Low Werner syndrome protein expression is associated with aggressive molecular phenotype and worse survival in patients with sporadic breast cancer

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Abstract

Purpose

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 characterised by premature aging and cancer predisposition. Malignancies that are commonly linked to WS are thyroid carcinoma, melanoma, breast cancer, meningioma, soft tissue and bone sarcomas. Currently, the clinicopathological significance of WRN in sporadic breast cancer is largely unknown.

Methods

We investigated the clinicopathological significance of WRN protein expression in invasive sporadic breast cancers in the Nottingham series comprising of two cohorts (n=1902). We correlated WRN protein expression to clinicopathological characteristics, DNA repair protein expression and survival outcomes.

Results

There is strong evidence of association between low WRN nuclear and cytoplasmic co-expression and low levels of KU70/KU80, DNA-PK, DNA Pol-B, CKD18, cytoplasmic RECQL4 and nuclear BLM protein expression (adjusted p-values <0.05). Tumours with low nuclear or cytoplasmic WRN expression have worse overall breast cancer-specific survival (BCSS) (adjusted p-values <0.05). In Topoisomerase I overexpressed tumours, low WRN nuclear expression was associated with poor BCSS (p-value <0.05). Interestingly, there was no evidence association between WRN protein expression and clinicopathological parameters (p-values>0.05).

Conclusions

Low WRN protein expression is associated with poor BCSS in patients with sporadic breast cancer. This can be used to optimise the risk stratification for personalised treatment.

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 which 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 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, inversions and reciprocal translocations with missense mutations being uncommon [9-11].

RECQ helicases have a highly conserved RECQ C-terminal group that interacts with DNA damage response proteins [1, 12, 13]. 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 [14]. This was supported by Opresko et. al who demonstrated that deletion of WRN resulted in growth arrest at G2/M cell cycle phases, DNA damage and increased tumour cell death rate [12]. Additionally, the surviving proliferative clones overexpressed WRN protein which indicates that WRN plays an important role not only in carcinogenesis but also in tumour growth [15].

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

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 four 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, USA). MDA-MB-436 and MDA-MB-468 cells were cultured in minimum essential amino acids medium supplemented with 1% L-glutamine and 1% non-essential amino acids. MCF-7 and MDA-MB-231 cells were grown in RPMI medium. All media were supplemented with 10% FBS and 1% penicillin streptomycin. 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 antibody used was anti-WRN rabbit polyclonal antibody (Novus Biological, cat.no NBP1-87143) at 1:1500 dilution. 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 two breast cancer patient cohorts. The first cohort consisted of 1650 primary invasive breast carcinomas who were diagnosed between 1986 and 1999 and entered into the Nottingham Tenovus Primary Breast Carcinoma series. Patient demographics are summarised in Supplementary Tables S1 and S2. This is a well-characterised series of patients with long-term follow-up that have been investigated in a wide range of biomarker studies [16-18]. 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 pre-menopausal patients with NPI scores of ≥ 3.4 (high risk), classical Cyclophosphamide, Methotrexate, and 5-Fluorouracil (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 breast cancer-specific survival (BCSS), disease-free

survival (DFS), and development of loco-regional and distant metastases (DM), were 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.

The second cohort was an independent series of 252 ER-negative primary invasive breast cancers diagnosed and treated at Nottingham University Hospitals between 1999 and 2007. All patients were primarily treated with surgery, followed by Radiotherapy and anthracycline chemotherapy.

Ethical approval was obtained from the Nottingham Research Ethics Committee (Reference number C202313). Tumour Marker Prognostics Studies (REMARK) criteria, recommended by McShane et al [19], were followed throughout this project.

Tissue microarray and immunohistochemistry

Breast tumours were arrayed in tissue microarrays (TMAs) constructed with two replicate 0.6 mm cores from the centre and periphery of the tumours. 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 h 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 tumour cores were evaluated by AA and an expert pathologist blinded to the clinicopathological 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, 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 tumour (<15% tumour). As our data were non-parametric, we used median cut off to dichotomise H score expression of WRN into low and high expression. A median H score of ≥ 116 was taken as the cut-off for high WRN nuclear expression and a median H-score of ≥20 was taken as cut off for high WRN cytoplasmic expression.

Statistical analysis

Data analysis was performed using SPSS (SPSS, version 22 Chicago, IL). Where appropriate, Pearson's Chi-square, Fisher's exact, Student's t and ANOVA one-way tests were used. Cumulative survival probabilities were estimated using the Kaplan–Meier method, and differences between survival rates were tested for significance using the log-rank test. Multivariate analysis for survival was performed using the Cox proportional hazard model. The proportional hazards assumption was tested using standard log–log plots. Hazard ratios (HR) and 95% confidence intervals (95% CI) were estimated for each variable. All tests were two-sided with a 95% CI and a p value < 0.05 considered significant. For multiple comparisons, p values were adjusted according to Holm-Bonferroni correction method [20].

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 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, MDA-MB-436, 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 WRN protein levels in clinical breast carcinoma samples.

WRN nuclear expression in tumour tissue is lower than normal breast tissue

We also evaluated the expression of WRN protein in 20 tumour-associated normal breast tissue slides. We observed both nuclear and cytoplasmic localisation of WRN in normal and tumour breast tissue. (Figure 1C). We also observed that WRN nuclear expression was lower in tumour tissue (Mean H-Score = 116) as compared to normal breast tissue (Mean H score = 220). For WRN cytoplasmic expression, in tumour tissue mean H score was 20 as compared to a mean H score of 170 in normal breast tissue (Figure 1D).

WRN nuclear expression is associated with other DNA repair proteins but not with clinicopathological features in breast cancer

A total of 720 tumours were suitable for WRN protein expression analyses. There was no statistically significant association between WRN nuclear expression and clinicopathological features in breast cancer (Table 1). However, when we correlated WRN nuclear expression to other DNA repair protein and regulators, low nuclear WRN was significantly associated with low KU70/KU80 levels ($p=0.039$), low DNA PKc ($p=0.019$), low cytoplasmic FEN1 ($p=0.016$) and low DNA Pol-b ($p=0.019$). In addition, reduced WRN protein expression was associated with low CDK18 levels ($p=0.015$). There was also a strong association with low expression levels of other DNA helicases such as nuclear and cytoplasmic RECQL4 (p values<0.05), and RECQL5 ($p=0.013$).

WRN cytoplasmic expression is not associated with clinicopathological features in breast cancer

There was no statistically significant association between WRN cytoplasmic expression and clinicopathological features in breast cancer (Table 2). However, when we correlated WRN cytoplasmic expression to other DNA repair protein and regulators, low nuclear WRN cytoplasmic was significantly associated with low DNA PKc ($p=0.039$) and low nuclear BLM ($p=0.026$).

