Exploring RECQ Helicase landscape in Breast Cancer

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Dedicated to the loving memory of my father, Professor Surinder Pal Arora

(1945-1996)

Abstract

Background

Breast cancer is the second most common cause of cancer death in females in the UK. Approximately 20 percent of breast cancers diagnosed are triple negative (TNBC) intrinsic subtype, which are aggressive high-grade tumours carrying a poor prognosis. About 20% of TNBC harbour a mutation in breast cancer gene (BRCA). BRCA plays important role in homologous recombination. There is evidence to suggest that non-familial breast cancers harbour homologous recombination repair defects that may be useful targets for therapy.

The RECQ protein family is a highly conserved group of DNA helicases, which play diverse roles in various DNA metabolic processes such as DNA replication and recombination repair. In humans, five RECQ helicases (RECQL1, BLM, WRN, RECQL4, and RECQL5) have been identified and three of them namely, WRN, BLM, and RECQL4 have been associated with autosomal recessive disorders which are characterized by genomic instability, premature aging, and predisposition to various cancers. The role of RECQ helicase expression in sporadic breast cancer pathogenesis is unknown. We hypothesized that dysregulation in RECQ helicase expression may have an impact on breast cancer pathogenesis and survival. The aim of this thesis was to have a comprehensive assessment of the prognostic, predictive and clinicopathological significance of five RECQ helicases at the transcriptome and protein level.

Methods

Investigation of the protein expression of RECQ helicases in breast cancer was performed in two patient cohorts. The first cohort was a series of 1650 primary invasive breast cancer patients who were diagnosed between 1986 and 1999 and entered into the Nottingham tenovus series. The second cohort used for evaluation was an independent series of 252 ER-negative invasive breast cancers diagnosed and managed at Nottingham University Hospitals between 1999 and 2007. Standard immunohistochemistry technique was used for staining the breast TMA (tissue microarray) and assessment of staining was done using H-scoring. Evaluation of the mRNA expression was done in the METABRIC dataset (1977 cases). Data analysis was performed using SPSS (SPSS, version 22 Chicago, IL). Frequency histogram distributions and X tile (Version 3.6.1) were used to establish the cut-offs for expression values such that the resulting subgroups have significantly different survival courses. Cumulative survival probabilities were estimated using the Kaplan–Meier method. Statistical significant differences between survival outcomes were tested using the log-rank test. P values for each test were adjusted with (Benjamini & Hochberg, 1995) multiple P-value adjustments and an adjusted p-value of <0.05 was considered significant. Multivariate analysis with survival Cox proportional hazard model was performed where a statistically significant survival outcome was seen in the univariate analysis. Cytotoxicity of Cisplatin in BLM Ctr and BLM KD Hela cells was assessed using clonogenic and MTS cell survival assays. Functional consequences of Cytotoxicity in BLM KD Hela cell lines was determined using

flow cytometry technique. Weasel (Victoria, Australia) flow cytometry analysis software was utilized for data analysis. Graphical visualisation and statistical analysis were carried out in GraphPad Prism 6 (GraphPad, La Jolla, USA). Two way ANOVA was used to determine the statistically significant differences between the variables.

Results

Low RECQL1 expression showed a statistically significant association with aggressive breast cancer phenotypes. Furthermore, low RECQL1 was linked to poor breast cancer survival ($p<0.05$) in the whole cohort and ER-positive patients including those who had adjuvant endocrine therapy, suggesting the prognostic and predictive significance of RECQL1 in sporadic breast cancers.

High RECQL5 mRNA expression and high RECQL5/low RAD51 protein expression revealed a statistically significant association with aggressive breast cancer phenotypes and poor survival which was more pronounced in ER-positive subgroup. This suggests the predictive and prognostic significance of RECQL5 in breast cancer. RECQL5 remained an independent predictor of survival on multivariate analysis.

RECQL4 amplification/gain in copy numbers, high RECQL4 mRNA, expression and low nuclear/high cytoplasmic RECQL4 protein expression were associated with aggressive breast cancer phenotypes, and poor survival ($p<0.05$). In tumours with high nuclear RECQL4 protein expression, c-MYC could stratify

patients into prognostic groups ($p<0.05$).This confirms the clinicopathological, predictive and prognostic significance of RECQL4 in breast cancer.

Low WRN mRNA levels were significantly associated with poor breast cancer-specific survival in the whole cohort and ER-positive patients including those who had adjuvant endocrine therapy, informing the prognostic and predictive significance. At the protein level, WRN protein expression remained an independent predictor of survival on multivariate analysis. ER-positive tumours, including those who had endocrine therapy, with high TOPO1/high WRN expression showed poor breast cancer-specific survival ($p<0.05$).

High BLM mRNA levels were significantly associated with aggressive breast cancer phenotypes and poor breast cancer-specific survival in the whole cohort and ER-positive tumour including those who had endocrine therapy, showing predictive and prognostic significance. Low nuclear BLM/low nuclear RAD51 protein levels were associated with poor survival($p<0.05$) in the whole cohort and ER-negative patients including those who had chemotherapy showing resistance to chemotherapy. BLM mRNA expression remained an independent predictor of survival on multivariate analysis. Moreover, preliminary cell line work showed that BLM-KD HeLa cells were sensitive to platinum chemotherapy.

Conclusion

RECQ helicases expression in breast cancer clearly has predictive, prognostic, and clinicopathological significance. RECQ helicases could have a synthetic lethality relationship with PARP inhibitors or other DNA repair inhibitors. It is important to do further cell line work in breast cancer cell lines through BLM knockdown with siRNA and Knock out with CRISPR cas-9.

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List of frequently used abbreviations

APE1	Apurinic/apyrimidinic endonuclease
AT	Adjuvant therapy
ATM	Ataxia-telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia and Rad3-related protein
BCSS	Breast cancer-specific survival
BER	Base excision repair
BLM	Bloom
BRCA	Breast cancer gene
BS	Bloom's syndrome
cDNA	Complementary DNA
C-Myc	c-mycproto-oncogene
CDK18	Cyclin Dependent Kinase 18
Chk	Checkpoint kinase
Ct	Threshold cycle
CT	Computed Tomography
DCIS	Ductal carcinoma in situ
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNA PKcs	DNA-dependent protein kinase, catalytic subunit
DSB	Double-strand breaks
Exo1	Exonuclease 1
ER	Oestrogen-receptor
ERCC1	Excision Repair Cross-Complementing
FEN1	5'-flap endonuclease 1
FISH	Fluorescence in situ hybridization
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HER2	Human epidermal growth factor receptor 2
HIER	Heat-induced epitope retrieval
HJ	Holliday junction

HNPCC	Hereditary nonpolyposis colorectal cancer
HR	Homologous recombination
HRR	Homologous recombination repair
ICL	Inter-strand cross-links
IHC	Immunohistochemistry
KD	Knockdown
Kda	Kilodalton
LABC	Locally advanced breast cancer
LN	Lymph node
MMR	Mismatch repair
MRN	MRE11/RAD50/NBN complex
MRI	Magnetic Resonance Imaging
mRNA	Messenger RNA
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
NLS	Nuclear localization sequence
NPI	Nottingham Prognostic Index
NTS	Nottingham Tenovus Series
NTC	No Template Control
P53	Phosphoprotein 53
PARP	poly-ADP ribose polymerase
PCR	Polymerase chain reaction
PR	Progesterone-receptor
POL-B	DNA polymerase β
PR	Progesterone-receptor
RNAi	RNA interference
RPA	Replication protein A
RT	Room temperature
RT Control	Reverse transcriptase control
RTS	Rothmund-Thomson syndrome
SCE	Sister chromatid exchange
SDSA	Synthesis dependant strand annealing
ssDNA	Single stranded DNA
SMUG1	Single-Strand Monofunctional Uracil- Glycosylase1

TMA	Tumour microarray
TNBC	Triple negative breast cancer
TOPO2A	Topoisomerase 2A
TOP3	Topoisomerase 3
WRN	WERNER
WS	Werner's syndrome
XRCC	X-ray repair cross-complementing protein
µg	Microgram
µl	Microliter
µM	Micromolar

CHAPTER 1

Background

1 Background

1.1 Epidemiology

There are around 55,000 new breast cancer cases in the UK annually, which is approximately 150 new cases diagnosed every day (2013-2015). Breast cancer is the second most common cancer among females in the UK, accounting for 15% of all new cancer cases (UK, 2019). Since 1990, breast cancer incidence rates have increased by a fifth (20%) in the UK. Incidence rates for breast cancer are expected to rise by 2% in the UK between 2014 and 2035, to 210 cases per 100,000 females by 2035. Breast cancer is more common in Caucasian females than in Asian or Black females (Cancer Research UK breast Cancer incidence statistics). Breast cancer in males is rare. The incidence has increased after the introduction of better imaging modalities and screening (McTiernan, 2010), and it continues to grow with the aging of the population. Prevalence is also increasing, as a result of rising incidence and due to better treatment options and outcomes. In the UK, Breast cancer is the 2nd most common cause of cancer death in females, with around 11,500 deaths in 2016. The mortality rates for breast cancer are expected to fall by 26% in the UK between 2014 and 2035, to 31 deaths per 100,000 females by 2035 (Cancer Research UK breast Cancer mortality statistics). The mortality rate is reducing because of early detection and improved treatment options (Allemani et al, 2018; Autier et al, 2010)

1.2 Breast Cancer risk factors

The most important risk factors include genetic predisposition, endogenous and exogenous oestrogen exposure, ionizing radiation, low parity, history of atypical hyperplasia, obesity and the consumption of alcohol. For male breast cancer, the major risk factors include diseases causing hormonal imbalances (especially gynaecomastia and cirrhosis), radiation exposure, positive family history and genetic predisposition (Ottini et al, 2010).

