Original Study

Werner Syndrome Protein Expression in Breast Cancer

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Abstract

Werner protein (WRN) is a DNA helicase involved in genomic stability and commonly inactivated in breast tumors. Its clinicopathologic significance was investigated in a cohort of clinically annotated series of sporadic (n = 1650) and BRCA-mutated (n = 75) invasive breast tumors. Low WRN expression was associated with worse survival and aggressive molecular phenotype. Low WRN expression in topoisomerase-I-overexpressed tumors was also associated with poor survival. These findings can be used to optimize personalized treatment.

Introduction: Werner protein (WRN) plays an important role in DNA repair, replication, transcription, and consequently genomic stability via its DNA-helicase and exonuclease activity. Loss of function of WRN is associated with Werner syndrome (WS), which is characterized by premature aging and cancer predisposition. Malignancies that are commonly linked to WS are thyroid carcinoma, melanoma, breast cancer, meningioma, and soft tissue and bone sarcomas. Currently, the clinicopathologic significance of WRN in breast cancer is largely unknown. **Patients and Methods:** We investigated the clinicopathologic and prognostic significance of WRN protein expression in a cohort of clinically annotated series of sporadic (n = 1650) and BRCA-mutated (n = 75) invasive breast cancers. We correlated WRN protein expression to clinicopathologic characteristics, DNA repair protein expression, and survival outcomes. **Results:** There is strong evidence of association between low nuclear and cytoplasmic WRN co-expression and low levels of KU70/KU80, DNA-PK, DNA Pol-B, CKD18, cytoplasmic RECQL4, and nuclear BLM protein expression (adjusted *P*-values $< .05$). Tumors with low nuclear or cytoplasmic WRN expression have worse overall breast cancer-specific survival (BCSS) (adjusted *P*-values $< .05$). In topoisomerase I overexpressed tumors, low WRN nuclear expression was associated with poor BCSS (*P*-value $< .05$). In BRCA-mutated tumors, low WRN cytoplasmic expression conferred shortest BCSS ($P < .05$). **Conclusions:** Low WRN protein expression is associated with poor BCSS in patients with breast cancer. This can be used to optimize the risk stratification for personalized treatment.

Clinical Breast Cancer, Vol. ■, No. ■, ■-■
Keywords: Biomarker, Breast Cancer, Helicase, Werner Syndrome Protein, WRN

Introduction

Werner (WRN) enzyme, also known as Recombinase Q like helicase 2 (RECQL2), has a DNA-helicase and exonuclease activity towards double-stranded DNA.[1,2] The gene that encodes WRN protein is located in chromosome 8p12, and its role is to unwind the DNA and remove abnormal structures in an ATP-dependent and directionally specific manner.[1,3,4] WRN protein has been shown to play an important role in DNA repair, replication, transcription, telomere maintenance, and, consequently, genomic stability.[1,5,6] WRN co-localizes and shows direct interaction with topoisomerase I (TOPO I). WRN enhances the ability of TOPO I to relax negatively supercoiled DNA.[7]

Mutations in the human WRN gene leading to the loss of WRN gene product are associated with Werner syndrome (WS).[3] WS is a rare autosomal recessive disease that is characterized by chromosomal instability, premature aging, and propensity to malignancies.[1,8] The most common neoplasms in patients with WS are soft tissue sarcoma, osteosarcoma, thyroid cancer, malignant melanoma, breast cancer, benign meningioma, and myeloid disorders.[8] Frequent molecular alterations that are seen in WS include nonsense, splicing or frameshift mutations, extensive deletions,

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Submitted: Apr 18, 2020; Revised: Jul 9, 2020; Accepted: Jul 16, 2020

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1526-8209/$ - see frontmatter
https://doi.org/10.1016/j.clbc.2020.07.013

inversions, and reciprocal translocations, with missense mutations being uncommon.[9-11] Hence, the involvement of WRN in genome stability makes WRN gene function as a tumor-suppressor gene.[12]

RECQ helicases have a highly conserved RECQ C-terminal group that interacts with DNA damage response proteins.[1,13,14] Knockout of WRN in primary fibroblasts using RNA interference led to increased oxidative DNA damage and early cellular senescence, indicating that WRN regulates oxidative stress homeostasis and DNA repair.[15] This was supported by a study by Opresko et al, who demonstrated that deletion of WRN resulted in growth arrest at G2/M cell cycle phases, DNA damage, and increased tumor cell death rate.[13] Additionally, the surviving proliferative clones overexpressed WRN protein, which indicates that WRN plays an important role not only in carcinogenesis but also in tumor growth.[16]

To date, there is no clear evidence about the clinicopathologic and prognostic significance of WRN protein in breast cancer. In this study, we investigated the clinicopathologic and prognostic significance of WRN protein expression in patients with invasive breast cancer.

Patients and Methods

Tissue Culture and Western Blot Analysis

Western blot analysis was used to evaluate the specificity of anti-WRN antibody before using them for immunohistochemistry (IHC). WRN protein expression was assessed in 4 breast cancer cell lines: MCF7, MDA-MB-231, MDA-MB-436, and MDA-MB-468. Cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA). MDA-MB-436 and MDA-MB-468 cells were cultured in Minimum Essential amino acids Medium (Sigma), supplemented with 1% L-glutamine and 1% non-essential amino acids. MCF-7 and MDA-MB-231 cells were grown in RPMI medium (Sigma). All media were supplemented with 10% FBS (Sigma), 5 mL of 1% penicillin/streptomycin (10,000 units penicillin and 10 mg streptomycin/mL; Sigma). All cell lines were maintained in a humidified incubator at 37°C with 5% carbon dioxide and grown as an adherent culture.

Protein samples were prepared by lysing cells in RIPA buffer (Sigma-Aldrich) containing protease inhibitor (Sigma) and phosphatase inhibitor cocktail 1 and 2 (Sigma). Samples were run on SDS-PAGE gel (4%-12%) bis-tris. The antibodies used were anti-WRN rabbit polyclonal antibody (Novus Biological, cat. no. NBP1-87143) at 1:1500 dilution, and anti−β-actin mouse monoclonal antibody (Sigma, cat. no. A2228 Clone AC-74) at 1:10,000 dilution. All equipment and reagents for Western blot were purchased from Thermo Fisher Scientific except for the protein standard (Precision Plus Protein All Blue Pre-stained Protein Standard, BioRad) and the secondary antibody solution (IRDye 800CW Donkey Anti-Rabbit IgG and IRDye 680CW Donkey Anti-Mouse IgG, Licor, Biosciences). Protein detection and quantification were determined by scanning the membranes on Licor-Odyssey's scanner (Licor, Biosciences) at the predefined intensity fluorescence.

Patient Selection for Protein Data

Comprehensive evaluation of the protein expression of WRN in breast cancer was performed in a consecutive series of sporadic (n = 1650) and BRCA-mutated (n = 75) invasive breast tumors.

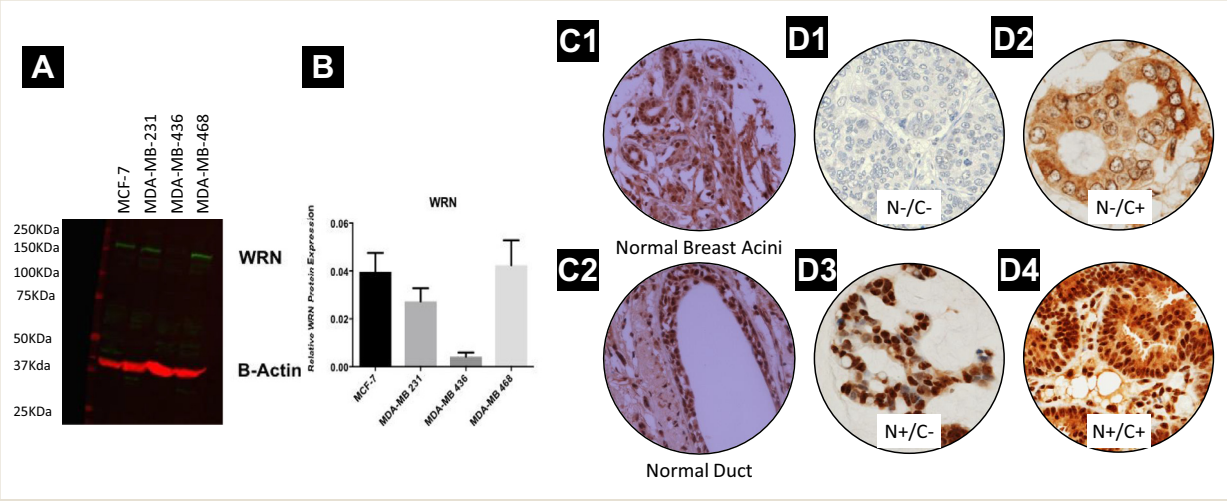
A total of 1650 unselected primary operable (stage I-III) sporadic invasive breast carcinomas from patients aged ≤ 70 years who were diagnosed between 1986 and 1999 were included in the Nottingham Tenovus Primary Breast Cancer series. Women older than 70 years were excluded from the study because of the increased confounding factor of death from other causes and because primary treatment protocols for these patients often differed from those for younger women. Patients diagnosed before 1986 and after 1999 have been excluded because major changes in diagnosis and treatment were applied to patients before and after these time points. The patients' clinical history and tumor characteristics including family history and outcomes were obtained from the database that is prospectively maintained. Patient demographics are summarized in Supplemental Table 1 (in the online version). This is a well-characterized series of patients with long-term follow-up that have been investigated in a wide range of biomarker studies.[17-19]

Genetic testing for BRCA1 and BRCA2 germline mutations was performed in those patients with breast cancer from the Nottingham Tenovus series who were deemed to be a high risk of being a carrier (young age < 45 years; triple negative breast cancers or family history of breast and/or ovarian cancer). Seventy-five confirmed germline deficient breast cancer tumors for BRCA1 or BRCA2 were identified and included into an independent BRCA-mutated cohort. Supplemental Table 2 (in the online version) summarizes the baseline characteristics of the BRCA-mutated cohort.

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), estrogen 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 CMF (cyclophosphamide 100 mg/m^2 orally on days 1−14 or 600 mg/m^2 intravenously (IV) on days 1 and 8, methotrexate 40 mg/m^2 IV on days 1 and 8, and 5-fluorouracil 600 mg/m^2 IV on days 1 and 8 every 28 days) chemotherapy was given; patients with ER-α-positive tumors were also offered endocrine therapy. Postmenopausal patients with NPI scores of ≥ 3.4 and ER-α positivity were offered endocrine therapy, whereas ER-α-negative patients received classical CMF chemotherapy. The 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 loco-regional and distant metastases (DMs), 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 (LN) relapse, or DM relapse. BCSS was defined as the number of months from diagnosis to the occurrence of BC-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.