WRN nuclear and cytoplasm co-expression is associated with impaired DNA repair and cell cycle regulation

15.4% of tumours had low nuclear/high cytoplasmic expression, 30.4% of tumours had low nuclear/low cytoplasmic expression, 36% of tumours had high nuclear/high cytoplasmic expression and 18.2% of tumours 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 between low WRN nuclear and cytoplasmic co-expression and low levels of KU70/KU80 (p=0.004), DNA-PK (p=0.003), DNA Pol-B (p=0.05), CKD18 (p=0.029), cytoplasmic RECQL4 (p=0.031), and nuclear BLM protein expression (p=0.044) (Table 3).

These results suggests that low WRN protein expression is associated with impaired DNA repair and cell cycle regulation in patients with sporadic breast cancer.

WRN nuclear and TOPO1 co-expression is not associated with clinicopathological features in breast cancer

As discussed previously WRN interacts with TOPO1 and enhances the ability of TOPO1 to relax the supercoiled DNA. Hence, we performed WRN nuclear and TOPO1 co-expression analysis in the sporadic breast but there was no statistically significant associations with clinicopathological variables.

Low WRN nuclear expression is associated with worse breast cancer specific survival

In univariate analysis, patients whose tumour had low WRN nuclear expression had significantly (p=0.02) worse overall breast cancer-specific survival (BCSS) (Figure 2A). Furthermore, a statistically significant worse BCSS was observed in the ER-negative cohort (p=0.012) (Figure 2C).

Low cytoplasmic WRN expression is associated with worse breast cancer specific survival

Tumours with low WRN cytoplasmic expression had a poor BCSS which was statistically significant (p=0.017). There was no statistically significant impact on BCSS in ER+ and ER- cohorts (Figures 3D-3F).

Low nuclear/cytoplasmic WRN co-expression is associated with poor breast cancer specific survival

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=0.04) suggesting that low expression has prognostic significance (Figure 3A). In the ER+ and ER- cohorts, WRN nuclear and cytoplasmic co-expression did not have any statistically significant impact on BCSS (Figure 3B & 3C).

Low WRN and high TOPO1 co-expression is associated with poor breast cancer specific survival

In WRN nuclear and TOPO1 nuclear co-expression analysis, tumours with low WRN nuclear expression and high TOPO1 expression had poor BCSS in the whole cohort (Figure 3D). Nevertheless, there was no statistically significant impact on BCSS in the subgroup analysis.

WRN nuclear and cytoplasmic expression are independent predictors of breast cancer specific survival

In multivariate analysis (Table 4), WRN nuclear and cytoplasmic expression was independent prognostic factor for BCSS (p=0.039 and 0.032, respectively). Tumour stage and grade were also independently associated with BCSS.

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 catalyses DNA-dependent reactions. WRN acts on various DNA structures to help with DNA repair through its enzymatic functions. Germline mutations in WRN leads to defects in DNA repair, premature aging and cancer susceptibility [21-23]. Genetic epidemiological studies identified certain polymorphisms of the WRN gene that are associated with increased risk of breast cancer [24-26]. Specifically, the CC genotype of WRN rs1346044 has been associated with the 2-fold risk of developing breast cancer [26]. In addition, a meta-analysis evaluated seven epidemiological studies and demonstrated that the CC genotype of Cys1367Arg polymorphism was also associated with 1.43 times increased risk of breast cancer [24]. A case-control study in Chinese women that included approximately 4000 patients also showed that the variant genotype of WRN Leu1074Phe was associated with 1.36 times higher risk of breast cancer [25].

We have previously shown, at transcriptomic level, that low WRN mRNA expression was associated with aggressive clinicopathological features such as high grade, lymph node stage and Her-2 overexpression and distinct aggressive molecular phenotypes as described by [27] including PAM50.Her2, PAM50.LumB, Genufu subtype (ER+/Her2-/High proliferation) and Genufu subtype (Her2 positive) breast tumours [28]. Low WRN mRNA level was also associated with poor BCSS [28]. At the protein level, we observed complex staining patterns with tumours showing negative, nuclear and/or cytoplasmic WRN staining. Similar to the WRN mRNA

expression data [28], low cytoplasmic and low nuclear WRN protein levels were correlated to poor BCSS. However, low WRN protein expression was not significantly linked to clinicopathological 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 tumours with the highest frequency in colorectal cancer [37.9% (69/182)] and a prevalence of 17.2% (10/58) in breast tumours [29]. Nevertheless, authors did not describe any clinicopathological associations in this study. In our previous study [28], we have found that WRN mRNA expression level was low in 326/1977 of breast tumours (16.5%) which are in accordance with the findings of with Agrelo et al study.

Our findings indicate that low WRN expression in human tumours may lead to a 'mutator phenotype' expressed as aggressive breast cancers. Inactivation of WRN protein makes tumour cells susceptible to topoisomerase 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 [30-32]. In colorectal tumours, hypermethylation of WRN promoter CpG island was correlated with good response and better overall survival after treatment with irinotecan [29]. Specifically, WRN knockdown and Camptothecin treatment both induce DNA damage and cause increased p21 expression and SA-β-gal activity in colon cancer [30]. On the other hand, the rescue of WRN in tumour cells treated with Camptothecin enhanced the efficiency of DNA damage response to eliminate cytotoxic DNA lesions [28].

In view of the interaction between WRN and TOPO1, we carried out combined WRN and TOPO1 analysis and showed that low WRN nuclear expression in TOPO1-overexpressed tumours is associated with worse BCSS. This is consistent with our previously published data at mRNA level where we demonstrated that low WRN expression in TOPO1-high tumours is associated with poor BCSS in the whole cohort [28]. Interestingly, at transcriptomic level, high WRN and high TOPO1 co-expression was associated with worse BCSS compared to low WRN and low TOPO1 co-expression in ER positive tumours. Nevertheless, this was not statistically significant at protein level. TOPO1 plays a vital role during replication and proliferation. We speculate that highly proliferative ER positive breast tumours (PAM50. Lum B phenotype) may be displaying endocrine resistance, hence leading to poor survival. As WRN is involved in various DNA repair pathways, it is possible that it promotes the DNA repair ability of established tumour 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 [33].

In conclusion, we provide compelling evidence that WRN protein expression can influence the clinical outcomes in patients with sporadic breast cancer. We have also shown the prognostic significance of low WRN expression in TOPO1-overexpressed tumours as these patients might benefit from Topoisomerase I poisons.