1.3 Diagnosis & Pathology

The diagnosis of breast cancer is made on the basis of clinical examination (palpation of breast and locoregional lymph nodes and also assessment for distant metastasis) and imaging (mammogram, Ultrasound +/- CT or MRI). CT scan and bone scan should be considered for high-risk patients such as patients with positive axillary nodes, large tumours (e.g. ≥5 cm), aggressive histological features or signs and symptoms suggestive of metastatic disease. MRI is only needed in some circumstances such as lobular disease, BRCA mutated breast cancer, breast implants and a discrepancy between clinical examination and conventional imaging. Diagnosis is confirmed by pathological assessment of the tumour sample obtained by image-guided needle core biopsy. It is also important to assess the involvement of axillary lymph nodes by doing fine needle aspiration cytology if lymph nodes are seen on imaging or by doing sentinel lymph node biopsy if imaging is clear. Breast cancer is classified into various histologic types. Infiltrating or invasive ductal cancer is the most common histologic type

(70% to 80% of all cases). Histologic types with favourable outcome include mucinous, medullary, and tubular carcinomas. The pathological report should also include the tumour grade, assessment of proliferation marker Ki67, oestrogen receptor (ER) status (using a standardized immunohistochemistry methodology such as Allred or H-score) and IHC evaluation of progesterone receptor (PR) and human epidermal growth factor 2 receptor (HER2) gene expression. If IHC is borderline for HER2 then the sample should be sent for FISH (Fluorescence in situ hybridization) test. For prognostication and treatment decision making, tumours should be classified into prognostic subgroups using valid prognostic tools such as the Nottingham Prognostic Index (NPI). NPI is calculated using the formula: NPI = [0.2 x S] + N + G Where: S is the size of the tumour in centimetres ; N is the lymph node status: 0 nodes = 1, 1-3 nodes = 2, >3 nodes = 3; and G is the grade of tumour: Grade I =1, Grade II =2, Grade III =3. Table 1.1 shows predicted ten-year breast cancer-specific survival and categories of the Nottingham Prognostic Index (Fong et al, 2015).

Table 1.1 Predicted 10-year breast cancer-specific survival based on NPI score

NPI	Score	Cancer-specific ten-year survival	All-cause ten-year survival
I (Excellent)	≤2.4	96%	88%
II (Good)	>2.4 but ≤3.4	93%	86%
III (Moderate)	>3.4 but ≤5.4	78%	74%
IV (Poor)	>5.4	44%	42%

Gene profile testing by microarray assay or reverse transcription-polymerase chain reaction such Oncotype DX should be used in decision making for systemic therapy. The Oncotype DX 21 gene assay is the gene profile test which is clinically validated and routinely used. It applies to hormone receptor-positive breast cancer. A 21-gene recurrence score is generated based on the level of expression of each of the 21 genes and patients are categorized into low risk (Recurrence score <18); intermediate-risk (Recurrence score ≥18 and <31) and (high-risk Recurrence score ≥31). Recently, results from a randomized controlled TAILORx trial showed that chemotherapy is unlikely to provide

significant benefit to patients > 50 years old with ER and PR positive and node-negative tumours and 21-gene recurrence score of 11 to 25 (Sparano et al, 2018).

1.4 Molecular intrinsic subtypes of breast cancer

Breast tumours should be categorized into surrogate intrinsic subtypes, defined by routine histology and IHC data (Coates et al, 2015). Genomic profiling has revealed heterogeneity of breast cancer and has classified invasive breast cancers into biologically and clinically distinct subtypes that have become known as Luminal A, Luminal B, HER2-Enriched (HER2-E) and Basal-like (Triple negative).

1.4.1 Luminal A tumours

About 40 percent of all breast cancers, usually have high expression of ER-related genes, low expression of the HER2 cluster of genes, and low expression of proliferation-related genes (Hu et al, 2006). Luminal A tumours are the most common subtype and in general, carry the best prognosis of all breast cancer subtypes (Sørlie et al, 2001).

1.4.2 Luminal B tumours

About 20 percent of breast tumours usually have relatively lower (although still present) expression of ER-related genes, variable expression of the HER2 cluster, and higher expression of the proliferation cluster. Luminal B tumours carry a worse prognosis than luminal A tumours (Voduc et al, 2010).

1.4.3 HER2-enriched

The HER2-enriched subtype constitutes about 10 to 15 percent of breast cancers (Yersal & Barutca, 2014) and it is characterized by overexpression of the HER2 and proliferation gene clusters, and low expression of the luminal and basal clusters. These tumours are most often negative for ER and PR and positive for HER2. Moreover, the HER2-enriched subtype is not the same as clinically HER2-positive breast cancer. About 30 percent of HER2-enriched tumours are clinically HER2-negative.

1.4.4 Triple-negative breast cancer (TNBC)

Although triple-negative and basal breast cancers are not the same, the triple negative phenotype is mostly made up of the basal-like molecular subtype. Basal breast cancers show the genomic expression of the "basal cluster" (a unique cluster of genes that includes the epidermal growth factor receptor or HER1, basal cytokeratins 5/6, and c-Kit; the proliferation cluster; and low expression of the hormone-receptor and HER2-related genes) (Korsching et al, 0000; Livasy et al, 2006). Approximately 20 percent of breast cancers diagnosed worldwide are TNBC. Studies suggest that TNBC tend to behave more aggressively than other types of breast cancer i.e. ER-positive, HER2-positive subtypes. TNBC is usually high grade, and the most common histology is infiltrating ductal carcinoma. There are no targeted treatments available, other than the administration of chemotherapy. Patients with triple-negative breast cancer have a poorer prognosis (Lin et al, 2012) which may be attributed to biologic

characteristics, such as BRCAness and the proliferative rate, of TNBC's. Despite available treatments, the prognosis of (TNBC) Triple-negative breast cancer [lack of expression of oestrogen receptor (ER), progesterone receptor (PR) & human epidermal growth factor receptor 2 (HER2)] and metastatic breast cancer patients remain dismal.

1.5 Staging of Breast Cancer

Breast cancer is treated after discussion in a multidisciplinary meeting involving surgeons, radiation oncologist, and medical oncologist. This approach has been linked to a reduction in breast cancer mortality (Kesson et al, 2012). Despite available treatments, the prognosis of TN breast cancer and metastatic breast cancer patients remain dismal. According to Tumour, Nodes, Metastases (TNM) stage (Table 1.2) , five-year relative survival rates by stage for patients presenting with stage I, IIA, IIB, IIIA, IIIB, and IV disease were 95, 85, 70, 52, 48, and 18 percent, respectively (Newman, 2009).

Table 1.2 TNM Staging in Breast Cancer (Hortobagyi et al, 2017)

T Category	**Criteria**
Tx	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tisb	DCIS
Tis (Paget)	Paget disease of the nipple NOT associated with invasive carcinoma and/or DCIS in the underlying breast parenchyma.
T1	Tumour ≤2 cm (greatest dimension)
T1mi	Tumour ≤1 mm in greatest dimension.
T1a	Tumour between >0.1 cm and ≤ 0.5 cm
T1b	Tumour >0.5 cm but ≤1 cm
T1c	Tumour >1 cm but ≤20 cm
T2	Tumour >2 cm but ≤5 cm
T3	Tumour >5 cm

T4	Tumour of any size with direct extension to the chest wall and/or to the skin (ulceration or macroscopic nodules
T4a	Extension to the chest wall; invasion
T4b	Ulceration and/or ipsilateral macroscopic satellite nodules and/or oedema (including peau d'orange) of the skin that does not meet the criteria for inflammatory carcinoma.
T4c	Both T4a and T4b are present.
T4d	Inflammatory carcinoma
N Category	**Criteria**
Nx	Regional Lymph nodes can't be assessed
N0	No regional lymph node metastases
N1	Metastases to movable ipsilateral Level I, II axillary lymph nodes(s)
N1mid	Micro-metastases (approximately 200 cells, >0.2 mm, but ≤2.0 mm)

cN2	Metastases in ipsilateral Level I, II axillary lymph nodes that are clinically fixed or matted; or in ipsilateral internal mammary nodes in the absence of axillary lymph node metastases
N2a	Metastases in ipsilateral Level I, II axillary lymph nodes fixed to one another (matted) or to other structures.
N2b	Metastases only in ipsilateral internal mammary nodes in the absence of axillary lymph node metastases.
cN3	Metastases in ipsilateral infraclavicular (Level Ill axillary) lymph node(s) with or without Level l, II axillary lymph node involvement; or in ipsilateral internal mammary lymph node(s) with Level l, II axillary lymph node metastases; or metastases in ipsilateral supraclavicular lymph node(s) with or without axillary or internal mammary lymph node involvement.

N3a	Metastases in ipsilateral infraclavicular lymph node(s).
N3b	Metastases in ipsilateral internal mammary lymph node(s) and axillary lymph node(s).
N3c	Metastases in ipsilateral supraclavicular lymph node(s).
M Category	**Criteria**
M0	No clinical or radiographic evidence of distant metastases
M1	Distant detectable metastases as determined by classic clinical and radiographic means and/or histologically proven >0.2 mm

1.6 Management of Breast cancer

1.6.1 Early stage breast cancer

This includes stage I, IIA, or a subset of stage IIB disease (T2N1). The mainstay of treatment for patients with early-stage breast cancer is to undergo primary surgery (lumpectomy or mastectomy) to the breast and regional lymph nodes

with or without adjuvant radiotherapy. Following definitive local treatment, adjuvant systemic therapy is considered depending on primary tumour characteristics, such as tumour size, grade, number of involved lymph nodes, the status of oestrogen (ER) and progesterone (PR) receptors, expression of the human epidermal growth factor 2 (HER2) receptor and Oncotype DX recurrence score.

1.6.2 Locally advanced breast cancer (LABC)

This includes stage IIB – stage IIIC disease. LABC is managed by using a multimodality approach including systemic and locoregional therapy. Most patients with LABC receive neoadjuvant systemic therapy to induce a tumour response before surgery and enable breast conservation. All patients normally undergo surgery (lumpectomy or mastectomy) following neoadjuvant systemic therapy regardless of response to chemotherapy. After surgery, adjuvant systemic therapy is considered based on the patient's tumour characteristics. For patients who received the full course of planned neoadjuvant chemotherapy, endocrine therapy is used for up to 10 years in hormone receptor-positive breast cancer and adjuvant trastuzumab is used for one year in patients with HER2-positive breast cancer. Adjuvant endocrine therapy reduces the risk of breast cancer recurrence and breast cancer-related mortality (Group, 2011) and one year of trastuzumab significantly improve disease-free survival and OS (Moja et al, 2012).