This retrospective study was performed on formalin-fixed paraffin-embedded (FFPE) archival tumor tissues collected immediately after surgery prior to any adjuvant oncologic treatment. The

Figure 1 A, Western Blot of WRN Protein Expression in Breast Cancer Cell Lines. B, Relative WRN Protein Expression in Breast Cancer Cell Lines. C, Microphotographs of WRN Protein Expression in Normal Breast Tissue. D, Microphotographs of WRN Protein Expression in Breast Tumors



Abbreviations: N−/C− = nuclear negative and cytoplasmic negative; N+/C+ = nuclear negative and cytoplasmic positive; N+/C− = nuclear positive and cytoplasmic negative; N−/C+ = nuclear negative and cytoplasmic positive; WRN = Werner protein.

tissue blocks were stored in a secure, purpose-built facility in Nottingham University Hospitals NHS Trust, which is accessible to only authorized Tissue Bank staff according to the National Health Service Research Authority guidelines. Representative tumor tissues were prepared as tissue microarrays (TMAs).

Tumour Marker Prognostics Studies (REMARK) criteria, recommended by McShane et al,[20] were followed throughout this project.

TMAs and IHC

Breast tumors were arrayed in TMAs constructed with 2 replicate 0.6-mm cores from the center and periphery of the tumors. Optimal concentration and conditions for staining were ascertained for WRN antibody 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. The WRN antibody (rabbit antibody, polyclonal) was purchased from Novus Biological (NBP1-87143). 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 (Whirlpool JT359 Jet Chef 1000W). A set of slides were incubated at 18 hours at room temperature at a dilution of 1:100. 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 tumor cores were evaluated by A.A. and an expert pathologist blinded to the clinicopathologic characteristics of patients. Whole field inspection of the core was scored, and intensities of nuclear and cytoplasmic 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. Not all cores within the TMA were suitable for IHC analysis as some cores were missing or lacked tumor (< 15% tumor). As our data were non-parametric, we used the median cutoff to dichotomize H score expression of WRN into low and high expression. A median H score of $\geq$ 116 was taken as the cutoff for high WRN nuclear expression, and a median H-score of $\geq$ 20 was taken as the cutoff for high WRN cytoplasmic expression.

Statistical Analysis

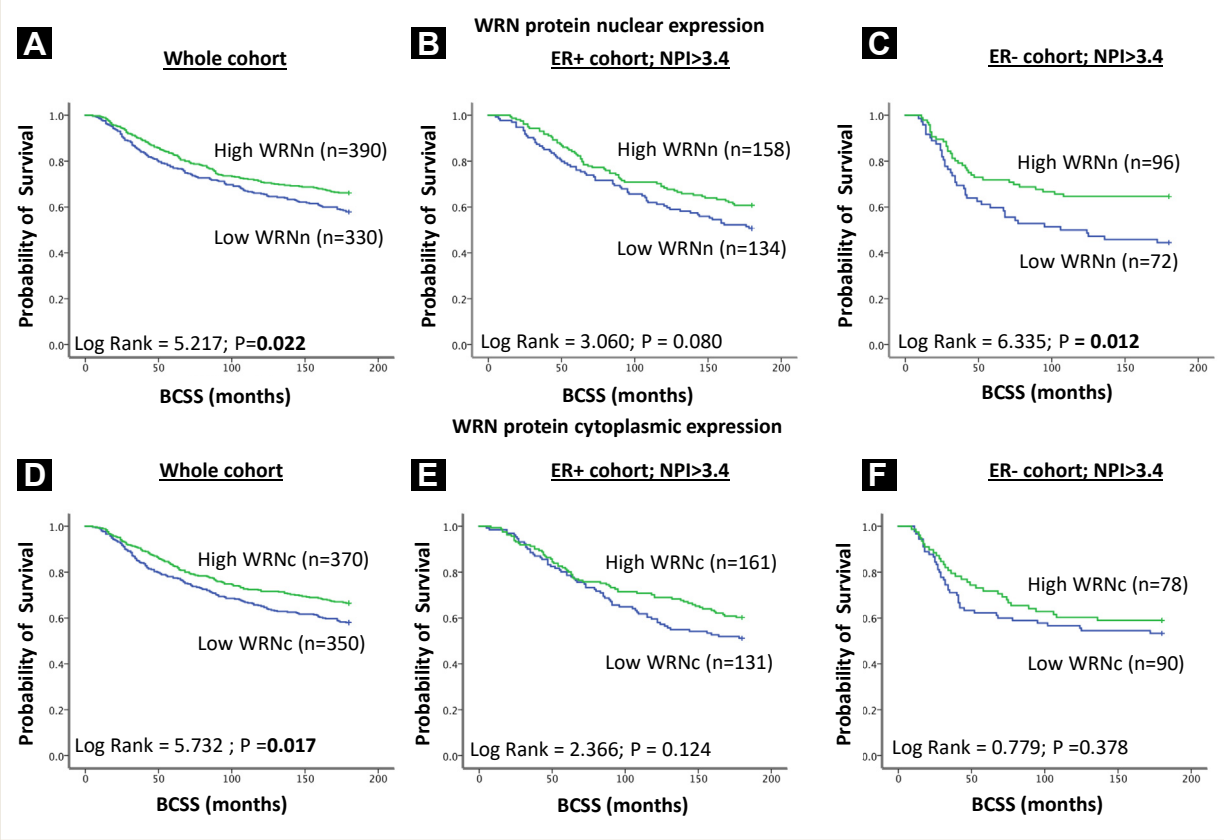
Data analysis was performed using SPSS (SPSS, version 22; Chicago, IL). Where appropriate, the Pearson χ^2, Fisher exact, Student *t*, and 1-way analysis of variance tests were used. Cumulative survival probabilities were estimated using the Kaplan-Meier method, and differences between survival rates 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 (HRs) and 95% confidence intervals (95% CIs) were estimated for each variable. All tests were 2-sided with a 95% CI and a *P* value < .05 considered significant. For multiple comparisons, *P* values were adjusted according to Benjamini and Hochberg multiple *P* value adjustment method.[21]

Results

WRN Protein Expression in Breast Cancer

We initially assessed WRN protein expression in a panel of breast cancer cell lines to confirm the specificity of antibodies for IHC in

Figure 2 Kaplan-Meier Curves Showing BCSS in WRN Nuclear (WRNn) and Cytoplasmic (WRNc) Expression at Protein Level in the Nottingham Tenovus Primary Breast Carcinoma Series



Abbreviations: BCSS = breast cancer-specific survival; ER = estrogen receptor; NPI = Nottingham Prognostic Index; WRN = Werner protein.

the current study. As shown in Figures 1A and 1B, the anti-WRN antibody was not only specific but also showed that MCF-7, MDA-MB231, and MDA-MB-468 have a robust expression of WRN protein. In contrast, MDA-MB-436 has the least WRN expression. We then proceeded to evaluate WRN protein levels in clinical breast carcinoma samples.

WRN Nuclear Expression in Tumor Tissue is Lower Than Normal Breast Tissue

We also evaluated the expression of WRN protein in 20 tumor-associated normal breast tissue slides. We observed both nuclear and cytoplasmic localization of WRN in normal and tumor breast tissue. We also observed that WRN nuclear expression was lower in tumor tissue (mean H-score, 116) as compared with normal breast tissue (mean H score, 220). For WRN cytoplasmic expression, the mean H score in tumor tissue was 20 as compared with a mean H score of 170 in normal breast tissue (Figures 1C1-2 and 1D1-4).

WRN Protein Expression is Associated With Low Levels of DNA Repair Proteins in Breast Cancer

A total of 933 sporadic tumors were suitable for WRN nuclear and 954 sporadic tumors for WRN cytoplasmic protein expression analyses. When we correlated WRN nuclear expression with other DNA repair proteins and regulators, low nuclear WRN was significantly associated with low KU70/KU80 levels ($P = .039$), low DNA PKc ($P = .019$), and low DNA Pol-b ($P = .019$). In addition, reduced WRN protein expression was associated with low CDK18 levels ($P = .015$). There was also a strong association with low expression levels of other DNA helicases, such as nuclear and cytoplasmic RECQL4 (P values $< .05$) and RECQL5 ($P = .013$). In addition, when we correlated WRN cytoplasmic expression with other DNA repair proteins and regulators, low nuclear WRN cytoplasmic was significantly associated with low DNA PKc ($P = .039$) and low nuclear BLM ($P = .026$). Nevertheless, there was no statistically significant association between WRN nuclear or cytoplasmic expression and clinicopathologic features in breast cancer (Tables 1 and 2).

A total of 70 BRCA-mutated tumors were suitable for WRN nuclear and WRN cytoplasmic protein expression analyses. Similarly to the sporadic breast tumors, there was no evidence of association between WRN protein expression and clinicopathologic parameters in the BRCA-mutated cohort (see Supplemental Tables 3 and 4 in the online version).

WRN Nuclear and Cytoplasm Co-Expression is Associated With Low Levels of DNA Repair Proteins in Breast Cancer. A total of 954 sporadic tumors were suitable for WRN nuclear and cytoplasmic protein

Table 1 Werner Nuclear Protein Expression in Sporadic Breast Cancer

	Werner Nuclear Protein Expression		*P* Value	
	Low, N (%)	High, N (%)	Unadjusted	Adjusted
Pathologic parameters				
Tumor size, cm[a]			.740	.848
< 1 (T1a+b)	7 (5.9)	13 (8.5)		
> 1-2 (T1c)	50 (42.4)	62 (40.5)		
> 2-5 (T2)	60 (50.8)	75 (49)		
> 5 (T3)	1 (0.8)	3 (2.0)		
Tumor stage			.330	.585
1	53 (44.9)	64 (41.8)		
2	48 (40.7)	74 (48.4)		
3	17 (14.4)	15 (9.8)		
Tumor grade[b]			.642	.807
G1	6 (5.1)	5 (3.3)		
G2	49 (41.5)	70 (45.8)		
G3	63 (53.4)	78 (51)		
Mitotic index			.785	.850
M1 (low; mitoses < 10)	33 (28.4)	47 (30.9)		
M2 (medium; mitoses 10-18)	30 (25.9)	42 (27.6)		
M3 (high; mitosis > 18)	53 (45.7)	63 (41.4)		
Tubule formation			.401	.680
1 (> 75% of definite tubule)	1 (0.9)	2 (1.3)		
2 (10%-75% definite tubule)	40 (34.5)	41 (27.0)		
3 (< 10% definite tubule)	75 (64.7)	109 (71.7)		
Pleomorphism			.462	.667
1 (small-regular uniform)	1 (0.9)	1 (0.7)		
2 (moderate variation)	42 (36.2)	66 (43.7)		
3 (marked variation)	73 (62.9)	84 (55.6)		
Tumor type			.730	.862
IDC-NST	70 (59.8)	86 (56.6)		
Tubular carcinoma	19 (16.2)	22 (14.1)		
Medullary carcinoma				
ILC	19 (16.2)	33 (21.7)		
Others				
Mixed NST/lobular/special type	9 (7.7)	11 (7.2)		
Lymph node status			.530	.712
Negative	51 (44.7)	64 (42.7)		
Positive (1-3)	50 (43.9)	74 (49.3)		
Positive (> 3)	13 (11.4)	12 (8.0)		
Aggressive phenotype				
HER2 overexpression			.525	.731
No	105 (91.3)	141 (93.4)		
Yes	10 (8.7)	10 (6.6)		
Triple negative				
No	103 (87.3)	123 (80.4)		
Yes	15 (12.7)	30 (19.6)	.130	.298
NPI				
≤ 3.4	7 (6.5)	7 (4.8)		
> 3.4	100 (93.5)	140 (95.2)	.539	.700