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Compliance with ethical standards

Conflict of interest

The authors declare no conflicts of interests.

Ethical approval

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.

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Legends

Table 1. Werner protein nuclear expression in sporadic breast cancer.

Table 2. Werner protein cytoplasmic expression in sporadic breast cancer.

Table 3. WRN protein nuclear and cytoplasmic co-expression in sporadic breast cancer.

Table 4. Multivariate analysis of WRN protein expression in sporadic breast cancer.

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 tumours.

Figure 2. Kaplan-Meier curves showing BCSS in WRN nuclear and cytoplasmic expression at protein level.

Figure 3. A-C. Kaplan-Meier curves showing BCSS in WRN nuclear and cytoplasmic co-expression at protein level. D. Kaplan-Meier curve showing BCSS in WRN and TOPO1 co-expression at protein level.

Table 1. Werner protein nuclear expression in sporadic breast cancer.

	Werner Nuclear Protein Expression		**P value**	
	Low N (%)	High N (%)	Unadjusted	Adjusted
A) Pathological Parameters				
Tumour Size				
<1cm	7 (5.9)	13 (8.5)		
>1-2cm	50 (42.4)	62 (40.5)	0.740	0.848
>2-5cm	60 (50.8)	75 (49)		
>5cm	1 (0.8)	3 (2.0)		
Tumour Stage				
1	53 (44.9)	64 (41.8)		
2	48 (40.7)	74 (48.4)	0.330	0.585
3	17 (14.4)	15 (9.8)		
Tumour Grade				
G1	6 (5.1)	5 (3.3)		
G2	49 (41.5)	70 (45.8)	0.642	0.807
G3	63 (53.4)	78 (51)		
Mitotic Index				
M1 (low; mitoses < 10)	33 (28.4)	47 (30.9)		
M2 (medium; mitoses 10-18)	30 (25.9)	42 (27.6)	0.785	0.850
M3 (high; mitosis >18)	53 (45.7)	63 (41.4)		
Tubule Formation				
1 (>75% of definite tubule)	1 (0.9)	2 (1.3)		
2 (10%-75% definite tubule)	40 (34.5)	41 (27.0)	0.401	0.680
3 (<10% definite tubule)	75 (64.7)	109 (71.7)		
Pleomorphism				
1 (small-regular uniform)	1 (0.9)	1 (0.7)		
2 (Moderate variation)	42 (36.2)	66 (43.7)	0.462	0.667
3 (Marked variation)	73 (62.9)	84 (55.6)		
Tumour Type				
IDC-NST	70 (59.8)	86 (56.6)		
Tubular Carcinoma	19 (16.2)	22 (14.1)		
Medullary Carcinoma			0.730	0.862
ILC	19 (16.2)	33 (21.7)		
Others				
Mixed NST/lobular/Special Type	9 (7.7)	11 (7.2)		
Lymph Node Status				
Negative	51 (44.7)	64 (42.7)		
Positive (1-3)	50 (43.9)	74 (49.3)	0.530	0.712
Positive (>3)	13 (11.4)	12 (8.0)		

B) Aggressive Phenotype				
HER2 overexpression No Yes	 105 (91.3) 10 (8.7)	 141 (93.4) 10 (6.6)	0.525	0.731
Triple Negative No Yes	 103 (87.3) 15 (12.7)	 123 (80.4) 30 (19.6)	0.130	0.298
NPI ≤ 3.4 >3.4	 7 (6.5) 100 (93.5)	 7 (4.8) 140 (95.2)	0.539	0.700
C) Hormone Receptors				
ER Negative Positive	 110 (26.3) 309 (73.7)	 139 (27) 375 (73)	0.786	0.828
PgR Negative Positive	 34 (29.6) 81 (70.4)	 33 (22.6) 113 (77.4)	0.201	0.392
D) DNA Repair Proteins				
ATM Low High	 139 (53.9) 119 (46.1)	 160 (50.6) 156 (49.4)	0.439	0.658
ATR (Nuclear) Low High	 75 (59.1) 52 (40.9)	 102 (46.6) 117 (53.4)	**0.025**	0.886
RAD51 (Cytoplasmic) Low High	 14 (5.8) 228 (94.2)	 16 (5.8) 259 (94.2)	0.987	38.49
RAD51 (Nuclear) Low High	 139 (57.2) 104 (42.8)	 132 (47.8) 144 (52.2)	**0.033**	0.107
BRCA1 Low High	 55 (17.5) 260 (82.5)	 57 (13.8) 356 (86.2)	0.175	0.359
PARP1 Low High	 173 (53.1) 153 (46.9)	 196 (50.0) 196 (50.0)	0.413	0.671
KU70/KU80 Low High	 62 (23.3) 204 (76.7)	 34 (11.3) 267 (88.7)	**<0.001**	**0.039**

DNA PKc Low High	50 (22.0) 202 (78.0)	37 (11.9) 274 (88.1)	**0.001**	**0.019**
ERCC1 Low High	100 (50.3) 99 (49.7)	107 (43.5) 139 (56.5)	0.155	0.335
XRCC1 Low High	62 (19.6) 255 (80.4)	50 (14.0) 307 (86.2)	**0.053**	0.137
SMUG Low High	143 (50.2) 142 (49.8)	167 (51.7) 156 (48.3)	0.707	0.861
DNA Pol-B Low High	78 (22.3) 272 (77.7)	55 (11.9) 346 (86.3)	**0.002**	**0.019**
FEN1 (Cytoplasmic) Low High	158 (51.6) 148 (48.4)	179 (51.4) 169(48.6)	**0.003**	**0.016**
FEN1 (Nuclear) Low High	225 (73.5) 81 (26.5)	252 (72.4) 96 (27.6)	0.749	0.834
<u>E) Cell Cycle and Apoptosis Regulators</u>				
P53 Low High	287 (69.7) 125 (30.3)	367 (72.1) 142 (27.9)	0.417	0.650
CDK18 Low High	171 (56.6) 131 (43.4)	154 (44.3) 194 (55.7)	**0.002**	**0.015**
Chk1 (Cytoplasmic) Low High	169 (41.7) 236 (58.3)	170 (34.4) 324 (65.6)	**0.024**	0.093
Chk1 (Nuclear) Low High	346 (85.4) 59 (14.6)	407 (82.4) 87 (17.6)	0.218	0.404
CHK2 Low High	63 (26.6) 174 (73.4)	56 (18.9) 240 (81.1)	**0.035**	0.105
RECQL5 (Nuclear) Low High	175 (56.6) 134 (43.4)	142 (39.1) 221 (60.9)	**<0.001**	**0.013**
RECQL4 (Nuclear) Low High	177 (66.6) 89 (33.5)	179 (56.1) 140 (43.9)	**0.010**	**0.048**
RECQL4 (Cytoplasmic) Low High	146 (55.1) 119 (44.9)	134 (42.3) 183 (57.7)	**0.002**	**0.013**