1.6.3 Metastatic Breast cancer

Approximately 5 percent of patients are diagnosed with stage 4 metastatic disease on initial presentation. The main aims of systemic treatment for metastatic breast cancer are improved survival, alleviation of symptoms, and maintenance or improvement in the quality of life, despite the toxicity associated with treatment. The median survival for metastatic breast cancer is 18 to 24 months (Lee et al, 1992), though this varies widely based on the subtype of a tumour, sites of metastatic involvement, and burden of metastatic disease, and some patients experience long-term survival. Most patients with metastatic breast cancer are treated with systemic medical therapy including chemotherapy, endocrine therapy, and/or targeted therapies. Patients who are not fit for systemic therapy , they get best supportive care measures to palliate their symptoms.

1.7 DNA Repair defects in breast cancer

About 20% of TNBC harbour a breast cancer gene (BRCA) mutation, particularly in BRCA1 (Gonzalez-Angulo et al, 2011). BRCA1 plays important role in DNA damage response and cell cycle checkpoint control via homologous recombination repair pathway (Kastan & Bartek, 2004). The presence of HRR defects in sporadic breast cancer come from functional studies. Germline mutated BRCA1 breast cancers and sporadic triple-negative breast cancers share various common characteristics, which has given rise to the term “BRCAness” phenotype in sporadic breast cancer (Turner et al, 2004b). There is emerging evidence that approximately 10-20% of non-familial breast cancers harbour

homologous recombination repair defects that may be useful targets for therapy (Hannemann et al, 2008).

The defects in homologous recombination DNA repair pathways can be exploited to eradicate tumours by blocking back up DNA repair pathways. This approach is called synthetic lethality. One good example of this approach is the use of DNA repair inhibitors against poly (ADP) ribose polymerase (PARP) in BRCA deficient tumours, which are defective in HR (Bryant et al, 2005; Farmer et al, 2005). Phase I and Phase II trials of PARP inhibitors that enhance the effects of anticancer treatments suggest that tumours that are deficient in HRR are susceptible to PARP blockade (Curtin, 2012).

1.7.1 The brief overview of DNA damage and DNA repair pathways

The maintenance of genomic stability is critical for every living organism. Cells have evolved diverse and highly efficient DNA repair pathways to counteract the effects of both endogenous and exogenous insults from genotoxic agents. These deleterious effects are encountered by continuous genome surveillance by various DNA repair pathways such as BER (Base excision repair), NER (Nucleotide excision repair), DSB (Double-strand break repair), MMR (Mismatch repair) and mitochondrial DNA repair. On the one hand, efficient DNA repair machinery in an organism leads to genomic stability and protects the genome from harmful effects of stress. On the other hand, defective DNA repair machinery would ultimately lead to genomic instability, cancer predisposition, and premature aging.

There are various cellular response mechanisms to DNA damage which include: (a) activation of DNA damage checkpoints and regulation of cell cycle progression to allow time for repair and to prevent transmission of damaged or incompletely replicated chromosomes; (b) tolerance of damage; (c) initiation of DNA repair and removal of damage; (d) transcriptional response, which causes changes in the transcriptional profile that may be beneficial to the cell; and/or (e) induction of apoptosis to eliminate heavily damaged cells.

Several discrete DNA repair pathways have evolved to preserve the integrity of the human genome. These repair pathways are so critical that defects in DNA damage repair mechanisms are known to increase cancer predisposition. Various syndromes have been defined which are associated with specific DNA repair defects such as hereditary non-polyposis carcinoma coli (HNPCC) or BRCA-deficient breast and ovarian cancer syndromes (which are important in Homologous recombination) (Hoeijmakers, 2001; Sweasy et al, 2006). Polymorphisms within DNA repair genes may also confer suboptimal DNA repair capacity, hence influencing cancer susceptibility and prognosis (Frosina, 2004; Goode et al, 2002). Loss of efficiency of one or more DNA repair mechanisms, whether by germ line inheritance or sporadic mutation, accelerates the rate of accumulation of subsequent mutations by 100 to 1000 times, with selective pressure favouring those mutations that drive carcinogenesis – the 'mutator phenotype' (Loeb et al, 1974).

On the one hand, DNA damage in healthy cells may pose a significant threat to cell survival and genomic stability. On the other hand, the cytotoxicity of radiotherapy and many anticancer agents such as cisplatin and carboplatin is directly related to their ability to produce genomic DNA damage. The ability of cytotoxic agents and radiotherapy to cause DNA damage in tumour cells has been exploited in cancer treatment. However, tumour cells can often recognize this damage and instigate DNA repair leading to treatment failure or resistance (Madhusudan & Wilson III, 2013). Thus the development of drugs with the ability to selectively block repair of DNA damage in tumour cells may represent an exciting strategy to improve the effectiveness of cytotoxic agents and improve patient outcomes.

Multiple DNA repair pathways as described in Table 1.3 (Madhusudan & Wilson III, 2013) exist in humans, each of which is able to process specific DNA lesions. The key pathways include base excision repair; nucleotide excision repair (NER); O6-methylguanine DNA methyltransferase (MGMT) (direct repair); mismatch repair (MMR); homologous/non-homologous end-joining (HR/NHEJ); and inter-strand crosslink repair. The presence of multiple repair pathways means that should one pathway fail often a second pathway is able to take over.

Table 1.3 Summary of the main DNA repair pathways in human cells

Repair Pathway	**Primary DNA Substrate**	**Relevant Anticancer agent(s)**
Base Excision Repair (BER)	Apurinic/apyrimidinic (AP) sites; oxidative, alkylative or spontaneous base damage; single strand breaks	Ionizing radiation, antimetabolites, alkylating agents, topoisomerase inhibitors.
Nucleotide excision repair (NER)	Bulky DNA adducts, UV induced photoproducts, intra-strand cross-links	alkylators, crosslinking agents
Mismatch repair (MMR)	Mis-paired nucleotides, insertions/deletion loops; and certain base modifications	alkylators, crosslinking agents, antimetabolites

Direct Repair/MGMT	O^6 Methylguanine	Alkylators such as temozolomide
Recombination Repair -Homologous recombination (HR) -Non-homologous end joining repair (NHEJ)	DNA double-strand breaks; collapsed replication forks Double strand breaks	Radiation, alkylating agents, crosslinking agents, topoisomerase poisons, failure to repair single-strand break

1.8 RECQ helicases

RECQ helicase is a major family of proteins that are actively involved in genome surveillance. The RECQ protein family is a highly conserved group of DNA helicases, which play diverse roles in various DNA metabolic processes such as DNA replication and recombination repair (Bachrati & Hickson, 2003; Brosh & Bohr, 2007; Hanada & Hickson, 2007a; Sharma et al, 2006a). This helicase family plays important roles in various DNA repair pathways including double-strand break repair, inter-strand cross-links (ICLs), recovery of stalled or broken replication forks and suppression of illegitimate recombination (Chakraverty & Hickson, 1999; Hickson, 2003; Shen & Loeb, 2000a).

The overexpression of many RECQ DNA helicases is seen in rapidly proliferating tumours and transformed cells. This up-regulation is important for cancer cells growth and for developing resistance to DNA damaging cytotoxic agents (Brosh, 2013a; Futami et al, 2010b; Kawabe et al, 2000; Su et al, 2010a; Turley et al, 2001). This further suggests that in the rapidly proliferating tumours, there is a requirement of increased DNA damage response due to the lesions formed secondary to replicative stress induced by rapid proliferation. On the contrary, under-expression of DNA helicases in hereditary helicase disorders lead to genomic instability and induce carcinogenesis. Thus DNA helicases act as a prominent class of genome caretakers. (Brosh, 2013a)

1.8.1 Structure of RECQ Family

RECQ family is defined by seven highly conserved domains. The helicase domain is usually centrally located in the protein. The biochemical analysis has shown that RECQ1, WRN and BLM helicases contain DNA unwinding and ATPase activities. WRN protein has exonuclease activity in its NH2 terminal region. All five helicases migrate to the nucleus to perform their biological role. WRN, BLM, RECQL5 and RECQL1 proteins have nuclear localization signals (NLS) composed of basic amino acids. One study suggested that RECQL4 localizes to the nucleolus in response to oxidative stress (Woo et al, 2006). However, NLS of RECQL4 helicase has not been identified yet. Figure 1.1 shows the structure of RECQ family helicases (Croteau et al, 2012b).

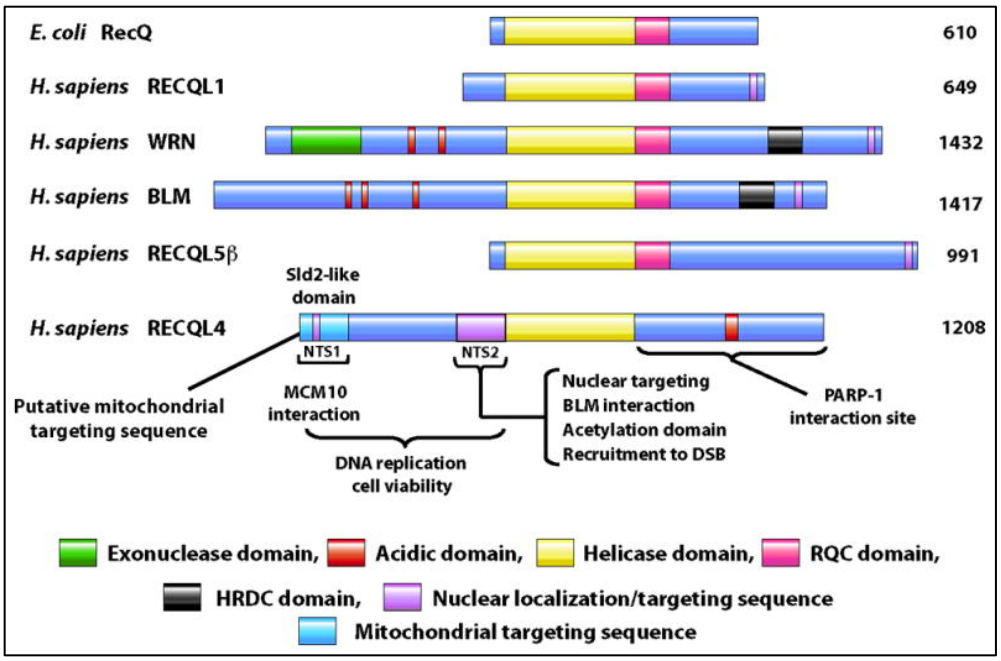


Figure 1.1 RECQ Family Structure

Structure of human RecQ helicases: All the RecQ helicase members have a conserved helicase domain (yellow). RECQL4 has two nuclear targeting sequences (NTS1 and NTS2), however all others helicases have the nuclear localization signal (*purple*). The RQC (pink) & helicase and RNase C-terminal (HRDC) domain (black) are present at the C-terminus of WRN and BLM helicase. The WRN protein is the only human RecQ helicase possessing an exonuclease domain (green). A mitochondrial targeting sequence is located at the N-terminus of RECQL4 (light blue).