Table 1 Continued

	Werner Nuclear Protein Expression		*P* Value	
	Low, N (%)	High, N (%)	Unadjusted	Adjusted
Hormone receptors				
ER				
Negative	110 (26.3)	139 (27)		
Positive	309 (73.7)	375 (73)	.786	.828
PgR				
Negative	34 (29.6)	33 (22.6)		
Positive	81 (70.4)	113 (77.4)	.201	.392
DNA repair proteins				
ATM			.439	.658
Low	139 (53.9)	160 (50.6)		
High	119 (46.1)	156 (49.4)		
ATR (Nuclear)			**.025**	.886
Low	75 (59.1)	102 (46.6)		
High	52 (40.9)	117 (53.4)		
RAD51 (Cytoplasmic)				
Low	14 (5.8)	16 (5.8)		
High	228 (94.2)	259 (94.2)	.987	38.49
RAD51 (Nuclear)			**.033**	.107
Low	139 (57.2)	132 (47.8)		
High	104 (42.8)	144 (52.2)		
BRCA1			.175	.359
Low	55 (17.5)	57 (13.8)		
High	260 (82.5)	356 (86.2)		
PARP1			.413	.671
Low	173 (53.1)	196 (50.0)		
High	153 (46.9)	196 (50.0)		
KU70/KU80			**<.001**	**.039**
Low	62 (23.3)	34 (11.3)		
High	204 (76.7)	267 (88.7)		
DNA PKc			**.001**	**.019**
Low	50 (22.0)	37 (11.9)		
High	202 (78.0)	274 (88.1)		
ERCC1			.155	.335
Low	100 (50.3)	107 (43.5)		
High	99 (49.7)	139 (56.5)		
XRCC1			**.053**	.137
Low	62 (19.6)	50 (14.0)		
High	255 (80.4)	307 (86.2)		
SMUG			.707	.861
Low	143 (50.2)	167 (51.7)		
High	142 (49.8)	156 (48.3)		
DNA Pol-B				
Low	78 (22.3)	55 (11.9)		
High	272 (77.7)	346 (86.3)	**.002**	**.019**
FEN1 (Cytoplasmic)			**.960**	**1.00**
Low	158 (51.6)	179 (51.4)		
High	148 (48.4)	169 (48.6)		

Table 1 Continued

	Werner Nuclear Protein Expression		*P* Value	
	Low, N (%)	High, N (%)	Unadjusted	Adjusted
FEN1 (Nuclear)				.834
Low	225 (73.5)	252 (72.4)		
High	81 (26.5)	96 (27.6)	.749	
Cell cycle and apoptosis regulators				
P53			.417	.650
Low	287 (69.7)	367 (72.1)		
High	125 (30.3)	142 (27.9)		
CDK18			**.002**	**.015**
Low	171 (56.6)	154 (44.3)		
High	131 (43.4)	194 (55.7)		
Chk1 (Cytoplasmic)			**.024**	.093
Low	169 (41.7)	170 (34.4)		
High	236 (58.3)	324 (65.6)		
Chk1 (Nuclear)			.218	.404
Low	346 (85.4)	407 (82.4)		
High	59 (14.6)	87 (17.6)		
CHK2			**.035**	.105
Low	63 (26.6)	56 (18.9)		
High	174 (73.4)	240 (81.1)		
RECQL5 (Nuclear)			**<.001**	**.013**
Low	175 (56.6)	142 (39.1)		
High	134 (43.4)	221 (60.9)		
RECQL4 (Nuclear)			**.010**	**.048**
Low	177 (66.6)	179 (56.1)		
High	89 (33.5)	140 (43.9)		
RECQL4 (Cytoplasmic)			**.002**	**.013**
Low	146 (55.1)	134 (42.3)		
High	119 (44.9)	183 (57.7)		
RECQL1			**.037**	.103
Low	137 (54.8)	128 (45.7)		
High	113 (45.2)	152 (54.3)		
BLM (Nuclear)			.118	.287
Low	100 (29.8)	90 (24.5)		
High	236 (70.2)	277 (75.5)		
BLM (Cytoplasmic)			**.017**	.073
Low	255 (75.9)	246 (67.8)		
High	81 (24.1)	117 (32.2)		
C-MYC			.845	.867
Low	140 (48.1)	131 (47.3)		
High	151 (51.9)	146 (52.7)		

Bold indicates statistically significant.
Unadjusted *P* values were calculated using the Pearson χ^2 test. The Fisher exact test was used to obtain *P* values where one or more of cells has an expected frequency of 5 or less. Adjusted *P* values were calculated using the Benjamini-Hochberg false discovery rate method to adjust for multiple testing.
Abbreviations: ER = estrogen receptor; HER2 = human epidermal growth factor receptor 2; IDC-NST = invasive carcinoma of no special type; ILC = invasive lobular carcinoma; NPI = Nottingham Prognostic Index; PgR = progesterone receptor; WRN = Werner protein.
[a]Tumor size as defined by TNM Classification of Malignant Tumours (8th edition).
[b]Grade as defined by Nottingham Grading System.

Table 2 Werner Cytoplasmic Protein Expression in Sporadic Breast Cancer

	Werner Cytoplasmic Protein Expression		*P* Value	
	Low, N (%)	High, N (%)	Unadjusted	Adjusted
Pathologic parameters				
Tumor size, cm[a]			.432	.673
< 1 (T1a+b)	52 (11.2)	61 (12.4)		
> 1-2 (T1c)	234 (50.4)	233 (47.6)		
> 2-5 (T2)	175 (37.7)	188 (38.4)		
> 5 (T3)	3 (0.6)	8 (1.6)		
Tumor stage			.335	.593
1	300 (64.7)	304 (61.9)		
2	120 (25.9)	147 (29.9)		
3	44 (9.5)	40 (8.1)		
Tumor grade[b]			.330	.612
G1	83 (17.9)	84 (17.1)		
G2	149 (32.1)	180 (36.7)		
G3	232 (50.0)	227 (46.2)		
Mitotic index			.213	.639
M1 (low; mitoses < 10)	158 (35.7)	191 (39.7)		
M2 (medium; mitoses 10-18)	72 (16.3)	87 (18.1)		
M3 (high; mitosis > 18)	212 (48.0)	203 (42.2)		
Tubule formation			.926	.976
1 (> 75% of definite tubule)	24 (5.4)	28 (5.8)		
2 (10%-75% definite tubule)	140 (31.7)	156 (32.4)		
3 (< 10% definite tubule)	278 (62.9)	297 (61.7)		
Pleomorphism			.123	.533
1 (small-regular uniform)	15 (3.4)	8 (1.7)		
2 (moderate variation)	166 (37.7)	202 (42.2)		
3 (marked variation)	259 (58.9)	269 (56.2)		
Tumor type			.079	.385
IDC-NST	261 (57.1)	269 (55.6)		
Tubular carcinoma	92 (20.1)	108 (22.3)		
Medullary carcinoma	18 (3.9)	5 (1.0)		
ILC	55 (12.0)	62 (12.8)		
Others	8 (1.8)	8 (1.7)		
Mixed NST/lobular/special type	23 (5.0)	32 (6.6)		
Lymph node status			.253	.616
Negative	246 (62.6)	285 (62.5)		
Positive (1-3)	113 (28.8)	144 (31.6)		
Positive (>3)	34 (8.7)	27 (5.9)		
Aggressive phenotype				
HER2 overexpression			.266	.546
No	395 (86.2)	422 (88.7)		
Yes	63 (13.8)	54 (11.3)		
Triple negative			.493	.739
No	393 (84.0)	405 (82.3)		
Yes	75 (16.0)	87 (17.7)		
NPI			.986	38.45
≤ 3.4	146 (33.0)	155 (33.0)		
> 3.4	296 (67.0)	315 (67.0)		

Table 2 Continued

	Werner Cytoplasmic Protein Expression		*P* Value	
	Low, N (%)	High, N (%)	Unadjusted	Adjusted
Hormone receptors				
ER				
Negative	129 (28.4)	120 (25.1)		
Positive	326 (71.6)	358 (74.9)	.262	.567
PgR				
Negative	206 (46.7)	184 (40.3)		
Positive	235 (53.3)	273 (59.7)	.051	.331
DNA repair proteins				
ATM				
Low	146 (51.8)	153 (52.4)		
High	136 (48.2)	139 (47.6)	.881	1.01
ATR (Nuclear)			.619	.832
Low	78 (52.7)	99 (50.0)		
High	70 (47.3)	99 (50.0)		
RAD51 (Cytoplasmic)				
Low	15 (5.7)	15 (5.9)		
High	249 (94.3)	238 (94.1)	.904	1.007
RAD51 (Nuclear)			**.002**	.078
Low	156 (58.9)	115 (45.3)		
High	109 (41.1)	139 (54.7)		
BRCA1				
Low	63 (17.9)	49 (13.0)		
High	288 (82.1)	328 (87.0)	.064	.356
PARP1				
Low	189 (51.8)	180 (51.0)		
High	176 (48.2)	173 (49.0)	.832	1.014
KU70/KU80				
Low	54 (18.7)	42 (15.1)		
High	235 (81.3)	236 (84.9)	.256	.587
DNA PKc				
Low	60 (21.5)	34 (11.7)		
High	219 (78.5)	257 (88.3)	**.002**	**.039**
ERCC1				
Low	106 (46.9)	101 (46.1)		
High	120 (53.1)	118 (53.9)	.868	1.025
XRCC1				
Low	58 (17.4)	54 (15.9)		
High	276 (82.6)	286 (84.1)	.605	.842
SMUG1				
Low	79 (25.2)	65 (22.0)		
High	234 (74.8)	230 (78.0)	.353	.598
DNA Pol-B				
Low	72 (19.4)	61 (16.1)		
High	300 (80.6)	318 (83.9)	.242	.629
FEN1 (Cytoplasmic)			.919	.995
Low	173 (51.3)	164 (51.7)		
High	164 (48.7)	153 (48.3)		

Table 2 Continued

	Werner Cytoplasmic Protein Expression		*P* Value	
	Low, N (%)	High, N (%)	Unadjusted	Adjusted
FEN1 (Nuclear)			.504	.728
Low	242 (71.8)	235 (74.1)		
High	95 (28.2)	82 (25.9)		
Cell cycle and apoptosis regulators				
P53			.131	.510
Low	312 (68.7)	342 (73.2)		
High	142 (31.3)	125 (26.8)		
CDK18			.272	.530
Low	169 (52.2)	156 (47.9)		
High	155 (47.8)	170 (52.1)		
Chk1 (Cytoplasmic)			.223	.621
Low	199 (45.3)	190 (41.3)		
High	240 (54.7)	270 (58.7)		
Chk1 (Nuclear)			.957	.982
Low	368 (83.8)	385 (83.7)		
High	71 (16.2)	75 (16.3)		
CHK2			.190	.673
Low	63 (24.8)	56 (20.1)		
High	191 (75.2)	223 (79.9)		
RECQL5 (Nuclear)			.767	.964
Low	159 (47.7)	158 (46.6)		
High	174 (52.3)	181 (53.4)		
RECQL4 (Nuclear)			.763	.991
Low	174 (61.5)	182 (60.3)		
High	109 (38.5)	120 (39.7)		
RECQL4 (Cytoplasmic)			**.014**	.109
Low	150 (53.4)	130 (43.2)		
High	131 (46.6)	171 (56.8)		
RECQL1 (Nuclear)			**.012**	.117
Low	151 (55.3)	114 (44.4)		
High	122 (44.7)	143 (55.6)		
BLM (Nuclear)			**.002**	**.026**
Low	111 (32.4)	79 (21.9)		
High	232 (67.6)	281 (78.1)		
BLM (Cytoplasmic)			.373	.606
Low	249 (73.2)	252 (70.2)		
High	91 (26.8)	107 (29.8)		
C-MYC			.208	.676
Low	153 (50.2)	118 (44.9)		
High	152 (49.8)	145 (55.1)		

Bold indicates statistically significant.
Unadjusted *P* values were calculated using the Pearson χ^2 test. The Fisher exact test was used to obtain *P* values where one or more of cells has an expected frequency of 5 or less. Adjusted *P* values were calculated using the Benjamini-Hochberg false discovery rate method to adjust for multiple testing.
Abbreviations: ER = estrogen receptor; HER2 = human epidermal growth factor receptor 2; IDC-NST = invasive carcinoma of no special type; ILC = invasive lobular carcinoma; NPI = Nottingham Prognostic Index; PgR = progesterone receptor; WRN = Werner protein.
[a]Tumor size as defined by TNM Classification of Malignant Tumours (8th edition).
[b]Grade as defined by Nottingham Grading System.