RECQL1 Low High	 137 (54.8) 113 (45.2)	 128 (45.7) 152 (54.3)	**0.037**	0.103
BLM (Nuclear) Low High	 100 (29.8) 236 (70.2)	 90 (24.5) 277(75.5)	0.118	0.287
BLM (Cytoplasmic) Low High	 255 (75.9) 81(24.1)	 246 (67.8) 117 (32.2)	**0.017**	0.073
C-MYC Low High	 140 (48.1) 151 (51.9)	 131 (47.3) 146 (52.7)	0.845	0.867

Abbreviations: Bold: Statistically significant; WRN: Werner; HER2: Human Epidermal Growth Factor Receptor 2; NPI: Nottingham Prognostic Index; 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.

Table 2. Werner protein cytoplasmic expression in sporadic breast cancer.

	Werner Cytoplasmic Protein Expression		**P Value**	
	Low N (%)	High N (%)	Unadjusted	Adjusted
A) Pathological Parameters				
Tumour Size <1cm >1-2cm >2-5cm >5cm	 52 (11.2) 234 (50.4) 175 (37.7) 3 (0.6)	 61 (12.4) 233 (47.6) 188 (38.4) 8 (1.6)	0.432	0.673
Tumour Stage 1 2 3	 300 (64.7) 120 (25.9) 44 (9.5)	 304 (61.9) 147 (29.9) 40 (8.1)	0.335	0.593
Tumour Grade G1 G2 G3	 83 (17.9) 149 (32.1) 232 (50.0)	 84 (17.1) 180 (36.7) 227 (46.2)	0.330	0.612
Mitotic Index M1 (low; mitoses < 10) M2 (medium; mitoses 10-18) M3 (high; mitosis >18)	 158 (35.7) 72 (16.3) 212 (48.0)	 191 (39.7) 87 (18.1) 203 (42.2)	0.213	0.639

Tubule Formation 1 (>75% of definite tubule) 2 (10%-75% definite tubule) 3 (<10% definite tubule)	 24 (5.4) 140 (31.7) 278 (62.9)	 28 (5.8) 156 (32.4) 297 (61.7)	 0.926	 0.976
Pleomorphism 1 (small-regular uniform) 2 (Moderate variation) 3 (Marked variation)	 15 (3.4) 166 (37.7) 259 (58.9)	 8 (1.7) 202 (42.2) 269 (56.2)	 0.123	 0.533
Tumour Type IDC-NST Tubular Carcinoma Medullary Carcinoma ILC Others Mixed NST/lobular/Special Type	 261 (57.1) 92 (20.1) 18 (3.9) 55 (12.0) 8 (1.8) 23 (5.0)	 269 (55.6) 108 (22.3) 5 (1.0) 62 (12.8) 8 (1.7) 32 (6.6)	 0.079	 0.385
Lymph Node Status Negative Positive (1-3) Positive (>3)	 246 (62.6) 113 (28.8) 34 (8.7)	 285 (62.5) 144 (31.6) 27 (5.9)	 0.253	 0.616
B) Aggressive Phenotype				
HER2 overexpression No Yes	 395 (86.2) 63 (13.8)	 422 (88.7) 54 (11.3)	 0.266	 0.546
Triple Negative No Yes	 393 (84.0) 75 (16.0)	 405 (82.3) 87 (17.7)	 0.493	 0.739
NPI ≤ 3.4 >3.4	 146 (33.0) 296 (67.0)	 155 (33.0) 315 (67.0)	 0.986	 38.45
C) Hormone Receptors				
ER Negative Positive	 129 (28.4) 326 (71.6)	 120 (25.1) 358 (74.9)	 0.262	 0.567
PgR Negative Positive	 206 (46.7) 235 (53.3)	 184 (40.3) 273 (59.7)	 0.051	 0.331
D) DNA Repair Proteins				

ATM Low High	 146 (51.8) 136 (48.2)	 153 (52.4) 139 (47.6)	 0.881	 1.01
ATR (Nuclear) Low High	 78 (52.7) 70 (47.3)	 99 (50.0) 99 (50.0)	 0.619	 0.832
RAD51 (Cytoplasmic) Low High	 15 (5.7) 249 (94.3)	 15 (5.9) 238 (94.1)	 0.904	 1.007
RAD51 (Nuclear) Low High	 156 (58.9) 109 (41.1)	 115 (45.3) 139 (54.7)	 **0.002**	 0.078
BRCA1 Low High	 63 (17.9) 288 (82.1)	 49 (13.0) 328 (87.0)	 0.064	 0.356
PARP1 Low High	 189 (51.8) 176 (48.2)	 180 (51.0) 173 (49.0)	 0.832	 1.014
KU70/KU80 Low High	 54 (18.7) 235 (81.3)	 42 (15.1) 236 (84.9)	 0.256	 0.587
DNA PKc Low High	 60 (21.5) 219 (78.5)	 34 (11.7) 257 (88.3)	 **0.002**	 **0.039**
ERCC1 Low High	 106 (46.9) 120 (53.1)	 101 (46.1) 118 (53.9)	 0.868	 1.025
XRCC1 Low High	 58 (17.4) 276 (82.6)	 54 (15.9) 286 (84.1)	 0.605	 0.842
SMUG1 Low High	 79 (25.2) 234 (74.8)	 65 (22.0) 230 (78.0)	 0.353	 0.598
DNA Pol-B Low High	 72 (19.4) 300 (80.6)	 61 (16.1) 318 (83.9)	 0.242	 0.629
FEN1 (Cytoplasmic) Low	 173 (51.3)	 164 (51.7)	 0.919	 0.995