1.8.2 Disease phenotypes associated with RECQ helicases

In humans, five RECQ helicases (RECQL1, BLM, WRN, RECQL4, and RECQL5) have been identified and three of them namely, WRN, BLM, and RECQL4 have been associated with autosomal recessive disorders which are characterized by genomic instability, premature aging and predisposition to

various cancers. This suggests the role of these helicases as tumour suppressor proteins. Werner syndrome (WS) is associated with defects in WRN protein, Bloom syndrome (BS) is associated with defects in BLM helicase, and Rothmund Thomson syndrome (RTS) is associated with defects in RECQL4 as summarised in (Table 1.4).

Werner's syndrome (WS) patients have short stature, juvenile cataracts, atrophy of the skin, greying and loss of hair, early hypogonadism, diabetes mellitus, arteriosclerosis, osteoporosis, and rare tumours (Goto et al, 1996). These patients die in the fourth or fifth decade, mainly due to death caused by malignant tumour or cardiovascular infarctions. The gene responsible for WS was identified in 1996 by positional cloning (Yu et al, 1996) . Bloom's syndrome (BS) patients have a short stature, skin disorders including hyperpigmentation, immunodeficiency, telangiectasia, male infertility, and various malignant neoplasia. Cells show a high rate of homologous chromosome recombination (sister chromatid exchanges) (German, 1993). The gene responsible for BS was identified in 1995 (Ellis et al, 1995). Rothmund-Thomson's syndrome (RTS) patients have growth deficiency, photosensitivity with poikilodermatous skin changes, early greying and hair loss, cataracts, and cancer predisposition , especially bone sarcomas(Lindor et al, 1996). The gene responsible for a subset of RTS patients, referred to here as RECQL4 or RTS, was identified in 1999 (Kitao et al, 1999).

Although no disease phenotypes have been associated with RECQL5 and RECQL1, the preclinical studies have suggested the role of RECQL1 and

RECQL5 in maintaining genomic stability (Singh et al, 2009). The poly-morphisms of RECQL1 are associated with the worst outcome in pancreatic cancer patients (Li et al, 2006a; Li et al, 2006b). Furthermore, in one preclinical study, RECQL5-deficient cells showed high frequencies of spontaneous DNA double-strand breaks and homologous recombination (HR) Moreover, these RECQL5 depleted cells were prone to replication stress induced chromosomal rearrangements and cancer susceptibility (Hu et al, 2007a).

Table 1.4 DNA helicase disease phenotypes

Gene/ Chromosomal location	**Disease**	**Cancer type**
WRN (8p12-11.2	Werner syndrome	Thyroid tumours, melanomas, haematological and lymphoid malignancies, meningiomas, and bone sarcomas.
BLM (15q26.1) SCE- 10 times elevated	Bloom syndrome	Adult epithelial tumours, haematological malignancies and paediatric tumours.
RECQL4 (8q24.3)	RTS*	Bone sarcomas, and lymphomas.

SCE- Normal or slightly elevated		

* Rothmund-Thomson's syndrome (Brosh Jr, 2013).

1.8.3 Role of RECQ helicases in DNA DSB repair & replication

Doubles strand breaks (DSBs) are very harmful to human genome, leading to genomic instability and cancer predisposition if left unrepaired. DSBs are repaired via at least two different pathways as shown in Figure 1.2 . The first pathway is called homologous recombination repair (HRR) which is a highly accurate, error-free repair mechanism requiring homologous sequences from the sister chromatids. HR plays the main role in late S-G2 phase of cell cycle. The other pathway is called non-homologous end joining (NHEJ) repair pathway. The NHEJ pathway is an error-prone mechanism as it is independent of terminal DNA sequence homology. NHEJ plays role in the G1 to early S-phase of the cell cycle although may be involved throughout the cell cycle. (Hefferin & Tomkinson, 2005; Liang et al, 1996; Lieber et al, 2003; Shrivastav et al, 2007; Takata et al, 1998; Thompson & Schild, 2002b)

The process of HRR is divided into four phases: 1). Resection of DNA ends; 2). Formation of RAD51 filament; 3). Strand invasion and Holliday junction (HJ) formation; and 4).Dissolution of HJs.

When DSBs are generated, they are first recognized by the MRE11-RAD50-NBS1 (MRN) complex. The MRN complex promotes resection either by

recruiting Top3 (topoisomerase 3) - Rmi1 complex to DNA ends or by recruiting CtIP to DNA ends. Top3 - Rmi1 complex together with RPA stimulates BLM helicase to unwind DNA ends. This leads to the generation of 5' flaps which then, in turn, are cleaved by Dna2 endonuclease. The MRN-CtIP complex also recruits Exo1 exonuclease to promote further resection. The ssDNA formed by resection is first recognized by ssDNA-binding protein RPA. BRCA2 also plays a critical role in the formation of RAD51 filament. It increases the binding of RAD51 to ssDNA and helps RAD51 to overcome the inhibition by RPA during binding to ssDNA. The Rad51 filament then enables the ssDNA overhangs to search for homologous sequences and formation of a displacement loop (D-loop). To prevent illegitimate recombination, RECQ helicases either disrupt the Rad51 filaments or prevent the formation of D-loop. HRR generates either crossover or non-cross over products depending upon how HJs are resolved. If they are resolved via synthesis-dependent strand annealing (SDSA), non-crossover products are formed. If they are resolved by BLM-TOPO3 complex, then crossover products are generated. Thus, RECQ helicases carry out important functions at various steps of the HR pathway including the initial step of end resection, disruption of Rad51 filament formation or D-loop formation to prevent illegitimate recombination, and resolution of double holiday junctions by branch migration activity. (KELLEY, 2012; Lisby et al, 2004; Sung et al, 2003; Wyman et al, 2004)

Another important pathway for DSB repair is NHEJ. The detection of DSB is carried out by a ring-shaped Ku heterodimer (Ku70/80) which tethers to the

broken ends of DNA. Ku then recruits DNA dependent protein kinase (DNA-PKcs) which is a member of the PIKK family of serine-threonine protein kinases. The final step of ligation is carried out by XRCC4/ligase IV.

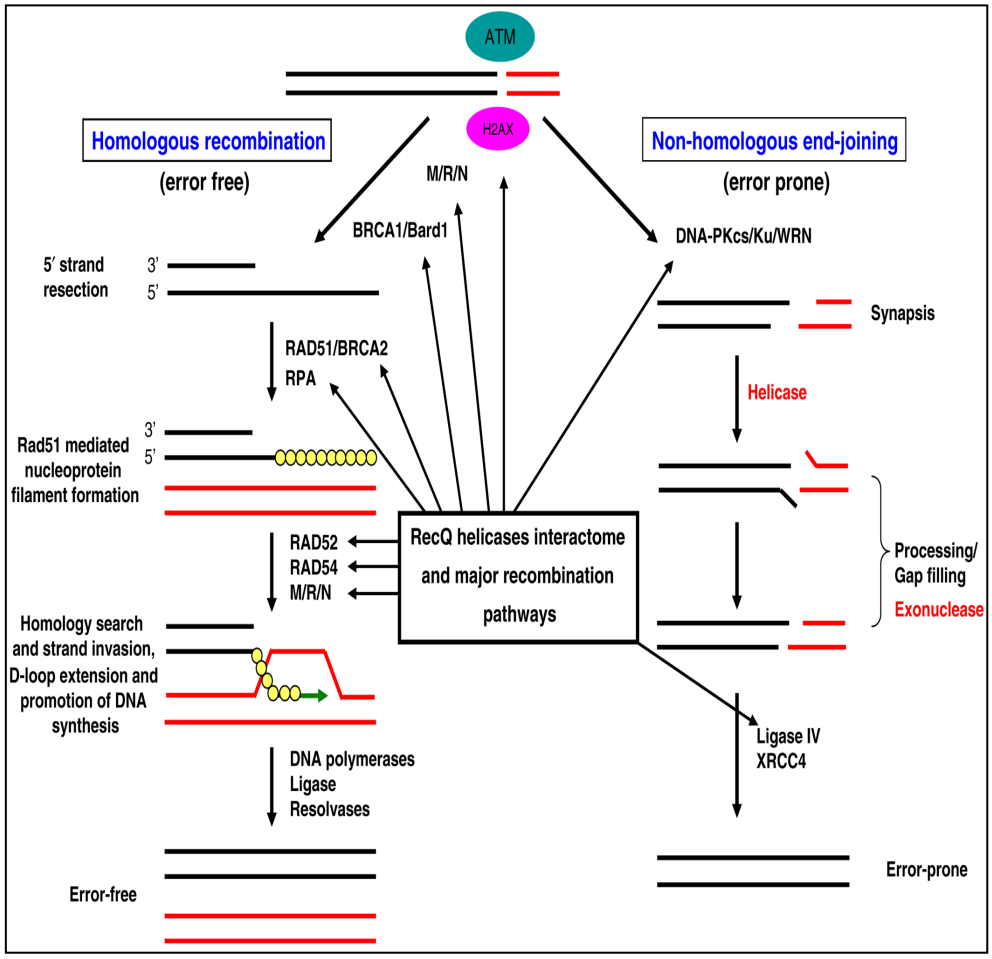


Figure 1.2 Double-strand break repair pathways (Homologous recombination & Non-homologous end joining)

1.8.4 Evidence supporting the role of RECQ helicases in DSB repair and replication

1.8.4.1 WRN

DNA interstrand cross-links (ICLs) create stalled replication forks leading to the formation of DSBs which are repaired by HR pathway. WRN depleted cells are hypersensitive to ICLs. WRN interacts with BRCA1 and both work in a coordinated fashion to repair ICLs. BRCA1 also stimulates WRN helicase activity on forked and Holliday junction substrates. (Cheng et al, 2006). WRN has been shown to be associated with the MRE11 complex via Nbs1 subunit. There is also an increased association between WRN and the Mre11 complex after cellular exposure to γ-irradiation (Cheng et al, 2004). WRN also interacts with ATR and RAD51, suggesting its role in the later HR steps of ICL-repair (Otterlei et al, 2006b). There is evidence to suggest that WRN modulates DNA replication checkpoint upstream of ATR-Chk1 (Cheng et al, 2008; Lee et al, 2010). One study suggested that WRN depletion in cells exposed to ICL induced double-strand breaks, resulted in checkpoint defect and impaired ATM activation(Cheng et al, 2008). WRN also plays an important role in the resolution of Holiday junctions (HJs), formed as an HR intermediate (Constantinou et al, 2000; Rodriguez-Lopez et al, 2007).