Table 3 WRN Nuclear and Cytoplasmic Protein Co-Expression in Sporadic Breast Cancer

	WRN Nuclear and Cytoplasmic Protein Co-Expression				*P* Value	
	WRNn−/WRNc−, N (%)	WRNn+/WRNc−, N (%)	WRNn−/WRNc+, N (%)	WRNn+/WRNc+, N (%)	Unadjusted	Adjusted
Pathologic parameters						
Tumor size, cm[a]					.382	.677
≤ 1 (T1a+b)	26 (9.0)	26 (14.9)	16 (11.3)	45 (12.9)		
> 1-2 (T1c)	144 (49.7)	90 (51.7)	68 (48.2)	165 (47.3)		
> 2-5 (T2)	119 (41.0)	56 (32.2)	54 (38.3)	134 (38.4)		
> 5 (T3)	1 (0.3)	2 (1.1)	3 (2.1)	5 (1.4)		
Tumor stage					.767	.830
1	185 (64.0)	115 (65.7)	84 (59.6)	220 (62.9)		
2	77 (26.6)	43 (24.6)	43 (30.5)	104 (29.7)		
3	27 (9.3)	17 (9.7)	14 (9.9)	26 (7.4)		
Tumor grade[b]					.664	.809
G1	53 (18.3)	30 (17.2)	27 (19.1)	57 (16.3)		
G2	89 (30.7)	60 (34.5)	54 (38.3)	126 (36.0)		
G3	148 (51.0)	84 (48.3)	60 (42.6)	167 (47.7)		
Mitotic index					.470	.789
M1 (low; mitoses < 10)	92 (33.9)	66 (38.6)	61 (43.6)	130 (38.1)		
M2 (medium; mitoses 10-18)	45 (16.6)	27 (15.8)	22 (15.7)	65 (19.1)		
M3 (high; mitosis > 18)	134 (49.4)	78 (45.6)	57 (40.7)	146 (42.8)		
Tubule formation					.981	38.25
1 (>75% definite tubule)	13 (4.8)	11 (6.4)	9 (6.4)	19 (5.6)		
2 (10%-75% definite tubule)	89 (32.8)	51 (29.8)	45 (32.1)	111 (32.6)		
3 (<10% definite tubule)	169 (62.4)	109 (63.7)	86 (61.4)	211 (61.9)		
Pleomorphism					.225	.461
1 (small-regular uniform)	11 (4.1)	4 (2.4)	3 (2.2)	5 (1.5)		
2 (moderate variation)	99 (36.5)	67 (39.6)	65 (46.8)	137 (40.3)		
3 (marked variation)	161 (59.4)	98 (58.0)	71 (51.1)	198 (58.2)		
Tumor type					.639	.859
IDC-NST	163 (56.8)	98 (57.6)	74 (53.6)	195 (56.4)		
Tubular carcinoma	59 (20.6)	33 (19.4)	32 (23.2)	76 (22.0)		
Medullary carcinoma	11 (3.8)	7 (4.1)	1 (0.7)	4 (1.2)		
ILC	33 (11.5)	22 (12.9)	19 (13.8)	43 (12.4)		
Others	4 (1.4)	4 (2.4)	3 (2.2)	5 (1.4)		
Mixed NST/lobular/special type	17 (5.9)	6 (3.5)	9 (6.5)	23 (6.6)		
Lymph node status					.668	.789
Negative	139 (61.0)	107 (64.8)	74 (60.7)	211 (63.2)		
Positive (1-3)	67 (29.4)	46 (27.9)	40 (32.8)	104 (31.1)		
Positive (>3)	22 (9.6)	12 (7.3)	8 (6.6)	19 (5.7)		
Aggressive phenotype						
HER2 overexpression					.641	.833
No	244 (85.6)	151 (87.3)	125 (89.9)	297 (88.1)		
Yes	41 (14.4)	22 (12.7)	14 (10.1)	40 (11.9)		
Triple negative					.599	.898
No	239 (82.4)	154 (86.5)	115 (81.6)	290 (82.6)		
Yes	51 (17.6)	24 (13.5)	26 (18.4)	61 (17.4)		
NPI					.964	1.016
≤ 3.4	90 (32.6)	56 (33.7)	46 (34.6)	109 (32.3)		
> 3.4	186 (67.4)	110 (66.3)	87 (65.4)	228 (67.7)		

Table 3 Continued

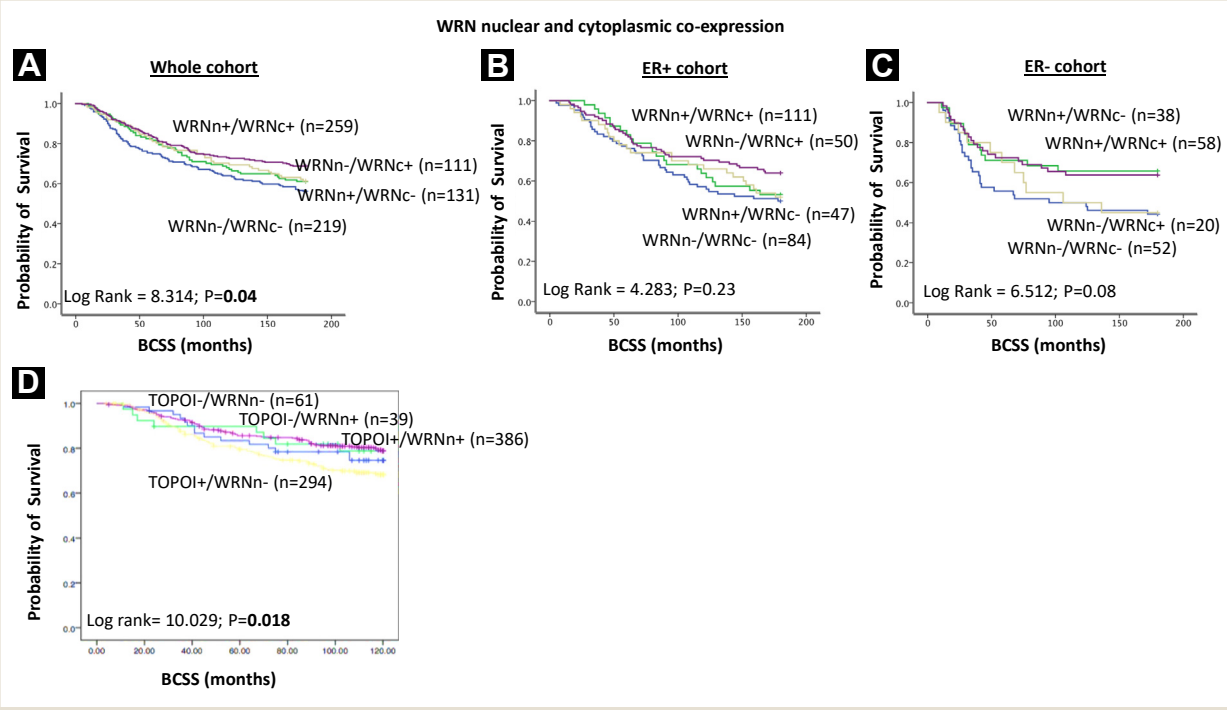
	WRN Nuclear and Cytoplasmic Protein Co-Expression				*P* Value	
	WRNn−/WRNc−, N (%)	WRNn+/WRNc−, N (%)	WRNn−/WRNc+, N (%)	WRNn+/WRNc+, N (%)	Unadjusted	Adjusted
Hormone receptors						
ER						
Negative	77 (27.3)	52 (30.1)	33 (24.1)	87 (25.5)		
Positive	205 (72.7)	121 (69.9)	104 (75.9)	254 (74.5)	.621	.865
PgR					.153	.397
Negative	133 (48.5)	73 (43.7)	51 (37.8)	133 (41.3)		
Positive	141 (51.5)	94 (56.3)	84 (62.2)	189 (58.7)		
DNA repair proteins						
ATM					.610	.881
Low	94 (55.0)	52 (46.8)	45 (51.7)	108 (52.7)		
High	77 (45.0)	59 (53.2)	42 (48.3)	97 (47.3)		
ATR					.142	.395
Low	45 (57.0)	33 (47.8)	30 (62.5)	69 (46.0)		
High	34 (43.0)	36 (52.2)	18 (37.5)	81 (54.0)		
RAD51 (Cytoplasmic)					.677	.776
Low	11 (6.6)	4 (4.1)	3 (3.9)	12 (6.8)		
High	155 (93.4)	94 (95.9)	73 (96.1)	165 (93.2)		
RAD51 (Nuclear)					**.011**	.53
Low	103 (61.7)	53 (54.1)	36 (47.4)	79 (44.4)		
High	64 (38.3)	45 (45.9)	40 (52.6)	99 (55.6)		
BRCA1					.247	.481
Low	40 (19.1)	23 (16.2)	15 (14.2)	34 (12.5)		
High	169 (80.9)	119 (83.8)	91 (85.8)	237 (87.5)		
PARP1					.758	.844
Low	117 (52.0)	72 (51.4)	56 (55.4)	124 (49.2)		
High	108 (48.0)	68 (48.6)	45 (44.6)	128 (50.8)		
Ku70/KU80					**<.001**	**.004**
Low	47 (25.7)	7 (6.6)	15 (18.1)	27 (13.8)		
High	136 (74.3)	99 (93.4)	68 (81.9)	68 (86.2)		
DNA PKc					**<.001**	**.003**
Low	47 (26.7)	13 (12.6)	10 (12.0)	24 (11.5)		
High	129 (73.3)	90 (87.4)	73 (88.0)	184 (88.5)		
ERCC1					.437	.74
Low	71 (51.1)	35 (40.2)	29 (48.3)	72 (45.3)		
High	68 (48.9)	52 (59.8)	31 (51.7)	87 (54.)		
XRCC1					.166	.404
Low	44 (20.7)	14 (11.6)	18 (17.3)	36 (15.3)		
High	169 (79.3)	107 (88.4)	86 (82.7)	200 (84.7)		
SMUG1					.973	.998
Low	100 (50.8)	60 (51.7)	43 (48.9)	107 (51.7)		
High	97 (49.2)	56 (48.3)	45 (51.1)	100 (48.3)		
DNA Pol-B					**.008**	**.05**
Low	58 (23.9)	14 (10.9)	20 (18.7)	41 (15.1)		
High	185 (76.1)	115 (89.1)	87 (81.3)	231 (84.9)		
FEN1 (Nuclear)					.208	.450
Low	154 (70.3)	88 (74.6)	71 (81.6)	164 (71.3)		
High	65 (29.7)	30 (25.4)	16 (18.4)	66 (28.7)		