High	164 (48.7)	153 (48.3)		
FEN1 (Nuclear) Low High	 242 (71.8) 95 (28.2)	 235 (74.1) 82 (25.9)	0.504	0.728
E) Cell cycle and Apoptosis Regulators				
P53 Low High	 312 (68.7) 142 (31.3)	 342 (73.2) 125 (26.8)	0.131	0.510
CDK18 Low High	 169 (52.2) 155 (47.8)	 156 (47.9) 170 (52.1)	0.272	0.530
Chk1 (Cytoplasmic) Low High	 199 (45.3) 240 (54.7)	 190 (41.3) 270 (58.7)	0.223	0.621
Chk1 (Nuclear) Low High	 368 (83.8) 71 (16.2)	 385 (83.7) 75 (16.3)	0.957	0.982
CHK2 Low High	 63 (24.8) 191 (75.2)	 56 (20.1) 223 (79.9)	0.190	0.673
RECQL5(Nuclear) Low High	 159 (47.7) 174 (52.3)	 158 (46.6) 181 (53.4)	0.767	0.964
RECQL4 (Nuclear) Low High	 174 (61.5) 109 (38.5)	 182 (60.3) 120 (39.7)	0.763	0.991
RECQL4 (Cytoplasmic) Low High	 150 (53.4) 131 (46.6)	 130 (43.2) 171 (56.8)	**0.014**	0.109
RECQL1 (Nuclear) Low High	 151 (55.3) 122 (44.7)	 114 (44.4) 143 (55.6)	**0.012**	0.117
BLM (Nuclear) Low High	 111 (32.4) 232 (67.6)	 79 (21.9) 281 (78.1)	**0.002**	**0.026**
BLM (Cytoplasmic) Low High	 249 (73.2) 91 (26.8)	 252 (70.2) 107 (29.8)	0.373	0.606
C-MYC Low High	 153 (50.2) 152 (49.8)	 118 (44.9) 145 (55.1)	0.208	0.676

Abbreviations: Bold: Statistically significant; WRN: Werner; HER2: Human Epidermal Growth Factor Receptor 2; NPI: Nottingham Prognostic Index; 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.

Table 3. WRN protein nuclear and cytoplasmic 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
A) Pathological Parameters						
Tumour Size						
≤1cm	26 (9.0)	26 (14.9)	16 (11.3)	45 (12.9)		
>1-2cm	144 (49.7)	90 (51.7)	68 (48.2)	165 (47.3)	0.382	0.677
>2-5cm	119 (41.0)	56 (32.2)	54 (38.3)	134 (38.4)		
>5cm	1 (0.3)	2 (1.1)	3 (2.1)	5 (1.4)		
Tumour Stage						
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)	0.767	0.830
3	27 (9.3)	17 (9.7)	14 (9.9)	26 (7.4)		
Tumour Grade						
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)	0.664	0.809
G3	148 (51.0)	84 (48.3)	60 (42.6)	167 (47.7)		
Mitotic Index						
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)	0.470	0.789
M3 (high; mitosis >18)	134 (49.4)	78 (45.6)	57 (40.7)	146 (42.8)		
Tubule Formation						
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)	0.981	38.25
3 (<10% definite tubule)	169 (62.4)	109 (63.7)	86 (61.4)	211 (61.9)		

Pleomorphism 1 (small-regular uniform) 2 (Moderate variation) 3 (Marked variation)	 11 (4.1) 99 (36.5) 161 (59.4)	 4 (2.4) 67 (39.6) 98 (58.0)	 3 (2.2) 65 (46.8) 71 (51.1)	 5 (1.5) 137 (40.3) 198 (58.2)	0.225	0.461
Tumour Type IDC-NST Tubular Carcinoma Medullary Carcinoma ILC Others Mixed NST/Lobular/Special Type	 163 (56.8) 59 (20.6) 11 (3.8) 33 (11.5) 4 (1.4) 17 (5.9)	 98 (57.6) 33 (19.4) 7 (4.1) 22 (12.9) 4 (2.4) 6 (3.5)	 74 (53.6) 32 (23.2) 1 (0.7) 19 (13.8) 3 (2.2) 9 (6.5)	 195 (56.4) 76 (22.0) 4 (1.2) 43 (12.4) 5 (1.4) 23 (6.6)	0.639	0.859
Lymph Node Status Negative Positive (1-3) Positive (>3)	 139 (61.0) 67 (29.4) 22 (9.6)	 107 (64.8) 46 (27.9) 12(7.3)	 74 (60.7) 40 (32.8) 8 (6.6)	 211 (63.2) 104 (31.1) 19 (5.7)	0.668	0.789
<u>B) Aggressive Phenotype</u>						
HER2 overexpression No Yes	 244 (85.6) 41 (14.4)	 151 (87.3) 22 (12.7)	 125 (89.9) 14 (10.1)	 297 (88.1) 40(11.9)	0.641	0.833
Triple Negative No Yes	 239 (82.4) 51 (17.6)	 154 (86.5) 24 (13.5)	 115 (81.6) 26 (18.4)	 290 (82.6) 61 (17.4)	0.599	0.898
NPI ≤3.4 >3.4	 90 (32.6) 186 (67.4)	 56 (33.7) 110 (66.3)	 46 (34.6) 87 (65.4)	 109 (32.3) 228 (67.7)	0.964	1.016
<u>C) Hormone Receptors</u>						

ER Negative Positive	77 (27.3) 205 (72.7)	52 (30.1) 121 (69.9)	33 (24.1) 104 (75.9)	87 (25.5) 254 (74.5)	0.621	0.865
PgR Negative Positive	133 (48.5) 141 (51.5)	73 (43.7) 94 (56.3)	51 (37.8) 84 (62.2)	133 (41.3) 189 (58.7)	0.153	0.397
DNA Repair Proteins						
ATM Low High	94 (55.0) 77 (45.0)	52 (46.8) 59 (53.2)	45 (51.7) 42 (48.3)	108(52.7) 97 (47.3)	0.610	0.881
ATR Low High	45 (57.0) 34 (43.0)	33 (47.8) 36 (52.2)	30 (62.5) 18 (37.5)	69(46.0) 81 (54.0)	0.142	0.395
RAD51 (Cytoplasmic) Low High	11 (6.6) 155 (93.4)	4 (4.1) 94 (95.9)	3 (3.9) 73 (96.1)	12 (6.8) 165 (93.2)	0.677	0.776
RAD51 (Nuclear) Low High	103 (61.7) 64 (38.3)	53 (54.1) 45 (45.9)	36 (47.4) 40 (52.6)	79 (44.4) 99 (55.6)	**0.011**	0.53
BRCA1 Low High	40 (19.1) 169 (80.9)	23 (16.2) 119 (83.8)	15 (14.2) 91 (85.8)	34 (12.5) 237 (87.5)	0.247	0.481
PARP1 Low High	117 (52.0) 108 (48.0)	72 (51.4) 68 (48.6)	56 (55.4) 45 (44.6)	124(49.2) 128 (50.8)	0.758	0.844
Ku70/KU80 Low High	47 (25.7) 136 (74.3)	7 (6.6) 99 (93.4)	15 (18.1) 68 (81.9)	27(13.8) 68 (86.2)	**<0.001**	**0.004**
DNA PKc Low High	47 (26.7) 129 (73.3)	13 (12.6) 90 (87.4)	10 (12.0) 73 (88.0)	24(11.5) 184 (88.5)	**<0.001**	**0.003**