WRN also plays a role in NHEJ pathway of DSB repair. WRN forms a complex with both Ku and DNA-PKcs as well as with the XRCC4/ ligase IV complex and regulates their functions (Cooper et al, 2000; Critchlow & Jackson, 1998;

Hartley et al, 1995; Kanaar et al, 1998; Kusumoto et al, 2008; Li & Comai, 2002). Ku and XRCC4-DNA ligase IV complex stimulate WRN exonuclease activity but not WRN helicase activity. (Cooper et al, 2000; Critchlow & Jackson, 1998; Hartley et al, 1995; Kanaar et al, 1998; Kusumoto et al, 2008; Li & Comai, 2002)

1.8.4.2 BLM

Bloom syndrome (BS) cells show markedly raised levels of sister chromatid exchanges (SCEs) (Chaganti et al, 1974). BLM perform critical roles at various steps of DSB repair such as stimulation of strand resection in association with Exo1, disruption of Rad51 filament formation, and dissolution of HJs by branch migration activity during the later stages of HR (Singh et al, 2012a). BLM is also implicated in affecting double Holliday junction (DHJ) dissolution processes of HR. To accomplish its various biological functions, BLM interacts with various proteins participating in HR such as Exo1 and RAD51 (Wu et al, 2001). It also interacts with other DNA repair proteins e.g. ATM, RPA, Mus81, MLH1, and MSH6 (Beamish et al, 2002; Braybrooke et al, 2003; Pedrazzi et al, 2003; Sharma et al, 2006a; Singh et al, 2012a).

BLM also plays an integral part in the repair of stalled or collapsed replication fork during the S-phase of the cell cycle (Singh et al, 2009). When hydroxyurea-induced replicative stress occurs, BLM localizes to repair centres at stalled replication forks (Davalos et al, 2004). Studies have also shown that BLM interacts with a topoisomerase (Topo IIIα), both localize to PML nuclear bodies

and help in the resolution of recombination intermediates (Hu et al, 2001; Wu et al, 2000).

1.8.4.3 RECQL1, RECQL4, and RECQL5

There is evidence to suggest the involvement of RECQL4 in DSB repair. One study has shown that RECQL4 and RAD51 form complex after etoposide chemotherapy, indicating their role in DSB repair via HR pathway (Petkovic et al, 2005). RECQL1 also interact with RAD51 implicating its role in DSB repair via HR (Sharma & Brosh Jr, 2007). RECQL1 has also been shown to have HJ branch migration activity (LeRoy et al, 2005). In preclinical studies, RECQL5 depleted cells have shown increased susceptibility to develop malignant phenotype. RECQL5 also binds to RAD51 and displace it from ssDNA, inhibiting D-loop formation. Thus RECQL5 may reduce carcinogenesis by disrupting RAD51 filaments (Hu et al, 2007a).

1.8.5 RECQ Helicases and Telomere maintenance

Telomeres are protective caps at the end of chromosomes and in their absence, chromosome develops aberrations and genomic instability. Hence, Telomere maintenance is essential for genomic stability and RECQ helicases play important role in this process. Some cells avoid this crisis of telomere shortening by using an alternative lengthening of telomere (ALT) pathways. There is evidence to suggest that WRN and BLM proteins help to segregate G – quadruplex structures in telomeres (Mohaghegh et al, 2001). TRF2 protein, a part of telomere nucleoprotein complex as shown in Figure 3, interact with

WRN, BLM and RECQL4 helicases at telomeric D-loop structures and increase helicase activity (Opresko et al, 2005). WRN has also been shown to repress recombination at telomeres by interacting with the Ku 70/80 heterodimer (Karmakar et al, 2002). The role of different RECQ helicases in various processes at telomere (Singh et al, 2012a) is shown in Figure 1.3.

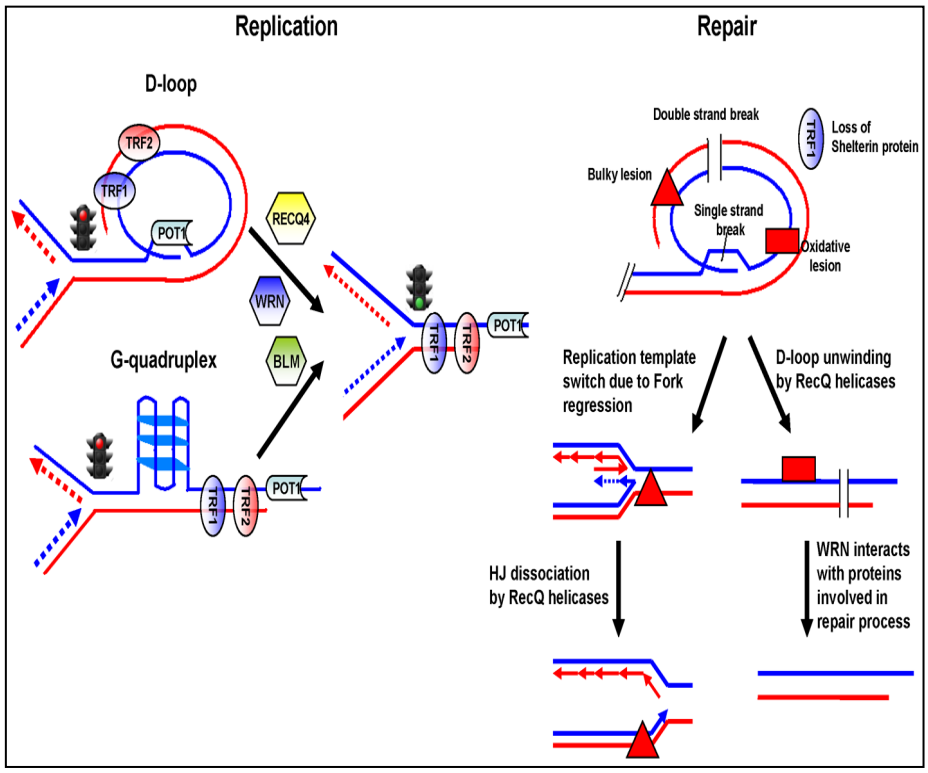


Figure 1.3 Role of RECQL4, WRN & BLM helicases in the maintenance of telomeres

1.8.6 Effects of RECQ helicase expression on tumour proliferation and growth

There is evidence to suggest that diminished or absent helicase expression leads to reduced cell proliferation and tumour growth. Arai, A., et al. demonstrated

that RECQL1 and WRN proteins are highly expressed in hypopharyngeal carcinomas and siRNA-mediated gene silencing of RECQL1 and WRN reduced tumour growth in vitro (Arai et al, 2011). Mendoza-Maldonado, R., et al. observed that depletion of RECQ1 by RNAi in T98G and U-87 glioblastoma cells resulted in marked reduction of cellular proliferation (Mendoza-Maldonado et al, 2011a). Moser, R., et al. showed that the conditional silencing of WRN expression in c-MYC overexpressing non-small cell lung cancer mouse xenografts reduced cancer growth and proliferation (Moser et al, 2012). Mao, F. J., et al. observed in an in-vitro study that WRN and BLM depleted (by short hairpin RNA) U2OS cells showed reduced proliferation (Mao et al, 2010a). Su, Y., et al. showed reduced metastatic prostate cancer cells invasiveness and carcinogenicity in RECQL4 depleted nude mice (Su et al, 2010a). Ramamoorthy, M., et al. observed that RECQL5-targeted shRNA HeLa cells failed to proliferate at the same rate as their scrambled shRNA counterparts (Ramamoorthy et al, 2012a). There are other studies which suggest that RECQL1 and RECQL5 are required for cancer cell proliferation (Arai et al, 2011; Futami et al, 2008a; Futami et al, 2008b; Futami et al, 2010b; Mendoza-Maldonado et al, 2011a)

Therefore, on the basis of the above preclinical studies, we can conclude that expression of RECQ helicases is normally increased in the proliferating cells and by knocking down the expression of these helicases, in cancer cells, proliferation and growth can be reduced.

1.8.7 RECQ helicase expression and sensitivity to DNA damaging chemotherapies

The preclinical data suggest that helicase depleted cells show hypersensitivity to chemotherapy and radiotherapy. RECQ1 depleted glioblastoma T98G and U-87 cells are hypersensitive to hydroxyurea or temozolomide treatment (Mendoza-Maldonado et al, 2011a). Arai, A., et al. demonstrated that the enhanced antitumor activity of cisplatin in WRN depleted hypopharyngeal carcinoma xenograft (Arai et al, 2011). Mao, F. J., et al. observed in vitro study that WRN and BLM depleted (by short hairpin RNA) U2OS cells showed hypersensitivity to camptothecin, cisplatin or 5-FU chemotherapy(Mao et al, 2010a). RECQL5-depleted HeLa cells accumulate persistent 53BP1 foci followed by γ-irradiation (Popuri et al, 2012c). Therefore, on the basis of data available from preclinical studies, we can conclude that the low expression of helicase in cancer cells is associated with chemotherapy and radiotherapy sensitivity.

1.8.8 Helicases as targets for anticancer therapy

Developing approaches to target DNA repair to improve cancer therapy is currently an important area of research. We are entering an age where personalized anticancer therapies make use of existing deficiencies in DNA repair pathways in tumours (Curtin, 2012). It has been proposed that inhibition of DNA repair proteins may provide an opportunity to selectively target cancer by enhancing the cytotoxicity of conventional anticancer therapies such as DNA-damaging chemotherapeutics or ionizing radiation therapy. These

conventional therapies can impose problems because of their cytotoxic effects on normal cells. Moreover, the tumours cells also have the ability to develop resistance to these therapies (Bouwman & Jonkers, 2012).