Table 3 Continued

	WRN Nuclear and Cytoplasmic Protein Co-Expression				*P* Value	
	WRNn−/WRNc−, N (%)	WRNn+/WRNc−, N (%)	WRNn−/WRNc+, N (%)	WRNn+/WRNc+, N (%)	Unadjusted	Adjusted
FEN1 (Cytoplasmic)					.207	.474
Low	119 (54.3)	54 (45.8)	39 (44.8)	125 (54.3)		
High	100 (45.7)	64 (54.2)	48 (55.2)	105 (45.7)		
Cell cycle and apoptosis regulators						
p53					.457	.742
Low	195 (68.9)	117 (68.4)	92 (71.3)	250 (74.0)		
High	88 (31.1)	54 (31.6)	37 (28.7)	88 (26.0)		
CDK18					**.003**	**.029**
Low	124 (59.3)	45 (39.1)	47 (50.5)	109 (46.8)		
High	85 (40.7)	70 (60.9)	46 (49.5)	124 (53.2)		
Chk1 (Cytoplasmic)					.099	.297
Low	118 (43.1)	53 (32.1)	51 (38.9)	117 (35.6)		
High	156 (56.9)	110 (67.9)	80 (61.1)	212 (64.4)		
Chk1 (Nuclear)					.643	.808
Low	233 (85.0)	135 (81.8)	113 (86.3)	272 (82.7)		
High	41 (15.0)	30 (18.2)	18 (13.7)	57 (17.3)		
Chk2					.078	.253
Low	39 (25.5)	24 (23.8)	24 (28.6)	32 (16.4)		
High	114 (74.5)	77 (76.2)	60 (71.4)	163 (83.6)		
RECQL5 (Nuclear)					**<.001**	**.001**
Low	118 (55.9)	41 (33.6)	57 (58.2)	101 (41.9)		
High	93 (44.1)	81 (66.4)	41 (41.8)	140 (58.1)		
RECQL4 (Nuclear)					.067	.237
Low	120 (65.9)	54 (53.5)	57 (67.9)	125 (57.3)		
High	62 (34.1)	47 (46.5)	27 (32.1)	93 (42.7)		
RECQL4 (Cytoplasmic)					**.004**	**.031**
Low	107 (59.1)	43 (43.0)	39 (46.4)	91 (41.9)		
High	74 (40.9)	57 (57.0)	45 (53.6)	126 (58.1)		
RECQL1 (Nuclear)					**.039**	.169
Low	99 (56.6)	52 (53.1)	38 (50.7)	76 (41.8)		
High	76 (43.4)	46 (46.9)	37 (49.3)	106 (58.2)		
BLM (Nuclear)					**.008**	**.044**
Low	78 (34.7)	33 (28.0)	22 (19.8)	57 (22.9)		
High	147 (65.3)	85 (72.0)	89 (80.2)	192 (77.1)		
BLM (Cytoplasmic)					**.05**	.195
Low	174 (77.7)	75 (64.7)	81 (72.3)	171 (69.2)		
High	50 (22.3)	41 (35.3)	31 (27.7)	76 (30.8)		
C-MYC					.372	.690
Low	105 (51.5)	48 (47.5)	35 (40.2)	83 (47.2)		
High	99 (48.5)	53 (52.5)	52 (59.8)	93 (52.8)		

Abbreviations: ER = estrogen receptor; HER2 = human epidermal growth factor receptor 2; IDC-NST = invasive carcinoma of no special type; ILC = invasive lobular carcinoma; NPI = Nottingham Prognostic Index; PgR = progesterone receptor; WRN = Werner protein.
Bold indicates statistically significant.
Unadjusted *P* values were calculated using the Pearson χ^2 test. The Fisher exact test was used to obtain *P* values where one or more of cells has an expected frequency of 5 or less. Adjusted *P* values were calculated using the Benjamini-Hochberg false discovery rate method to adjust for multiple testing.
[a]Tumor size as defined by TNM Classification of Malignant Tumours (8th edition).
[b]Grade as defined by Nottingham Grading System.

Figure 3 Kaplan-Meier Curves Showing BCSS in WRN Nuclear (WRNn) and Cytoplasmic (WRNc) Co-Expression (A-C) and BCSS in WRN Nuclear (WRNn) and TOPOI Nuclear Co-expression at Protein Level (D), in the Nottingham Tenovus Primary Breast Carcinoma Series

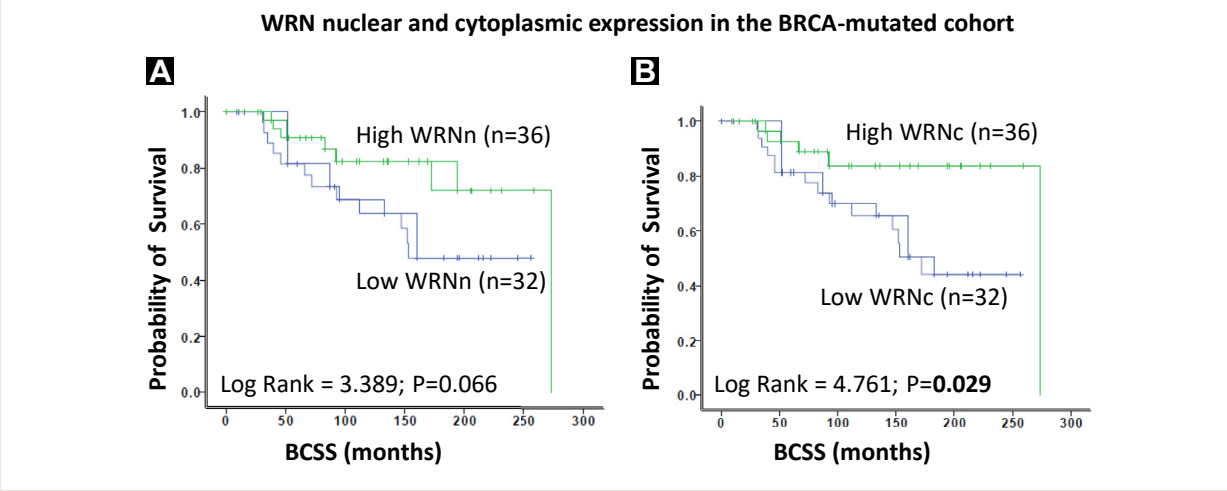


Abbreviations: BCSS = breast cancer-specific survival; TOPOI = topoisomerase I; WRN = Werner protein.

co-expression analyses. Of these tumors, 15.4% had low nuclear/high cytoplasmic expression, 30.4% had low nuclear/low cytoplasmic expression, 36% had high nuclear/high cytoplasmic expression, and 18.2% demonstrated high nuclear/low cytoplasmic expression.

When we combined the nuclear and cytoplasmic expression of WRN protein, low cytoplasmic and nuclear co-expression of WRN protein was statistically associated with an aggressive molecular phenotype. Specifically, there was strong evidence of association

Figure 4 Kaplan-Meier Curves Showing BCSS in WRN Nuclear (WRNn) and Cytoplasmic (WRNc) Expression at Protein Level in the BRCA-Mutated Cohort



Abbreviations: BCSS = breast cancer-specific survival; WRN = Werner protein.

Table 4 Cox Proportional Hazard Multivariate Analysis of WRN Protein Expression in Sporadic Breast Cancer

Breast cancer-specific survival	*P* Value	Exp (B)	95% CI of Exp (B)	
			Lower	Upper
Stage	**<.001**	1.954	1.442	2.649
Grade	**<.001**	2.473	1.636	3.738
HER2 overexpression	.242	1.313	0.832	2.073
NPI	.531	0.793	0.383	1.639
WRN (Cytoplasmic)	**.039**	0.677	0.467	0.980
WRN (Nuclear)	**.032**	0.672	0.466	0.967
TOPO I (Nuclear)	.270	1.234	0.849	1.794

Abbreviations: CI = confidence interval; HER2 = human epidermal growth factor receptor 2; NPI = Nottingham Prognostic Index; TOPO I = topoisomerase I; WRN = Werner protein.
Bold indicates statistically significant.

between low WRN nuclear and cytoplasmic co-expression and low levels of KU70/KU80 ($P = .004$), DNA-PK ($P = .003$), DNA Pol-B ($P = .05$), CKD18 ($P = .029$), cytoplasmic RECQL4 ($P = .031$), and nuclear BLM protein expression ($P = .044$) (Table 3).

These results suggest that low WRN protein expression is associated with low levels of DNA repair and cell cycle regulation in patients with breast cancer.

Low WRN Protein Expression is Associated With Worse BCSS

In univariate analysis, patients whose tumor had low WRN nuclear expression had significantly ($P = .02$) worse overall BCSS in the Nottingham Tenovus Primary Breast Carcinoma series (Figure 2A). Furthermore, a statistically significant worse BCSS was observed in the ER-negative cohort ($P = .012$) (Figure 2C). Tumors with low WRN cytoplasmic expression also demonstrated poor BCSS, which was statistically significant ($P = .017$). We then evaluated the impact of WRN nuclear and cytoplasmic co-expression on BCSS. In the whole cohort, patients with low nuclear/low cytoplasmic WRN expression had poor BCSS ($P = .04$), suggesting that low expression has prognostic significance (Figure 3A). The impact of WRN protein expression was also evaluated in the BRCA-mutated cohort, where low WRN cytoplasmic expression conferred the shortest BCSS ($P = .029$) (Figure 4B). Although low WRN nuclear expression showed a trend towards worse BCSS, this was not statistically significant ($P = .066$) (Figure 4A).

Low WRN and High TOPOI Co-expression is Associated With Poor BCSS

As discussed previously, WRN interacts with TOPOI and enhances the ability of TOPOI to relax the supercoiled DNA. In WRN nuclear and TOPOI nuclear co-expression analysis, tumors with low WRN nuclear expression and high TOPOI expression had poor BCSS in the whole cohort (Figure 3D).

WRN Nuclear and Cytoplasmic Expression are Independent Predictors of BCSS

In multivariate analysis, WRN nuclear and cytoplasmic expression were independent prognostic factors for BCSS ($P = .039$ and $P = .032$, respectively). Tumor stage and grade were also independently associated with BCSS (Table 4).