ERCC1 Low High	 71 (51.1) 68 (48.9)	 35 (40.2) 52 (59.8)	 29 (48.3) 31 (51.7)	 72 (45.3) 87 (54.)	0.437	0.74
XRCC1 Low High	 44 (20.7) 169 (79.3)	 14 (11.6) 107 (88.4)	 18 (17.3) 86 (82.7)	 36(15.3) 200 (84.7)	0.166	0.404
SMUG1 Low High	 100 (50.8) 97 (49.2)	 60 (51.7) 56 (48.3)	 43 (48.9) 45 (51.1)	 107(51.7) 100 (48.3)	0.973	0.998
DNA Pol-B Low High	 58 (23.9) 185 (76.1)	 14 (10.9) 115 (89.1)	 20 (18.7) 87 (81.3)	 41 (15.1) 231 (84.9)	**0.008**	**0.05**
FEN1 (Nuclear) Low High	 154 (70.3) 65 (29.7)	 88 (74.6) 30 (25.4)	 71 (81.6) 16 (18.4)	 164(71.3) 66 (28.7)	0.208	0.450
FEN1 (Cytoplasmic) Low High	 119 (54.3) 100 (45.7)	 54 (45.8) 64 (54.2)	 39 (44.8) 48 (55.2)	 125(54.3) 105 (45.7)	0.207	0.474
<u>Cell Cycle and Apoptosis Regulators</u>						
p53 Low High	 195 (68.9) 88 (31.1)	 117 (68.4) 54 (31.6)	 92 (71.3) 37 (28.7)	 250(74.0) 88 (26.0)	0.457	0.742
CDK18 Low High	 124 (59.3) 85 (40.7)	 45 (39.1) 70 (60.9)	 47 (50.5) 46 (49.5)	 109 (46.8) 124 (53.2)	**0.003**	**0.029**
Chk1 (Cytoplasmic) Low High	 118 (43.1) 156 (56.9)	 53 (32.1) 110 (67.9)	 51 (38.9) 80 (61.1)	 117(35.6) 212 (64.4)	0.099	0.297
Chk1 (Nuclear) Low High	 233 (85.0) 41 (15.0)	 135 (81.8) 30 (18.2)	 113 (86.3) 18 (13.7)	 272(82.7) 57 (17.3)	0.643	0.808

Chk2						
Low	39 (25.5)	24 (23.8)	24 (28.6)	32(16.4)	0.078	0.253
High	114 (74.5)	77 (76.2)	60 (71.4)	163 (83.6)		
RECQL5 (Nuclear)						
Low	118 (55.9)	41(33.6)	57 (58.2)	101(41.9)	**<0.001**	**0.001**
High	93 (44.1)	81 (66.4)	41 (41.8)	140 (58.1)		
RECQL4 (Nuclear)						
Low	120 (65.9)	54 (53.5)	57 (67.9)	125(57.3)	0.067	0.237
High	62 (34.1)	47 (46.5)	27 (32.1)	93 (42.7)		
RECQL4 (Cytoplasmic)						
Low	107 (59.1)	43 (43.0)	39 (46.4)	91(41.9)	**0.004**	**0.031**
High	74 (40.9)	57 (57.0)	45 (53.6)	126 (58.1)		
RECQL1 (Nuclear)						
Low	99 (56.6)	52 (53.1)	38 (50.7)	76(41.8)	**0.039**	0.169
High	76 (43.4)	46 (46.9)	37 (49.3)	106 (58.2)		
BLM (Nuclear)						
Low	78 (34.7)	33 (28.0)	22 (19.8)	57(22.9)	**0.008**	**0.044**
High	147 (65.3)	85 (72.0)	89 (80.2)	192 (77.1)		
BLM (Cytoplasmic)						
Low	174 (77.7)	75 (64.7)	81 (72.3)	171(69.2)	**0.05**	0.195
High	50 (22.3)	41 (35.3)	31 (27.7)	76 (30.8)		
C-MYC						
Low	105 (51.5)	48 (47.5)	35 (40.2)	83 (47.2)	0.372	0.690
High	99 (48.5)	53 (52.5)	52 (59.8)	93 (52.8)		

Abbreviations: Bold: Statistically significant; WRNn: Werner (nuclear); WRNc: Werner (cytoplasmic); HER2: Human Epidermal Growth Factor Receptor 2; NPI: Nottingham Prognostic Index; 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.

Table 4. Multivariate analysis of WRN protein expression in sporadic breast cancer.

	P value	Exp (B)	95% CI of Exp (B)	
			Lower	Upper
Breast Cancer Specific Survival				
Stage	**<0.001**	1.954	1.442	2.649
Grade	**<0.001**	2.473	1.636	3.738
HER2 overexpression	0.242	1.313	0.832	2.073
NPI	0.531	0.793	0.383	1.639
WRN (Cytoplasmic)	**0.039**	0.677	0.467	0.980
WRN (Nuclear)	**0.032**	0.672	0.466	0.967
TOPO1 (Nuclear)	0.270	1.234	0.849	1.794

Bold: statistically significant.