The overexpression of DNA helicases in rapidly proliferating cancer cells suggests their involvement in resistance to DNA-damaging chemotherapeutics. Thus the overexpression or reduced expression of helicase proteins may serve as a predictive biomarker for personalized cancer therapy (Curtin, 2012). The preclinical studies of helicases depletion using RNA interference strategy, as discussed earlier, further support the idea that DNA helicases influence cancer cell proliferation and survival after chemotherapeutic DNA damage. DNA helicases have gained significant interest in recent years as drug targets because of their involvement in the DNA damage repair in rapidly proliferating tumour cells.

1.8.9 How to target Helicases in cancer therapeutics

There are two main approaches which can be adapted to target helicases. These are RNAi (RNA interference) and small molecule inhibitors.

1.8.9.1 RNAi-based therapeutic approaches

RNAi-based therapeutic approaches can effectively silence gene expression with sequence specificity; hence it may represent the new frontier of pharmaceutical drug treatments. One research group investigated the hypothesis that depletion of DNA repair proteins may render cancer cells more sensitive to DNA

damaging agents used in anticancer therapy (Collis et al, 2003). siRNA down-regulated expression of the DNA damage response kinases ATM, ATR, and DNA-PKCS resulted in an increased sensitivity of human prostate cancer cells to DNA damaging agents or radiation. A number of studies have utilized RNA interference technology to evaluate the consequences of WRN deficiency on DNA damage response, telomere maintenance, and chromosomal stability. Knockdown of WRN expression by RNA interference in cell lines derived from c-myc transformed Burkitt's lymphomas also resulted in slow cell proliferation and increased apoptosis (Grandori et al, 2004).

1.8.9.2 Small-molecule helicase inhibitors

Small molecules offer a number of advantages for targeting DNA repair proteins since the activity of helicase can be turned on or off temporarily and may be targeted to certain tissues.

A small molecule inhibitor (NSC 19630) of WRN helicase was identified recently which inhibited proliferation of human cancer cells grown in culture. NSC 19630 sensitized cancer cells to PARP inhibitors and acted synergistically with topotecan chemotherapy to reduce cellular proliferation. (Aggarwal et al, 2011a). Recently a small molecular inhibitor (ML216) of BLM was found via high output screening (Nguyen et al, 2013).

Given the key role of RECQ helicases in genomic stability and their emergence as promising drug targets, we investigated the role of RECQ helicases as biomarkers in sporadic breast cancers.

1.9 Aims and Hypothesis

As discussed previously, there is a large body of preclinical and clinical evidence to suggest that RECQ helicases play an integral role in genomic surveillance. Their function is up-regulated in proliferating tumours and their depletion in cancer cells render them sensitive to anticancer therapies. Therefore, we hypothesized that RECQ helicase expression may be de-regulated in human sporadic breast cancers, contributing to the pathogenesis of breast cancer. We aimed to investigate the expression of five RECQ helicases (RECQL1, RECQL4, RECQL5, WRN, and BLM) at mRNA and protein level in large cohorts of breast cancers and correlate to clinicopathological variables and survival outcomes.

1.9.1 Aims and Hypothesis:

- To investigate the prognostic and predictive significance of DNA RECQ helicases mRNA expression in Breast cancer.
- To investigate the prognostic and predictive significance of DNA RECQ helicases protein expression in Breast cancer.
- To evaluate the potential to target BLM-proficient and BLM-deficient cell lines with Cisplatin to induce lethality.

Chapter 2

Material and Methods

2 Material and Methods

2.1 DNA helicase protein expression profiling by immunohistochemistry

Investigation of the protein expression of RECQL1, RECQL4, RECQL5, BLM and WRN in breast cancer was performed in two patient cohorts. The first cohort was a series of 1650 primary invasive breast cancer patients who were diagnosed between 1986 and 1999 and entered into the Nottingham tenovus series. The second cohort used for evaluation was an independent series of 252 ER-negative invasive breast cancers diagnosed and managed at Nottingham University Hospitals between 1999 and 2007. Breast tumours were arrayed in tissue microarrays (TMAs) constructed with two replicate 0.6 mm cores from centre and periphery of the tumours.

2.1.1 Construction of Tissue Microarray (TMA)

The construction of Breast TMA was done by tumour site specialised histopathologists who identified and marked formalin-fixed paraffin-embedded tissue blocks containing tumour sample on haematoxylin and eosin stained slides. Two replicate 0.6mm cores from the centre and periphery of the tumours were taken and arrayed into a recipient paraffin block using a tissue puncher/arrayer (Beecher Instruments, Silver Spring, MD, USA). Four-micron sections of the tissue array block were cut and placed on Superfrost Plus slides (Thermo Scientific) for immunohistochemical staining.

2.1.2 Immunohistochemistry technique

Immunohistochemical staining was performed using the Leica Novolink Max Polymer detection system (RE7280K-1250 tests), Thermo Scientific Shandon Sequenza chambers (REF:72110017), and the Leica Bond Primary antibody diluent (AR9352). Each of these was used as per the manufacturer's instructions (Leica Microsystems). To ensure that tissue will stick to the slide for the duration of the IHC protocol, slides were heated to 60°C for 10 minutes and then allowed to cool. The tissue was prepared first by removing the paraffin wax with xylene and then rehydrating it using decreasing concentrations of alcohol using the Leica Autostainer XL (Leica ST5010) (Program 1). To break the crosslinks, antigen retrieval was done by placing the slides in a bath of citrate buffer (pH 6) and heated to 95°C (20 minutes) in a microwave (Whirlpool JT359 Jet Chef 1000W). The slides were then cooled in water to bring them back to room temperature ready for mounting in the Shandon sequenza plate system. The slides were then loaded onto the sequenza plates while submerged. Once assembled the slide/plate was loaded into the Shandon sequenza system holder by firmly holding the two elements together until the plate engages with the holder, ensuring no air gets between them. Slides were then incubated with peroxidase block for 5 minutes to remove all the intrinsic peroxidase activity in the tissue, enhancing the final signal by removing any noise. This was followed by a protein block for 5 minutes to block the non-specific binding of the primary antibody and the polymer. For WRN, protein block step was replaced with Ultra V block (TA-060-UB; Thermo-Fischer Scientific). Slides were then exposed to

optimised primary antibody at the chosen concentration for 60 minutes (overnight for BLM and WRN), followed by post-primary block (Rabbit anti-Mouse IgG, used to detect mouse antibodies) for 30 minutes each to ensure that any alternative targets are masked preventing nonspecific binding of the polymer. This was then followed by polymer block (used to detect rabbit immunoglobulins including the post primary and primary rabbit antibody bound to tissues) for 30 minutes. DAB (3,3' – Diaminobenzidine) working solution (1:20, chromogen: DAB substrate) was applied for 5 minutes to provide the means of labelling the target site of the primary antibody and to produce a peroxidase reaction that results in the formation of a brown precipitate at the reaction site. Two Tris-buffered saline (TBS) 5-minute washes were performed between all incubation steps. Haematoxylin was used as a counterstain for 6 minutes and slides were then washed with distilled water to prevent further staining. 100µl of each reagent/antibody was used per slide for each step. Slides were dehydrated using the alcohol baths then cleared in xylene wash using the Leica Autostainer XL (Program 2). Sections were mounted under coverslips using DPX (a mixture of distyrene, plasticizer, and xylene) and allowed to dry overnight.

2.1.3 Primary antibodies

The TMAs were immunohistochemically profiled for RECQL1, RECQL4, RECQL5, BLM, WRN by myself , however the immunohistochemical profiling of TMAs for several other biological antibodies was done by other researchers at Nottingham University (Table 2.1). The final concentrations of Primary

antibodies used for immunohistochemistry (IHC) analysis are shown in Table 2.1. Western blot analysis as described in Section 2.3.2.2 was used to evaluate the specificity of antibodies, before using them for IHC.

Table 2.1 Antigens, primary antibodies, clone, source, optimal dilution and scoring system used for immunohistochemical markers used for analysis

Antigen	**Antibody/ Clonality**	**Clone**	**Source**	**Antigen Retrieval**	**Dilution / Incubation Time**	**Distribution**	**Scoring system**	**Cut-offs**
RECQL1	Rabbit Ab (Polyclonal)	A300-450A	BETHYL	Citrate pH6	1:1000 60 min (RT)	Nuclear	H-score	≥215 (High)
RECQL4	Rabbit Ab (Polyclonal)	25470002	Novus Biological	Citrate pH6	1:175 60 min (RT)	Nuclear Cytoplasm	H-score	≥30 (High) ≥100 (High)
RECQL5	Rabbit Ab (Polyclonal)	HPA-029971	Sigma	Citrate pH6	1:100 60 min (RT)	Nuclear	H-score	≥10 (High)
BLM	Rabbit Ab (Polyclonal)	NBP1-89929	Novus Biological	Citrate pH6	1:100 Overnight	Nuclear and Cytoplasm	H-score	≥50 (High)
WRN	Rabbit Ab (Polyclonal)	NBP1-87143	Novus Biological	Citrate pH6	1:100 Overnight	Nuclear Cytoplasm	H-score	≥116 (High) ≥20 (High)

ATR	Rabbit Ab (Monoclonal)	1E9	Novus Biological	Citrate pH6	1:20 18 hours	Nuclear	H-score	≥60 (High)
pChk1	Rabbit Ab (Monoclonal)	Ab58567	Abcam	Citrate pH6	1:140 60 min	Nuclear	H-score	≥50 (High)
DNA-PKcs	Mouse Ab (Monoclonal)	3H6	Abcam	Citrate pH6	1:1000 20 min	Nuclear	H-score	>260 (high)
XRCC1	Mouse Ab (Monoclonal)	33-2-5	Thermo-scientific	Citrate pH6	1:200 20 min	Nuclear	% of positive cells	≥10% (positive)
APE1	Rabbit Ab (Polyclonal)		Novus Biologicals	Citrate pH6	1:500 60 min	Nuclear	H-score	>100 (positive)
SMUG1	Goat Ab (Polyclonal)		Acris Antibodies	Citrate pH6	1:200 15 min	Nuclear	H-score	≤35 (negative)
FEN1	Rabbit Ab (Polyclonal)		Novus Biologicals	Citrate pH6	1:200 60 min	Nuclear and Cytoplasm	H-score	≤100 (negative)