Discussion

WRN is the largest family member of the human RECQ helicase protein. WRN is the only DNA RECQ helicase that contains a nuclease domain and catalyzes DNA-dependent reactions. WRN acts on various DNA structures to help with DNA repair through its enzymatic functions. Although WS cells demonstrate compromised survival after exposure to replication stress, certain cells with chromosomal abnormalities enter cell replication that increases the degree of mutations and, consequently, the level of genomic instability in survival cells, leading to malignant transformation.[12]

Germline mutations in WRN lead to defects in DNA repair, premature aging, and cancer susceptibility.[22-24] Genetic epidemiologic studies identified certain polymorphisms of the WRN gene that are associated with increased risk of breast cancer.[25-27] Specifically, the CC genotype of WRN rs1346044 has been associated with a 2-fold risk of developing breast cancer.[27] In addition, a meta-analysis evaluated 7 epidemiologic studies and demonstrated that the CC genotype of Cys1367Arg polymorphism was also associated with a 1.43-times increased risk of breast cancer.[25] A case-control study in Chinese women that included approximately 4000 patients also showed that the variant genotype of WRN Leu1074Phe was associated with a 1.36-times higher risk of breast cancer.[26]

We have previously shown, at a transcriptomic level, that low WRN mRNA expression was associated with aggressive clinicopathologic features such as high grade, lymph node stage, and human epidermal growth factor receptor 2 (HER2) overexpression and distinct aggressive molecular phenotypes as described by Curtis et al,[28] including PAM50.Her2, PAM50.LumB, Genufu subtype ($ER^+/HER2^-$/high proliferation), and Genufu subtype ($HER2^+$) breast tumors.[29] Low WRN mRNA level was also associated with poor BCSS.[29] At the protein level, we observed complex staining patterns, with tumors showing negative, nuclear, and/or cytoplasmic WRN staining. Similar to the WRN mRNA expression data,[29] low cytoplasmic and low nuclear WRN protein levels were associated with poor BCSS. However, low WRN protein expression was not significantly linked to clinicopathologic characteristics. The mechanism of regulation of WRN expression is not clearly understood. It has been previously shown that epigenetic inactivation of WRN is common in solid tumors, with the highest frequency in colorectal cancer (37.9%; 69/182) and a

prevalence of 17.2% (10/58) in breast tumors.[30] Nevertheless, the authors did not describe any clinicopathologic associations in this study. In our previous study,[29] we have found that WRN mRNA expression level was low in 326 (16.5%) of 1977 breast tumors, which is in accordance with the findings of the study from Agrelo et al.[30]

Wild type WRN protein contains a nuclear localization signal motif in the region of C-terminus.[31] This C-terminal sequence, which is often lost as a result of mutations, is necessary for nuclear localization of the WRN protein.[31] Most of the mutations of the WS gene, which are found in its coding region, lead to instability of WRN mRNA and truncation of the protein with loss of the nuclear localization signal.[32] Impaired nuclear import probably constitutes an important contributing factor in the development of WS.[31]Furthermore, the transcriptional status of the cells determines the localization of WRN protein.[33] WRN protein is accumulated in the nucleoli when rRNA transcription is stimulated, whereas the lack of transcriptional activity when cells enter G0 or during late G2 phase of the cell cycle leads to the release of WRN protein from the nucleoli to nucleoplasm.[33] During the metaphase, WRN protein is released from the condensed chromatic to the cytoplasm.[34] Moreover, WRN CpG island promoter hypermethylation undergoes hypermethylation in human cancer cells, leading to loss of WRN protein expression and hypersensitivity to topoisomerase inhibitors and DNA-damaging agents.[30] These findings suggest that mutations or epigenetic silencing may explain the different localization of WRN protein, which plays a key role in DNA damage response.

Our findings indicate that low WRN expression in human tumors may lead to a 'mutator phenotype' expressed as aggressive breast cancers. Inactivation of WRN protein makes tumor cells susceptible to TOPO I poison and DNA-damaging agents. Cellular senescence is increased in WRN-deficient cells, in the presence of constant DNA damage and after treatment with chemotherapeutic agents such as camptothecin.[35-37] In colorectal tumors, hypermethylation of WRN promoter CpG island was correlated with good response and better overall survival after treatment with irinotecan.[30] Specifically, WRN knockdown and camptothecin treatment both induce DNA damage and cause increased p21 expression and SA-β-gal activity in colon cancer.[35] On the other hand, the rescue of WRN in tumor cells treated with camptothecin enhanced the efficiency of DNA damage response to eliminate cytotoxic DNA lesions.[29]

There is evidence to suggest that RECQ helicases cooperate with each other to perform their vital biological roles so there may be functional redundancy between them.[38-41] Hence, we looked at correlation between WRN and RECQL1, RECQL4, RECQL5, and BLM helicases that showed a significant correlation between low nuclear RECQL4 and high nuclear WRN ($P < .05$). Further analysis among the 5 helicases demonstrated a significant correlation between low RECQL1 nuclear and high nuclear BLM ($P < .05$); low nuclear RECQL4 and high nuclear BLM ($P < .05$), and low nuclear RECQL5 and high nuclear BLM ($P < .05$) (see Supplemental Table 5 in the online version). These findings suggest that there is a possibility that low expression of one helicase in breast cancer might lead to compensatory increase in the expression of another helicase.

In view of the interaction between WRN and TOPO I, we carried out combined WRN and TOPO I analysis and showed that low WRN nuclear expression in TOPO I-overexpressed tumors is associated with worse BCSS. This is consistent with our previously published data at the mRNA level, where we demonstrated that low WRN expression in TOPO I-high tumors is associated with poor BCSS in the whole cohort.[29] TOPO I plays a vital role during replication and proliferation. As WRN is involved in various DNA repair pathways, it is possible that it promotes the DNA repair ability of established tumor cells to withstand DNA damage induced by endogenous and exogenous agents. A recent study identified NSC 19630 as a specific inhibitor of WRN, which synergistically inhibited cell proliferation and induced DNA damage with topotecan.[42]

Targeting DNA helicases for therapeutic purposes has gained interest with the development of other DNA repair inhibitors, such as poly (ADP) ribosylase (PARP) inhibitors used in synthetic lethality approaches to control carcinogenesis in homologous recombination-defective BRCA1/2-deficient tumors. A recent study that performed genome-scale CRISPR-Cas9 screen in 324 human cancer cell lines from 30 tumor types identified WRN as a potential synthetic lethality target for cancers with microsatellite instability.[43] Hence, we evaluated the prognostic impact of WRN expression in BRCA-mutated tumors that showed that low WRN expression is associated with worse BCSS. WRN and BRCA1 facilitate DNA damage response in a coordinated manner, as BRCA1 binds directly to WRN by stimulating its exonuclease and helicase activity.[44] This interaction is enhanced in HeLa cells exposed to DNA cross-linking agents, where WRN participates in the DNA repair via its helicase activity.[44] PARP1 inhibits both WRN exonuclease and helicase activities, an interaction that is influenced by the poly (ADP-ribosyl)ation status of PARP1.[45] WS cells are deficient in the poly (ADP-ribosyl)ation pathway after they are treated with agents that induce oxidative stress and DNA alkylation.[46] Further understanding of the functional interaction between WRN and PARP1 in BRCA-mutated tumors may lead to novel therapeutic approaches. Given the role of RECQ helicases in homologous recombination, it will be important to study the possibility that RECQ helicases could have a synthetic lethality relation with PARP inhibitors or other DNA repair inhibitors such as ATM/WEE1, which are currently under wide investigation in clinical trials.

In conclusion, we provide evidence that WRN protein expression can influence molecular phenotype and clinical outcomes in patients with breast cancer. We have also shown the prognostic significance of low WRN expression in TOPO I-overexpressed tumors as these patients might benefit from TOPO I poisons.

Clinical Practice Points

- WRN protein is a DNA helicase involved in genomic stability and commonly inactivated in breast tumors.
- We showed that low WRN protein expression is associated with worse survival and aggressive molecular phenotype in patients with sporadic breast cancer.
- WRN expression in TOPO I-overexpressed tumors is also associated with poor survival, indicating that these patients might benefit from TOPO I poisons.
- Low WRN expression is associated with worse BCSS in BRCA-mutated breast tumors. Further understanding of the functional interaction between WRN and PARP1 in BRCA-mutated tumors may lead to novel therapeutic approaches.
- These findings can be used to optimize the risk stratification for personalized treatment.

Acknowledgments

The authors thank the Nottingham Health Science Biobank and Breast Cancer Now Tissue Bank for the provision of tissue samples.

Disclosure

The authors have stated that they have no conflicts of interest.

Ethical Approval

Ethical approval was obtained from the Nottingham Research Ethics Committee (Reference number C202313). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent

All data were anonymized, and all personal identifiers were removed.

Supplemental Data

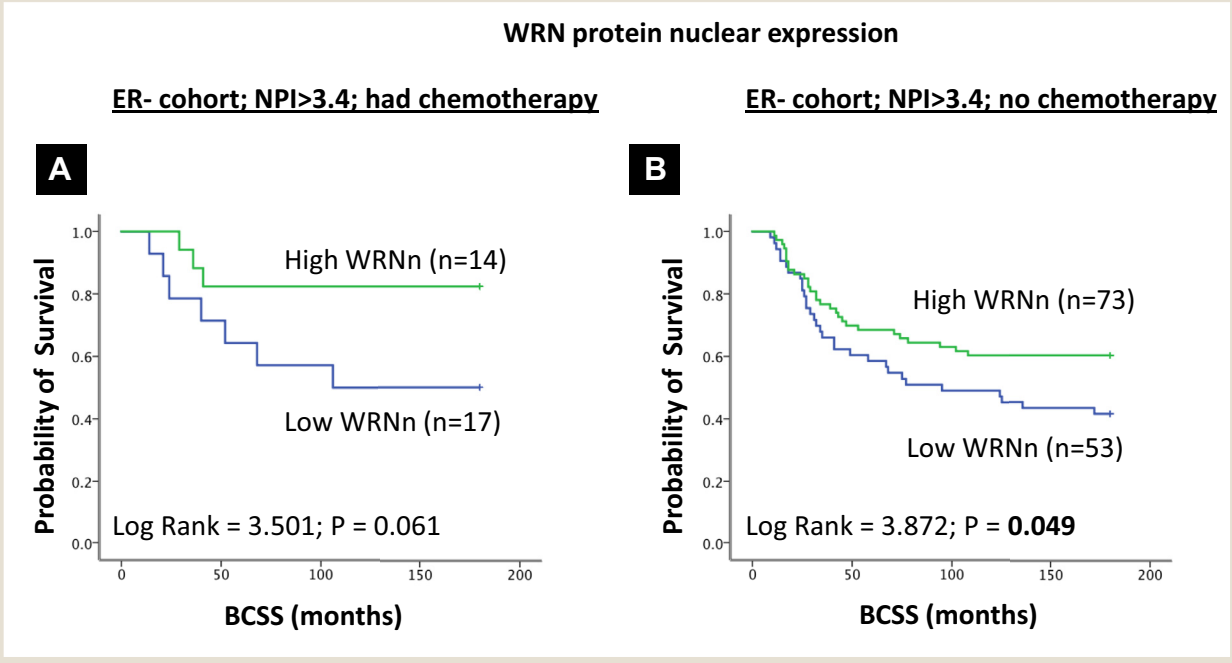
Supplemental tables and figures accompanying this article can be found in the online version at https://doi.org/10.1016/j.clbc.2020.07.013.