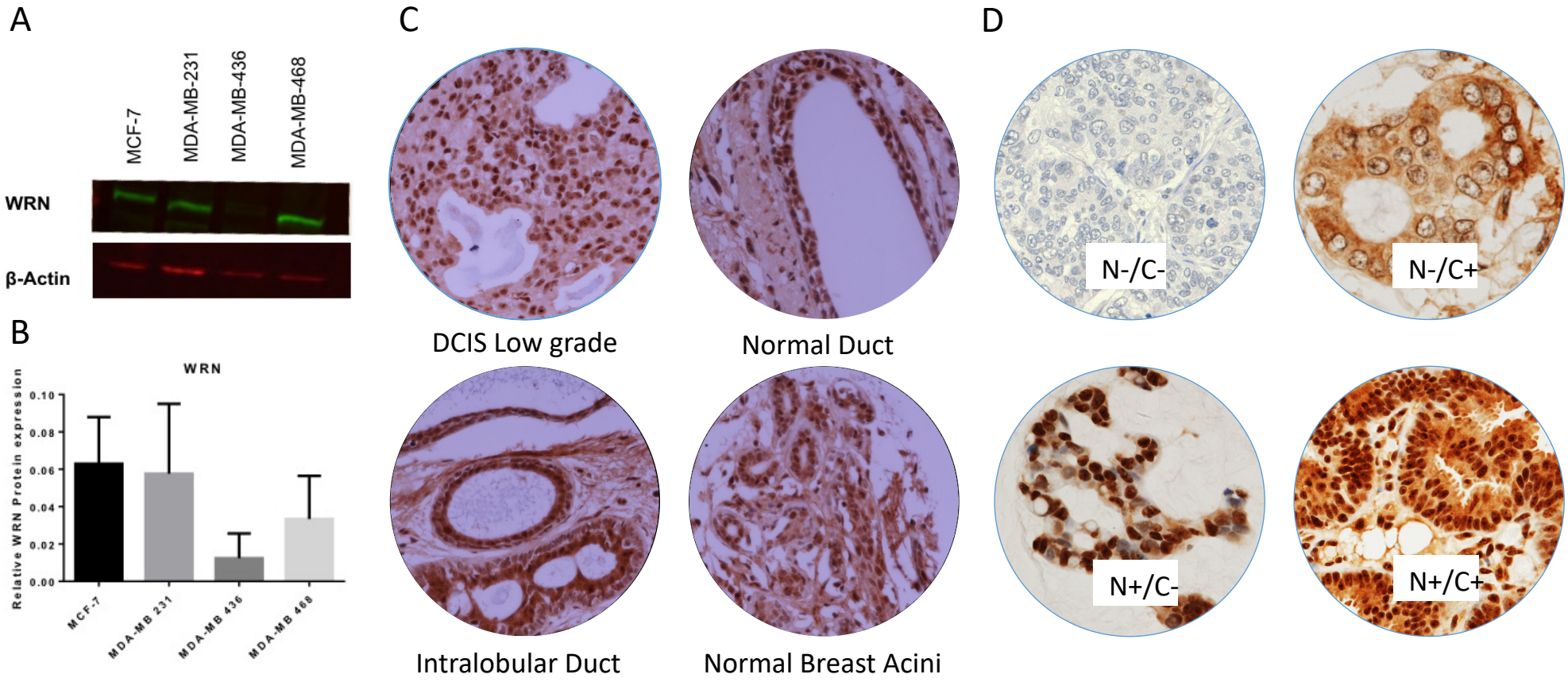

Figure 1. A) Western blot of WRN protein expression in breast cancer cell lines. B) Relative WRN protein expression in breast cancer cell lines. C) Microphotographs of WRN protein expression in normal breast tissue. D) Microphotographs of WRN protein expression in breast tumours.

N-/C- (Nuclear negative & cytoplasmic negative); N+/C+ (Nuclear negative & cytoplasmic positive); N+/C- (Nuclear positive & cytoplasmic negative); N-/C+ (Nuclear negative & cytoplasmic positive).

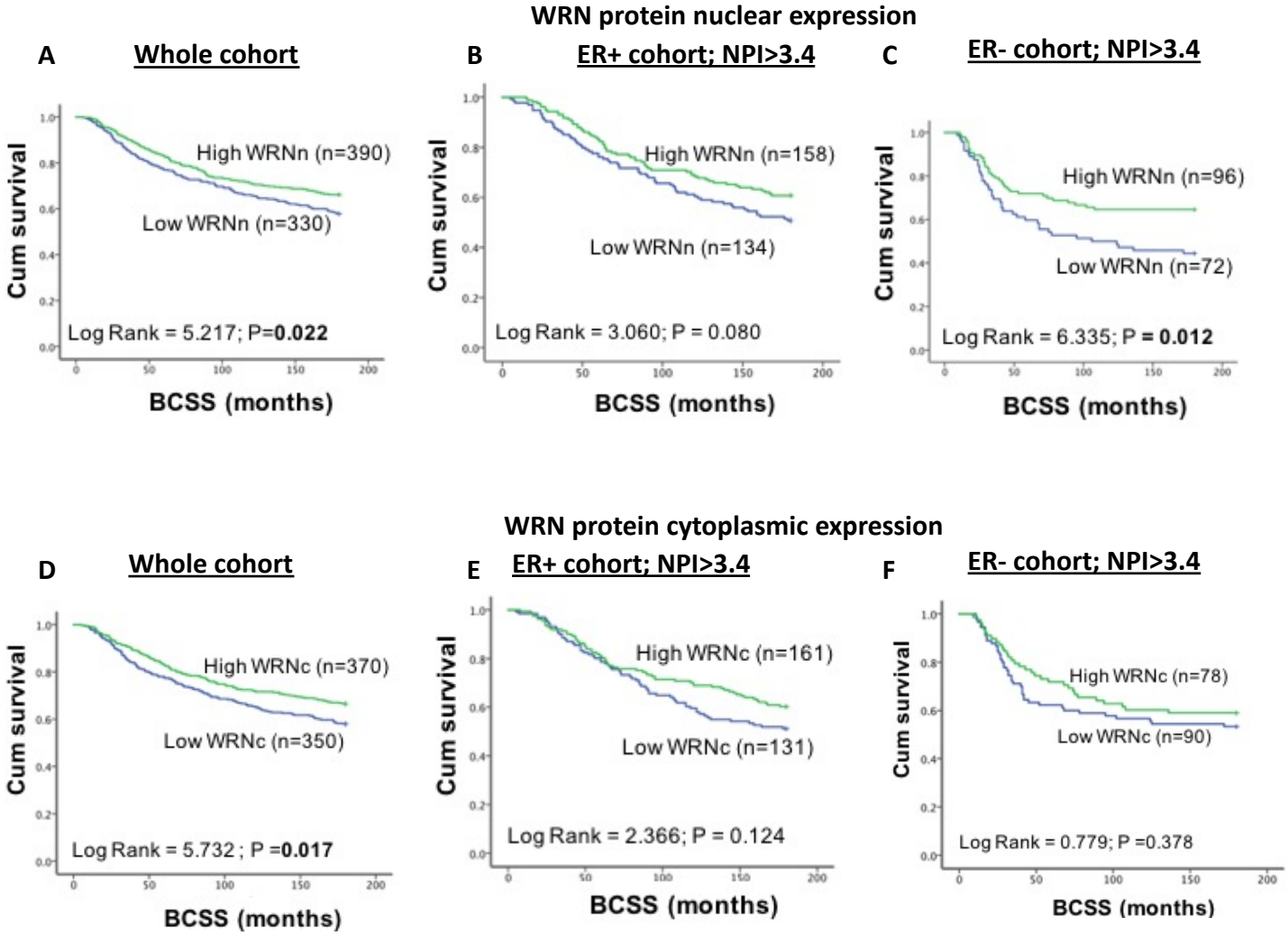

Figure 2. Kaplan-Meier curves showing BCSS in WRN nuclear and cytoplasmic expression at protein level.