P21	Mouse Ab (Monoclonal)	SW118	Dako-Cytomation	Citrate pH6	1:50 60 min	Nuclear	% of positive cells	≥10% (positive)
Ki67	Mouse Ab (Monoclonal)	MIB1	Dako-Cytomation	1:300	Nuclear	% of positive cells	H-score	< 10% (low) 10-30% (moderate) >30% (high)
P53	Mouse Ab (Monoclonal)	DO7	Novocastra	Citrate pH6	1: 50 60 min	Nuclear	% of positive cells	≤20% (low) >20% (High)
Bcl-2	Mouse Ab (Monoclonal)	124	Dako-Cytomation	Citrate pH6	1:100 60 min	Cytoplasm	% of positive cells	>10% (positive
ER	Mouse Ab (Monoclonal)	SP1	Dako-Cytomation	Citrate pH6	1:150 30 min	Nuclear	Allred score	≥3 (positive)

ER	Mouse Ab (Monoclonal)	EP1	Dako-Cytomation	Citrate pH6	1:80 30 min	Nuclear	% positive cells	≥1% positive
PR	Mouse Ab (Monoclonal)	PR636	Dako-Cytomation	Citrate pH6	1:125 30 min	Nuclear	% positive cells	≥1% positive
CK14	Mouse Ab (Monoclonal)	LL002	Novocastra	Citrate pH6	1:40 60 min	Cytoplasm	% of positive cells	≥10% (positive)
Ck5/6	Mouse Ab (Monoclonal)	D5/161B4	Dako-Cytomation	EDTA pH8	1:100 60 min	Cytoplasm	% of positive cells	≥10% (positive)
Ck17	Mouse Ab (Monoclonal)	E3	Dako-Cytomation	Citrate pH6	1:100 60 min	Cytoplasm	% of positive cells	≥10% (positive)
Ck18	Mouse Ab (Monoclonal)	DC10	Dako-Cytomation	Citrate pH6	1:100 60 min	Cytoplasm	% of positive cells	≥10% (positive)
HER2	Rabbit Ab (Polyclonal)	polyclonal	Dako-Cytomation	None	1:400 60 min	Membrane	See text	See text

TOP2A	Mouse Ab (Monoclonal)	KiS1	Dako-Cytomation	Citrate pH6	1:150	Nuclear/ cytoplasm	% of positive cells	>25% (positive)
RAD51	Mouse Ab (Polyclonal)		Abcam Ab88572	Citrate pH6	1:70 60 min	Nuclear/ cytoplasm	H-Score	≥8 / ≥80
ATM	Mouse Ab (Monoclonal)	Y170	Abcam Ab32420	Citrate pH6	1:100 18 Hr	Nuclear	% of positive cells	≥25% (positive)
Chk2	Rabbit Ab (Polyclonal)		Abcam Ab47433	Citrate pH6	1:100 60 min	Nuclear	H-Score	≥100 (median)

All sections were pre-treated with microwave antigen retrieval using 0.1% citrate buffer (pH 6) except for HER2 (no pre-treatment) and EGFR (pre-treated with protease for 10 minutes); RT (Room temperature).

2.1.4 Antibody Optimisation

Optimal dilutions and conditions for staining were determined for each antibody using standard IHC technique, as described in section 2.1.2. Each dilution was tested on the same type and version of tissue , to ensure the experimental conditions standardized. The online resource 'Human Protein Atlas' (www.proteinatlas.org) was consulted to identify tissues that are known to express the protein of interest and therefore would be suitable for antibody optimisation. The tissues and optimal antibody concentrations used for optimisation are shown in Table 2.2.

Table 2.2 List of tissues used for optimisation and antibody concentrations

Antibody	Tissue	Concentration	Final Dilution
RECQL1	Breast optimisation TMA*	1:500, 1:1000, 1:1500, 1:2000	1:1000
RECQL4	Breast optimisation TMA Gastric Cancer TMA	1:150, 1:175, 1:200, 1:225, 1:250	1:175
RECQL5	Breast TMA	1:50, 1:100, 1:150, and 1:200	1:100
WRN	Breast optimisation TMA	1:75,1:100,1:125, 1:150, 1:200	1:100
BLM	Breast optimisation TMA	1:50,1:75,1:100, 1:150, 1:200	1:100

*TMA = tissue microarrary

All optimisation slides were then evaluated by an expert histopathologist (MA, EAR or TMA) after each round of optimisation to decide if further optimisation was required or to identify the optimal concentration for staining the breast tumours. The optimal dilution which gave the best differential pattern of staining with minimum background / non-specific binding was selected for each marker.

2.1.5 Use of both positive and negative controls

A negative control was included by omitting primary antibody and exposing to antibody diluent only. This was to determine if the secondary antibody was binding non-specifically resulting in false positives. It will be ideal to use absorption control to test if the primary antibody binds specifically to the antigen of interest.

Beta 2 microglobulin (B2M) was used as a positive control because it is widely expressed in a variety of tissues and known to be highly expressed in breast cancer (Li et al, 2012). The same positive control section was included in each run of IHC staining to ensure evaluation of consistency.

2.1.6 Histopathological scoring of stained Breast TMAs

Scoring of all Breast TMAs was performed with an expert histopathologist (MA, EAR or TAF) blinded to the clinicopathological characteristics of patients. The first 150 cores were independently assessed by two scorers and the concordance between the two scorers was excellent ($k = 0.79$). The rest of the cores were

assessed by two scorers together. Whole field inspection of the core was scored and intensities of nuclear staining were grouped as follows: 0 = no staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining. The percentage of each category was estimated (0-100%). H-score (range 0-300) was determined by multiplying the intensity of staining and percentage of staining in the tumour core. Not all cores within the TMA were suitable for IHC analysis as some cores were missing or lacked tumour (<15% tumour). Frequency histogram distributions and X tile (Version 3.6.1) were used to establish the cut-offs for protein expression values such that the resulting subgroups have significantly different survival courses.

2.1.7 Patient demographics

The study was performed in a consecutive series of 1650 patients with primary invasive breast carcinomas who were diagnosed between 1986 and 1999 and entered into the Nottingham Tenovus Primary Breast Carcinoma series. Patient demographics are summarised in Table 2.4. This is a well-characterized series of patients with long-term follow-up that have been comprehensively evaluated in a wide range of biomarker studies (Abdel-Fatah et al, 2013b; Abdel-Fatah et al, 2013c; Abdel-Fatah et al, 2014b; Abdel-Fatah et al, 2014c; Abdel-Fatah et al, 2014d; Sultana et al, 2013). All patients were treated in a standard way in a single institution with standard surgery (mastectomy or wide local excision) with radiotherapy. Prior to 1989, patients did not receive systemic adjuvant treatment (AT). After 1989, AT was given based on prognostic and predictive factor status such as Nottingham Prognostic Index (NPI), oestrogen receptor-α (ER-α) status,

and menopausal status. Patients with NPI scores of <3.4 (classed as low risk) did not receive AT. In pre-menopausal patients with NPI scores of ≥3.4 (classed as 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 to 233 months). Survival data, including overall survival, disease-free survival (DFS), and development of loco-regional and distant metastases (DM), was maintained on a prospective basis. DFS was defined as the number of months from diagnosis to the occurrence of local recurrence, local lymph node (LN) relapse or DM (distant metastasis) relapse. Breast cancer-specific survival (BCSS) was defined as the number of months from diagnosis to the occurrence of breast cancer 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. In cases where the patient was still alive at the time of analysis, lost to follow-up, or died from other causes, survival was censored.

We also evaluated an independent series of 252 ER-negative invasive breast tumours diagnosed and managed at the Nottingham University Hospitals between 1999 and 2007. All patients were primarily treated with surgery, followed by adjuvant radiotherapy and adjuvant modern anthracycline chemotherapy (as compared to old fashioned CMF chemotherapy used in

primary NTS series). The characteristics of this cohort are summarised in Table 2.4.

Table 2.3 Patient demographics of the Nottingham IHC Cohort

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

Table 2.4 Patient demographics of the ER negative 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)

2.1.8 Ethical approval

Ethical approval was obtained from the Nottingham Research Ethics Committee (Reference number C202313, Approval date : 21/10/2010). Tumour Marker Prognostics Studies (REMARK) criteria, recommended by McShane et al (McShane et al, 2005) , were followed throughout this project.

2.1.9 Statistical analysis of immunohistochemistry data

Data analysis was performed using SPSS (SPSS, version 22 Chicago, IL). Where appropriate, Pearson's Chi-square (outcome binary and exposure binary or categorical comparing >2 groups), Fisher's exact (where more than 20% of cells contained less than five cases), $\chi 2$ for trend (where outcome was binary and exposure was ordered categorical), Student's t-test & ANOVA (outcome continuous normally distributed and exposure categorical comparing >2 groups) were performed using SPSS software (SPSS, version 20 Chicago, IL). As our data was non-parametric (not normally distributed), median survival was used. Cumulative survival probabilities were estimated using the Kaplan–Meier method which provides a graphic display of differences in survival rates between groups. Statistical significant differences between survival outcomes were tested using the log-rank test. P values for each test were adjusted with (Benjamini & Hochberg, 1995) multiple P-value adjustments and an adjusted p-value of <0.05 was considered significant, meaning there is 5% probability the observed difference is by chance. Multivariate analysis with survival Cox proportional

hazard model was performed where a statistically significant survival outcome was seen in the univariate analysis.

The log-rank test that is applied in survival analysis provides an estimate of the likelihood that an individual factor affects prognosis, however, it does not allow for the potential influence of confounding factors. In order to address this issue, multivariate analysis was done. It measures the interrelationship between multiple variables and give an estimate of the degree of effect called hazard ratio.

Xtile (Version 3.6.1, Yale University, USA) was used to dichotomise H-score expression of RECQL1 (low <215, high ≥215). Median cut off was used to dichotomise H score expression of RECQL4, RECQL5, BLM, and WRN into low and high expression. Nuclear RECQL4 (low <30, high ≥30), cytoplasmic RECQL4 (low <100, high ≥100), nuclear RECQL5(low <10, high ≥10), nuclear BLM (low <50, high ≥50), cytoplasmic BLM (low <50, high ≥50), nuclear WRN (low <116, high ≥116), and cytoplasmic WRN (low <20, high ≥20).

2.2 DNA helicase gene expression profiling in Breast Cancers

This work was performed in collaboration with Miss Devika Aggarwal and Professor Graham Ball (Department of Biosciences, Nottingham Trent University, Nottingham, UK).

