously, we showed a direct interaction between RECQL5 and Topoisomerase IIα (TOP2A) specifically stimulated the decatenation activity of TOP2A (29). TOP2A expression is a marker for proliferation and has been analyzed in a number of breast cancer studies in part because it lies in close proximity to the HER2 gene on chromosome 17. Therefore, we were interested

in evaluating if there were any expression correlations between RECQL5, which also resides on chromosome 17 (17q25.1), and TOP2A expression in our breast cancer cohorts. Interestingly, high grade tumours are more often scored as RECQL5– and TOP2A+, than any other expression pattern. This may reflect the fact that loss of RECQL5 promotes transcriptional stress. Additionally, while TOP2A is known as a marker for proliferation, loss of RECQL5 may make these cells more dependent on TOP2A to relieve transcriptional stress. Thus in this subgroup of patients, it would be interesting to evaluate the efficacy of TOP2A targeted therapies (such as doxorubicin that is routinely used in breast cancer therapy), if in fact these tumours are more dependent on topoisomerase activities than other tumour types cells.

In conclusion, our data provides evidence that RECQL5 could be a promising biomarker in breast cancer and needs further investigation as a potential drug target.

Supplementary material

Supplementary Tables 1–5 can be found at http://carcin.oxfordjournals.org/

Conflict of Interest Statement: None declared.

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