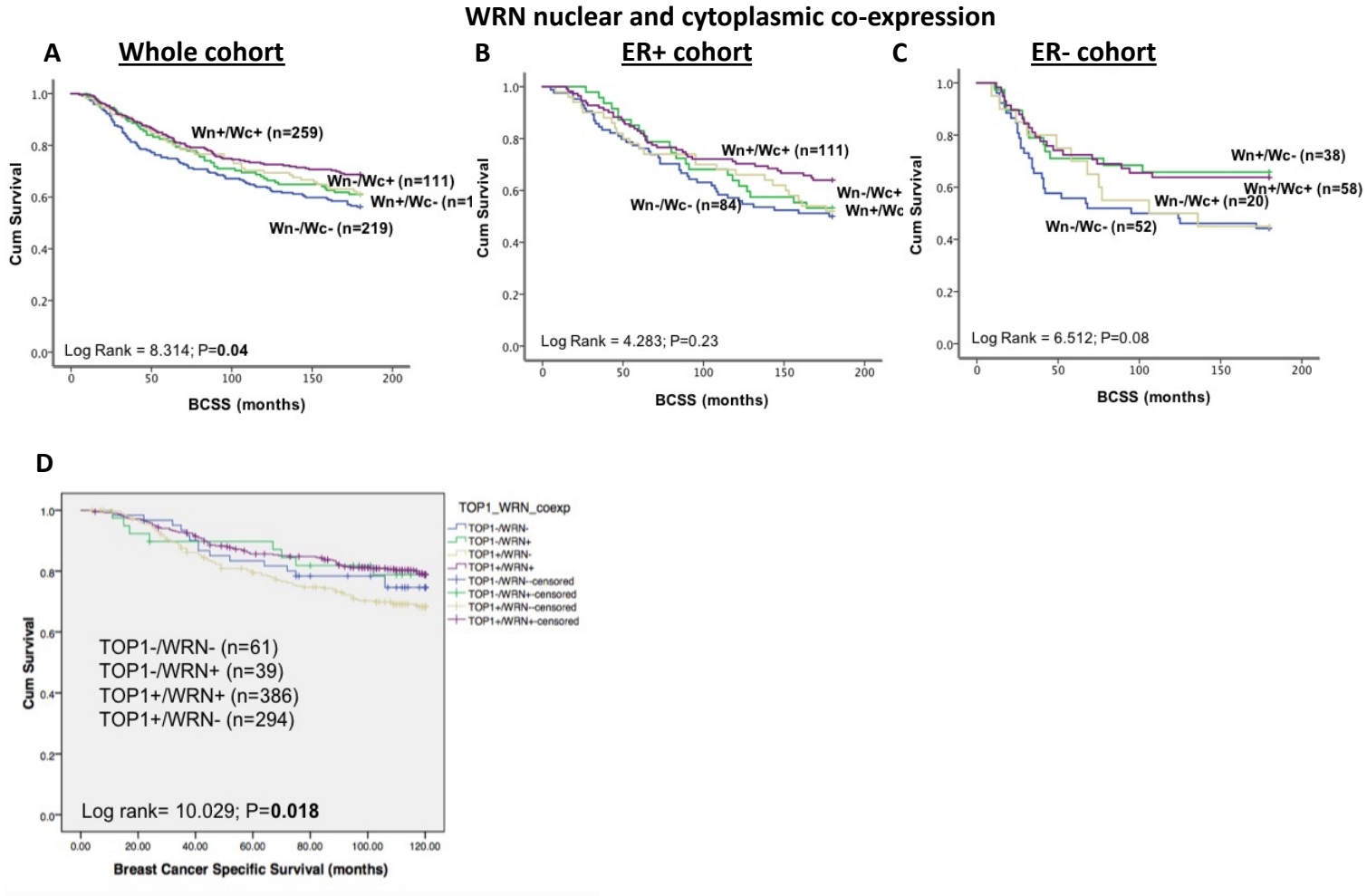

Figure 3. A-C. Kaplan-Meier curves showing BCSS in WRN nuclear and cytoplasmic co-expression at protein level. D. Kaplan-Meier curve showing BCSS in WRN and TOPO1 co-expression at protein level.

Supplementary Table S1. Clinicopathological characteristics of Nottingham Tenovus series.			
Variable	**n***	**Cases**	**(%)**
Menopausal status	1650		
Pre-menopausal		612	(37.0)
postmenopausal		1038	(63.0)
Tumour Grade (NGS)	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)
Tumour size (cm)	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)
Tumour 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(6.5–6.8)		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 (AT)			
No AT		665	(42.0)
Hormone therapy (HT)		642	(41.0)
Chemotherapy		307	(20.0)
Hormone + chemotherapy		46	(3.0)

* Number of cases for which data were available.

NPI; Nottingham prognostic index, PG; prognostic group

Supplemental Table S2. Clinicopathological characteristics of ER- cohort.

Variable	**n***	**Cases**	**(%)**
Menopausal status	252		
Pre-menopausal		122	(48.5)
postmenopausal		130	(51.5)
Tumour Grade (NGS)	252		
G1		1	(0.3)
G2		27	(10.6)
G3		224	(89.1)
Lymph node stage	252		
Negative		121	(48)
Positive (1-3 nodes)		86	(34)
Positive (>3 nodes)		45	(18)
Tumour size (cm)	252		

T1 a + b (≤1.0)		28	(11)
T1 c (>1.0 -2.0)		106	(42)
T2 (>2.0-5)		103	(41)
T3 (>5)		15	(6)
Tumour type	252		
IDC-NST		224	(89.0)
Tubular		5	(2.0)
ILC		8	(3.0)
Medullary (typical/atypical)		5	(2.0)
Others		0	(4.0)
NPI subgroups	252		
Excellent PG(2.08-2.40)	Low risk	0	(0.0)
Good PG(2.42-3.40)		0	(0.0)
Moderate I PG(3.42 to 4.4)	High risk	111	(44.0)
Moderate II PG(4.42 to 5.4)		81	(32.0)
Poor PG(5.42 to 6.4)		38	(15.0)
Very poor PG(6.5–6.8)		22	(9.0)
Survival at 5 years	252		
Alive and well		176	(70.0)
Dead from disease		73	(29.0)
Dead from other causes		3	(1.0)

* Number of cases for which data were available.

NPI; Nottingham prognostic index, PG; prognostic group

Supplementary Table and Figure legends

Supplementary Table 1: Clinicopathological characteristics of Nottingham Tenovus series.

Supplementary Table 2. Clinicopathological characteristics of ER- cohort.