References

1. Bohr VA. Rising from the RecQ-age: the role of human RecQ helicases in genome maintenance. *Trends Biochem Sci* 2008; 33:609-20.
2. Croteau DL, Popuri V, Opresko PL, Bohr VA. Human RecQ helicases in DNA repair, recombination, and replication. *Annu Rev Biochem* 2014; 83:519-52.
3. Goto M, Rubenstein M, Weber J, Woods K, Drayna D. Genetic linkage of Werner's syndrome to five markers on chromosome 8. *Nature* 1992; 355:735-8.
4. Schellenberg GD, Martin GM, Wijsman EM, Nakura J, Miki T, Ogihara T. Homozygosity mapping and Werner's syndrome. *Lancet* 1992; 339:1002.
5. Harrigan JA, Wilson DM 3rd, Prasad R, et al. The Werner syndrome protein operates in base excision repair and cooperates with DNA polymerase beta. *Nucleic Acids Res* 2006; 34:745-54.
6. Thompson LH, Schild D. Recombinational DNA repair and human disease. *Mutat Res* 2002; 509:49-78.
7. Laine JP, Opresko PL, Indig FE, Jarrigan JA, von Kobbe C, Bohr VA. Werner protein stimulates topoisomerase I DNA relaxation activity. *Cancer Res* 2003; 63:7136-46.
8. Lauper JM, Krause A, Vaughan TL, Monnat RJ Jr. Spectrum and risk of neoplasia in Werner syndrome: a systematic review. *PLoS One* 2013; 8:e59709.
9. Shen JC, Loeb LA. The Werner syndrome gene: the molecular basis of RecQ helicase-deficiency diseases. *Trends Genet* 2000; 16:213-20.
10. van Brabant AJ, Stan R, Ellis NA. DNA helicases, genomic instability, and human genetic disease. *Annu Rev Genomics Hum Genet* 2000; 1:409-59.
11. Yu CE, Oshima J, Wijsman EM, et al. Mutations in the consensus helicase domains of the Werner syndrome gene. Werner's Syndrome Collaborative Group. *Am J Hum Genet* 1997; 60:330-41.
12. Mukherjee S, Sinha D, Bhattacharya S, Srinivasan K, Abdisalaam S, Asaithamby A. Werner syndrome protein and DNA replication. *Int J Mol Sci* 2018; 19:3442.
13. Opresko PL, Calvo JP, von Kobbe C. Role for the Werner syndrome protein in the promotion of tumor cell growth. *Mech Ageing Dev* 2007; 128:423-36.
14. Sharma S, Doherty KM, Brosh RM Jr. Mechanisms of RecQ helicases in pathways of DNA metabolism and maintenance of genomic stability. *Biochem J* 2006; 398:319-37.
15. Szekely AM, Bleichert F, Nümann A, et al. Werner protein protects nonproliferating cells from oxidative DNA damage. *Mol Cell Biol* 2005; 25:10492-506.
16. Otterlei M, Bruheim P, Ahn B, et al. Werner syndrome protein participates in a complex with RAD51, RAD54, RAD54B and ATR in response to ICL-induced replication arrest. *J Cell Sci* 2006; 119:5137.
17. Arora A, Abdel-Fatah TMA, Agarwal D, et al. Clinicopathological and prognostic significance of RECQL5 helicase expression in breast cancers. *Carcinogenesis* 2016; 37:63-71.
18. Arora A, Agarwal D, Abdel-Fatah TM, et al. RECQL4 helicase has oncogenic potential in sporadic breast cancers. *J Pathol* 2016; 238:495-501.
19. Savva C, De Souza K, Ali R, Rakha EA, Green AR, Madhusudan S. Clinicopathological significance of ataxia telangiectasia-mutated (ATM) kinase and ataxia telangiectasia-mutated and Rad3-related (ATR) kinase in MYC overexpressed breast cancers. *Breast Cancer Res Treat* 2019; 175:105-15.
20. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM. Statistics Subcommittee of the NCI-EORTC Working Group on Cancer Diagnostics. Reporting recommendations for tumor marker prognostic studies (REMARK). *J Natl Cancer Inst* 2005; 97:1180-4.
21. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc Ser B (Methodological)* 1995; 57:289-300.
22. Chun SG, Yee NS. Werner syndrome as a hereditary risk factor for exocrine pancreatic cancer: potential role of WRN in pancreatic tumorigenesis and patient-tailored therapy. *Cancer Biol Ther* 2010; 10:430-7.
23. Ding SL, Yu JC, Chen ST, Hsu GC, Shen CY. Genetic variation in the premature aging gene WRN: a case-control study on breast cancer susceptibility. *Cancer Epidemiol Biomarkers Prev* 2007; 16:263-9.
24. Chun SG, Shaeffer DS, Bryant-Greenwood PK. The Werner's syndrome RecQ helicase/exonuclease at the nexus of cancer and aging. *Hawaii Med J* 2011; 70:52-5.
25. Wang B, Li G, Sun F, Dong N, Sun Z, Jiang D. Association between WRN Cys1367Arg (T>C) and cancer risk: a meta-analysis. *Technol Cancer Res Treat* 2014; 15:20-7.
26. Wang Z, Xu Y, Tang J, et al. A polymorphism in Werner syndrome gene is associated with breast cancer susceptibility in Chinese women. *Breast Cancer Res Treat* 2009; 118:169-75.
27. Zins K, Frech B, Taubenschuss E, Schneeberger C, Abraham D, Schreiber M. Association of the rs1346044 polymorphism of the Werner syndrome gene RECQL2 with increased risk and premature onset of breast cancer. *Int J Mol Sci* 2015; 16:29643-53.
28. Curtis C, Shah SP, Chin SF, et al. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature* 2012; 486:346-52.
29. Shamanna RA, Lu H, Croteau DL, et al. Camptothecin targets WRN protein: mechanism and relevance in clinical breast cancer. *Oncotarget* 2016; 7:13269-84.
30. Agrelo R, Cheng WH, Setien F, et al. Epigenetic inactivation of the premature aging Werner syndrome gene in human cancer. *Proc Natl Acad Sci U S A* 2006; 103:8822-7.
31. Matsumoto T, Shimamoto A, Goto M, Furuichi Y. Impaired nuclear localization of defective DNA helicases in Werner's syndrome. *Nat Genet* 1997; 16:335-6.
32. Smith JA, Ndoye AMN, Geary K, Lisanti MP, Igoucheva O, Daniel R. A role for the Werner syndrome protein in epigenetic inactivation of the pluripotency factor Oct4. *Aging Cell* 2010; 9:580-91.
33. Suzuki T, Shiratori M, Furuichi Y, Matsumoto T. Diverged nuclear localization of Werner helicase in human and mouse cells. *Oncogene* 2001; 20:2551-8.
34. Shiratori M, Sakamoto S, Suzuki N, et al. Detection by epitope-defined monoclonal antibodies of Werner DNA helicases in the nucleoplasm and their upregulation by cell transformation and immortalization. *J Cell Biol* 1999; 144:1-9.
35. Han Z, Wei W, Dunaway S, et al. Role of p21 in apoptosis and senescence of human colon cancer cells treated with camptothecin. *J Biol Chem* 2002; 277:17154-60.
36. Lu H, Fang EF, Sykora P, et al. Senescence induced by RECQL4 dysfunction contributes to Rothmund-Thomson syndrome features in mice. *Cell Death Dis* 2014; 5:e1226.
37. Rodier F, Coppé JP, Patil CK, et al. Persistent DNA damage signalling triggers senescence-associated inflammatory cytokine secretion. *Nat Cell Biol* 2009; 11:973-9.
38. Popuri V, Huang J, Ramamoorthy M, Tadokoro T, Croteau DL, Bohr VA. RECQL5 plays co-operative and complementary roles with WRN syndrome helicase. *Nucleic Acids Res* 2017; 45:1566.
39. Hu Y, Lu X, Barnes E, Yan M, Lou H, Luo G. Recql5 and Blm RecQ DNA helicases have nonredundant roles in suppressing crossovers. *Mol Cell Biol* 2005; 25:3431-42.
40. Otsuki M, Seki M, Inoue E, et al. Analyses of functional interaction between RECQL1, RECQL5, and BLM which physically interact with DNA topoisomerase IIIalpha. *Biochim Biophys Acta* 2008; 1782:75-81.
41. Singh DK, Popuri V, Kulikowicz T, et al. The human RecQ helicases BLM and RECQL4 cooperate to preserve genome stability. *Nucleic Acids Res* 2012; 40:6632-48.
42. Aggarwal M, Sommers JA, Shoemaker RH, Brosh RM Jr. Inhibition of helicase activity by a small molecule impairs Werner syndrome helicase (WRN) function in the cellular response to DNA damage or replication stress. *Proc Natl Acad Sci U S A* 2011; 108:1525-30.
43. Behan FM, Iorio F, Picco G, et al. Prioritization of cancer therapeutic targets using CRISPR–Cas9 screens. *Nature* 2019; 568:511-6.
44. Cheng WH, Kusumoto R, Opresko PL, et al. Collaboration of Werner syndrome protein and BRCA1 in cellular responses to DNA interstrand cross-links. *Nucleic Acids Res* 2006; 34:2751-60.
45. von Kobbe C, Harrigan JA, Schreiber V, et al. Poly(ADP-ribose) polymerase 1 regulates both the exonuclease and helicase activities of the Werner syndrome protein. *Nucleic Acids Res* 2004; 32:4003-14.
46. von Kobbe C, Harrigan JA, May A, et al. Central role for the Werner syndrome protein/poly(ADP-ribose) polymerase 1 complex in the poly(ADP-ribosyl)ation pathway after DNA damage. *Mol Cell Biol* 2003; 23:8601-13.

Supplemental Data

Supplemental Figure 1 Kaplan-Meier Curves Showing BCSS in WRNn at protein Level Nottingham Tenovus Primary Breast Cancer Series



Abbreviations: BCSS = breast cancer-specific survival; ER⁻ = estrogen receptor-negative; NPI = Nottingham Prognostic Index; WRN = Werner protein; WRNn = WRN nuclear expression.

Supplemental Table 1 Clinicopathologic Characteristics of Nottingham Tenovus Primary Breast Cancer Series

Variable	n[a]	Cases	%
Menopausal status	1650		
Pre-menopausal		612	37.0
Postmenopausal		1038	63.0
Tumor grade (NGS)[b]	1650		
G1		306	18.5
G2		531	32.2
G3		813	49.3
Lymph node stage	1650		
Negative		1056	64.0
Positive (1-3 nodes)		486	29.5
Positive (> 3 nodes)		108	6.5
Tumor size, cm[c]	1650		
T1 a + b (≤ 1.0)		187	11.0
T1 c (> 1.0-2.0)		868	53.0
T2 (> 2.0-5)		579	35.0
T3 (> 5)		16	1.0
Tumor type	1650		
IDC-NST		941	57
Tubular		349	21
ILC		160	10
Medullary (typical/atypical)		41	2.5
Others		159	9.5
NPI subgroups	1650		
Excellent PG (2.08-2.40)	Low risk	207	12.5
Good PG (2.42-3.40)		331	20.1
Moderate I PG (3.42 to 4.4)	High risk	488	29.6
Moderate II PG (4.42 to 5.4)		395	23.9
Poor PG (5.42 to 6.4)		170	10.3
Very poor PG (6.5 to 6.8)		59	3.6
Survival at 20 years	1650		
Alive and well		1055	64.0
Dead from disease		468	28.4
Dead from other causes		127	7.6
Adjuvant systemic therapy			
No adjuvant systemic therapy		665	42.0
Hormone therapy		642	41.0
Chemotherapy		307	20.0
Hormone + chemotherapy		46	3.0

Abbreviations: IDC-NST = invasive carcinoma of no special type; ILC = invasive lobular carcinoma; NPI = Nottingham Prognostic Index; PG = prognostic group.
[a]Number of cases for which data were available.
[b]Grade as defined by Nottingham Grading System.
[c]Tumor size as defined by TNM Classification of Malignant Tumours (8th edition).

Supplemental Table 2 Clinicopathologic Characteristics of the BRCA-Mutated Cohort[a]

Variable	BRCA1-Mutated Cancer (n = 48), N (%)	BRCA2-Mutated Cancer (n = 27), N (%)
Mean age at diagnosis, y (range)	40.66 (26-64)	45 (28-71)
Tumor size, cm		
< 2	24 (50.0)	11 (40.7)
≥ 2	24 (50.0)	16 (59.3)
Tumor type		
Ductal/NST	39 (81.2)	18 (66.7)
Lobular/mixed	1 (2.1)	7 (25.9)
Medullary (typical/atypical)	7 (14.6)	2 (7.4)
Other	1 (2.1)	0
Tumor grade (NGS)[b]		
1	1 (2.1)	0
2	4 (8.3)	7 (24.0)
3	43 (89.6)	20 (74.1)
Stage		
1	30 (65.2)	17 (63.0)
2	15 (32.6)	8 (29.6)
3	1 (2.2)	2 (7.4)
Vascular invasion		
No	29 (63.0)	20 (74.1)
Yes	17 (37.0)	7 (25.9)
NPI subgroups		
Good PG	3 (6.5)	6 (22.2)
Moderate PG	34 (73.9)	15 (55.6)
Poor PG	9 (19.6)	6 (22.2)
Estrogen receptor (ER)		
Negative	39 (83.0)	4 (14.8)
Positive	8 (17.0)	23 (85.2)
HER2		
Negative	45 (95.7)	26 (96.3)
Positive	2 (4.3)	1 (3.7)
Triple negative		
No	15 (32.6)	25 (92.6)
Yes	31 (67.4)	2 (7.4)
Recurrence		
No	41 (85.4)	22 (81.5)
Yes	7 (15.9)	5 (20.8)
Mean survival, mos (range)	121.5 (9-265)	87.3 (13-206)
Alive	33 (75.0)	16 (66.7)
Dead	11 (25.0)	8 (33.3)
Bilateral cancer		
No	33 (75.0)	20 (83.3)
Yes	11 (25.0)	4 (16.7)

Abbreviations: NPI = Nottingham Prognostic Index; PG = prognostic group.
[a]A total of 75 tumor samples from 68 patients with confirmed germline mutations for BRCA1 or BRCA2.
[b]Grade as defined by Nottingham Grading System.

Supplemental Table 3 Werner Nuclear Protein Expression in BRCA-mutated Breast Tumors

	Werner Nuclear Protein Expression		*P* Value	
	Low, N (%)	High, N (%)	Unadjusted	Adjusted
Tumor size, cm[a]			.467	1.87
< 1 (T1a+b)	4 (13.3)	1 (2.9)		
> 1-2 (T1c)	11 (36.7)	14 (41.2)		
> 2-5 (T2)	14 (46.7)	17 (5 .0)		
> 5 (T3)	1 (3.3)	2 (5.9)		
Tumor stage			.227	1.59
1	21 (65.6)	20 (58.8)		
2	11 (34.4)	11 (32.4)		
3	0	3 (8.8)		
Tumor grade[b]				
G2	4 (12.5)	5 (13.9)		
G3	28 (87.5)	31 (86.1)	.866	.866
Vascular invasion			.319	1.60
No	18 (58.1)	23 (6 .5)		
Probable	0	2 (5.3)		
Yes	13 (41.9)	10 (26.3)		
Tumor type			.161	1.45
IDC-NST	28 (87.5)	26 (65.7)		
Medullary carcinoma	2 (6.3)	5 (14.3)		
ILC	1 (3.1)	5 (14.3)		
Others	1 (3.1)	0		
HER2 overexpression				1.51
No	30 (93.8)	34 (97.1)		
Yes	2 (6.3)	1 (2.9)	.502	
Triple negative			.244	1.46
No	14 (45.2)	22 (62.9)		
Yes	17 (54.8)	13 (37.1)		
NPI			.574	1.15
≤ 3.4	2 (6.7)	1 (3.4)		
> 3.4	28 (93.3)	28 (96.6)		
ER			**.015**	.195
Negative	24 (75.0)	16 (45.7)		
Positive	8 (32.0)	19 (54.3)		
PgR			.093	1.02
Negative	21 (67.7)	16 (47.1)		
Positive	10 (32.3)	18 (52.9)		

Bold indicates statistically significant.
Unadjusted *P* values were calculated using the Pearson χ^2 test. The Fisher exact test was used to obtain *P* values where one or more of cells has an expected frequency of 5 or less. Adjusted *P* values were calculated using the Benjamini-Hochberg false discovery rate method to adjust for multiple testing.
Abbreviations: ER = estrogen receptor; HER2 = human epidermal growth factor receptor 2; IDC-NST = invasive carcinoma of no special type; ILC = invasive lobular carcinoma; NPI = Nottingham Prognostic Index; PgR = progesterone receptor; WRN = Werner protein.
[a]Tumor size as defined by TNM Classification of Malignant Tumours (8th edition).
[b]Grade as defined by Nottingham Grading System.

Supplemental Table 4 Werner Cytoplasmic Protein Expression in BRCA-Mutated Breast Tumors

	Werner Cytoplasmic Protein Expression		*P* Value	
	Low, N (%)	High, N (%)	Unadjusted	Adjusted
Tumor size, cm[a]			.382	1.91
< 1 (T1a+b)	4 (11.8)	1 (3.3)		
> 1-2 (T1c)	11 (32.4)	14 (46.7)		
> 2-5 (T2)	18 (52.9)	13 (43.3)		
> 5 (T3)	1 (2.9)	2 (6.7)		
Tumor stage			.148	1.48
1	23 (63.9)	18 (60.0)		
2	13 (36.1)	9 (30.0)		
3	0	3 (10.0)		
Tumor grade[b]				
G2	7 (19.4)	2 (6.3)		
G3	29 (80.6)	30 (93.8)	.157	1.41
Vascular invasion			.100	1.20
No	17 (48.6)	24 (74.4)		
Probable	1 (2.9)	1 (3.2)		
Yes	17 (48.6)	6 (19.4)		
Tumor type			.310	2.17
IDC-NST	31 (86.1)	23 (71.9)		
Medullary carcinoma	3 (8.3)	4 (12.5)		
ILC	1 (2.8)	5 (15.6)		
Others	1 (2.8)	0		
HER2 overexpression			1.00	1.00
No	34 (94.4)	30 (96.8)		
Yes	2 (5.6)	1 (3.2)		
Triple negative				
No	19 (54.3)	17 (54.8)		
Yes	16 (45.7)	14 (45.2)	.992	1.98
NPI				
≤ 3.4	3 (9.1)	0		
> 3.4	30 (90.9)	26 (100.0)	.115	1.27
ER				
Negative	24 (66.7)	16 (51.6)		
Positive	12 (33.3)	15 (48.4)	.210	1.68
PgR				
Negative	20 (58.8)	17 (54.8)		
Positive	14 (41.2)	14 (45.2)	.746	2.98

Bold indicates statistically significant.
Unadjusted *P* values were calculated using the Pearson χ^2 test. The Fisher exact test was used to obtain *P* values where one or more of cells has an expected frequency of 5 or less. Adjusted *P* values were calculated using the Benjamini-Hochberg false discovery rate method to adjust for multiple testing.
Abbreviations: ER = estrogen receptor; HER2 = human epidermal growth factor receptor 2; IDC-NST = invasive carcinoma of no special type; ILC = invasive lobular carcinoma; NPI = Nottingham Prognostic Index; PgR = progesterone receptor; WRN = Werner protein.
[a]Tumor size as defined by TNM Classification of Malignant Tumours (8th edition).
[b]Grade as defined by Nottingham Grading System.

Supplemental Table 5 Correlation Among 5 RECQ Helicases in the Nottingham Tenovus Primary Breast Cancer Series

	Low, N (%)	High, N (%)	Adjusted *P* Value[a]
RECQL1			
RECQL5			**<.001**
Low	201 (52.6)	140 (37.3)	
High	181 (47.4)	235 (62.7)	
RECQL4			**<.001**
Low	245 (65.9)	173 (50.6)	
High	127 (34.1)	169 (49.4)	
BLM			**<.001**
Low	120 (30.7)	71 (19.5)	
High	271 (69.3)	293 (80.5)	
WRN			**.037**
Low	137 (51.7)	113 (42.6)	
High	128 (48.3)	152 (57.4)	
RECQL4			
RECQL1			**<.001**
Low	245 (58.6)	127 (42.9)	
High	173 (41.4)	169 (57.1)	
RECQL5			**<.001**
Low	279 (57.2)	113 (32.7)	
High	204 (42.8)	233 (67.3)	
BLM			**.010**
Low	167 (32.9)	58 (16.6)	
High	341 (67.1)	291 (83.4)	
WRN			**<.001**
Low	177 (49.7)	89 (38.9)	
High	179 (50.3)	140 (61.1)	
RECQL5			
RECQL1		181 (43.5)	**<.001**
Low	201 (58.9)		
High	140 (41.1)	235 (56.5)	
RECQL4			**<.001**
Low	279 (71.2)	209 (47.3)	
High	113 (28.8)	233 (52.7)	
BLM			**<.001**
Low	170 (37.0)	85 (16.5)	
High	290 (63.0)	429 (83.5)	
WRN			**<.001**
Low	175 (55.2)	134 (37.7)	
High	142 (44.8)	221 (62.3)	
BLM			
RECQL1			**<.001**
Low	120 (62.8)	271 (48.0)	
High	71 (37.2)	293 (52.0)	
RECQL4			**<.001**
Low	167 (74.2)	341 (54.0)	
High	58 (25.8)	291 (46.0)	
RECQL5			**<.001**
Low	170 (66.7)	290 (40.3)	
High	85 (33.3)	429 (59.7)	

Supplemental Table 5 Continued

	Low, N (%)	High, N (%)	Adjusted *P* Value[a]
WRN			.118
Low	100 (52.6)	236 (46.0)	
High	90 (47.4)	233 (54.0)	
WRN			
RECQL1			**.037**
Low	137 (54.8)	128 (45.7)	
High	113 (45.2)	152 (54.3)	
RECQL4			**.010**
Low	177 (66.5)	179 (56.1)	
High	89 (33.5)	140 (43.9)	
RECQL5			**<.001**
Low	175 (56.6)	142 (39.1)	
High	134 (43.4)	221 (60.9)	
BLM			.118
Low	100 (29.8)	90 (24.5)	
High	236 (70.2)	277 (75.5)	

Bold indicates statistically significant.
[a]Adjusted *P* values were calculated using Benjamini-Hochberg false discovery rate method to adjust for multiple testing.