We investigated DNA RECQ helicase mRNA expression in METABRIC (Molecular Taxonomy of Breast Cancer International Consortium) cohort. METABRIC is a public dataset, Miss Devika Aggarwal downloaded the log

normalised processed data from the EMBL website, after requesting it from the EMBL data manager. As described in the supplementary information from the Curtis et al 2012 paper (Curtis et al, 2012b), there were two datasets, discovery, and validation. Two datasets were merged together to give a total of 1977 cases after removing the duplicates.

The METABRIC study protocol and the molecular profiling methodology in a cohort of 1977 breast cancer samples are detailed by Curtis et al (Curtis et al, 2012b). Patient demographics are summarised in Table 2.5. ER-positive and/or lymph node-negative patients did not receive adjuvant chemotherapy. ER-negative and/or lymph node positive patients received adjuvant chemotherapy. For this cohort, the mRNA expression was hybridized to Illumina HT-12 v3 platform (Bead Arrays), and the data were pre-processed and normalised as described previously in Curtis et al paper. Samples were classified into the intrinsic subtypes based on the PAM50 gene list. A description of the normalisation, segmentation, and statistical analyses as previously described (Curtis et al, 2012b).

To select the probes for the analysis, the annotation file of Curtis et al study was used. The library annotation file had probe quality described as perfect, good or bad. The probes which were regarded to have bad quality or all the 50 nucleotides of the probe being covered by the repeat mask were not used. The probe used for mRNA analysis of each helicase was a perfect match and quality for its target, having a GC content of 58%, 0 SNPs and it does not possess a poly

G tail at the end. The list of probes selected for analysis is summarised in Table 2.6.

Xtile (Version 3.6.1) was used to identify a cut-off in gene expression values such that the resulting subgroups have significantly different survival courses. Once we got the cut-off, the cases were split from x-tile, re-coded as 0 and 1 in SPSS and then plotted the KM plots in SPSS (version 22 Chicago, IL). The Chi-square test was used for testing association between categorical variables, and a multivariate Cox model was fitted to the data using as endpoint breast cancer-specific death.

Table 2.5 Clinicopathological characteristics in the METABRIC test dataset

Variables	**N (%)**
Age at diagnosis [Median (range)]	61.8 (21.93-96.29)
Tumour size [Median (range)]	23 (1, 182)
NPI [Median (95% CI)]	4.04 (3.99-4.09)
Survival [Median (Months, 95% Cl)]	149 (141-159)

Lymph nodes status	
0	1012
1	336
2	170
3	112
>3	316
ER status	
Positive	1485
Negative	437
PAM50 subtype	
Basal	322
HER2	238
Luminal A	714
Luminal B	484
Normal	188
Not classified	6
Adjuvant systemic therapy (AT)	
No AT	290
Hormone therapy (HT)	1014
Chemotherapy	226
Hormone + chemotherapy	192

Table 2.6 Probes selected in the METABRIC test dataset

DNA Helicase	Probe ID
RECQL1	ILMN_1692705
RECQL4	ILMN_1703092
RECQL5	ILMN_1697682
BLM	ILMN_1709484
WRN	ILMN_1679881

2.3 Cell line studies

2.3.1.1 Cell lines and culture requirements

2.3.1.1.1 Breast cancer cell lines

MCF-7, MDA-MB-231, MDA-MB-468, and MDA-MB-436 human breast cancer cell lines were purchased by DNA repair group at Nottingham, from American Type culture collection (ATCC, Manassas, USA) and were grown as per ATCC recommendations (Table 2.7). The MCF-7 cell line was grown in RPMI-1640 (Sigma, UK) with the addition of 10% FBS (Sigma, UK). The MDA-MB-231 cell line was maintained in Dulbecco's Modified Eagle Medium (DMEM with 4500mg/L glucose, L-glutamine, sodium pyruvate) with the addition of 10% FBS. MDA-MB-468 and MDA-MB-436 cell lines were grown

in Dulbecco's Modified Eagle Medium (DMEM with F-12 Nutrient Mixture-Ham and L-glutamine) with the addition of 10% FBS. All media were supplemented with 5ml of 1% penicillin/streptomycin (10,000 units penicillin and 10mg streptomycin/mL) (Sigma, UK). All cell lines were maintained in a humidified incubator at 37°C with 5% carbon dioxide and grown as an adherent culture.

A frozen stock of all cell lines was acquired at early passage. One million cells were harvested and re-suspended in 1ml of freezing medium. Freezing medium for all cell lines was composed of 10% dimethyl sulfoxide (DMSO) (Sigma, UK) and 90% standard growth media. Freezing cryovials were placed in Mr. Frosty™ freezing container (Thermo Fisher Scientific™) and transferred immediately to -80°C for at least 24-48 hours. Cryovials were then transferred to liquid nitrogen for long-term storage. All cell lines were sub-cultured two to three times per week in class II laminar flow hood under sterile conditions. Media was removed from the flask. Cells were then washed twice with Dulbecco's phosphate buffered saline (DPBS) (Sigma/Invitrogen, UK) to remove any dead cells or cell debris. 3ml – 5ml of Trypsin-EDTA solution (Sigma, UK) was added and incubated at 37°C for three to four minutes until all cells had detached from the bottom of the flask. Media was added at a ratio of 1:4 (trypsin-EDTA: media) to neutralise the trypsin. The suspension was centrifuged at 1000 rpm for five minutes to pellet the cells. The supernatant was removed and the cell pellet was re-suspended in 5ml of fresh normal culture medium and re-seeded into tissue culture flasks at a dilution between 1:5 to 1:10. 70-80% confluent cells were

used for all cell line-based experiments. All experiments were done in at least triplicate unless otherwise stated.

Table 2.7 Panel of breast cancer cell lines with identified, BRCA mutation and ER/PR/HER2 expression status; WT: wild type

Cell line	**BRCA1 mutation status** (Elstrodt et al, 2006)	**ER/ PR / HER2 protein expression (Riaz et al, 2013)**
MCF-7	WT	ER+/PR+/HER2-
MDA-MB-231	WT	ER-/PR-/HER2-
MDA-MB-468	WT	ER-/PR-/HER2-
MDA-MB-436	5396+1G>A (splice donor)	ER-/PR-/HER2-

2.3.1.1.2 BLM KD and Control HeLa cell lines

Cell line experiment was done in BLM knock-down cell lines as commercially available BLM Knock-down (BLM KD) HeLa cell line (BLM HeLa SilenciX, Cat number 00301-00026, passage 13, Adherent HeLa cells silenced for BLM, Accession number: NM_000057, clone BD1163/1) and BLM control (BLM CTR) Hela cell line (Control HeLa SilenciX, cat number 00301_00001, adherent HeLa cells transfected with control shRNA, passage 44) from tebu-bio laboratories were purchased by Prof. Madhusudan research group. These lines were tested free of bacterial contamination in antibiotic-free medium and tested

free of mycoplasma with MycoAlert Mycoplasma Detection Kit (cat number LT07-318, from Lonza, US) by tebu-bio laboratories.

BLM KD and BLM CTR HeLa cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM with 4500mg/L glucose, L-glutamine, 110mg/l sodium pyruvate) supplemented with 10% FBS, 1% penicillin and streptomycin (10,000 units penicillin and 10mg streptomycin/mL) (Sigma, UK) and 250µg/ml hygromycinB (cat number 10687-010,Invitrogen) as per tebu-bio user manual. Cell lines were preserved in a humidified incubator at a temperature of 37°C with 5% of carbon dioxide. A frozen stock of all cell lines was acquired at an early passage (BLM KD HeLa – passage 13 and BLM CTR HeLa – passage 44). One million cells were harvested and re-suspended in 1ml of freezing medium. Freezing medium was composed of 50% FBS, 50% D-PBS containing 20% cell culture tested DMSO (10% dimethyl sulfoxide (DMSO) final concentration in the freezing medium. Freezing cryovials were stored and sub-cultured (sub-cultivation ratio: 1:3) two to three times per week as discussed in the breast cancer cell line section. 70-80% confluent cells were used for all cell line-based experiments. All experiments were done in at least triplicate unless otherwise stated.

2.3.2 Cell line characterisation studies

2.3.2.1 RT Polymerase chain reaction

The specific sequences within a cDNA template can be amplified using the Polymerase chain reaction (PCR). In this study, Real-time PCR, which is a

reliable and sensitive method for gene expression analysis, was used to quantify differences in DNA repair gene expression between cell lines using the RT^2 DNA Profiler PCR array (#PAHS-042Z, Qiagen, Manchester, UK). Validation of BLM knock-down was also done by real-time PCR in BLM HeLa SilenciX cells, in comparison with Control HeLa SilenciX cells.

This experiment requires the extraction of RNA from the cell line of interest and the production of cDNA. This cDNA is then mixed with primers and a fluorescent dye prior to undergoing thermal cycling. In real-time quantitative PCR, the amount of product is measured after each cycle using fluorescent reporter dyes, such as SYBR® Green, a fluorescent signal is directly proportional to the number of PCR product amplicons generated.

2.3.2.1.1 RNA extraction

Total RNA was extracted from MCF-7, MDA-MB-231, MDA-MB-468, MDA-MB-436, BLM Ctr HeLa and BLM KD HeLa cells using RNeasy Mini kit (Qiagen, Venlo, Netherlands). $3x10^6$ cells were harvested and washed in PBS. The cell pellet was suspended in 350µl of Buffer RLT and then 350µl of 70% ethanol was added to the cell pellet. Cells were lysed by passing lysate through a 19-gauze needle attached to RNase free syringe at least 5 times. The homogenised mixture was transferred to an RNeasy Mini spin column and care was taken to avoid touching the membrane with the tip. The tube was spun at 10,000 rpm for 15 seconds and flow-through was discarded. 350µl Buffer RW1 was added to the column, the sample spun at 10,000 rpm for 15 seconds and

flow-through removed. DNase incubation mix was made by adding 10µl DNase I stock solution to 70µl Buffer RDD. The mix was transferred to an RNeasy column membrane and incubated at room temperature for 15 minutes. 350µl Buffer RW1 was then added, the sample centrifuged as before, and flow-through discarded. RNA precipitation was done by adding, 500µl of Buffer RPE to the membrane, the sample spun as before and then this step was repeated one more time. Fresh collection tube was taken and the column was spun at full speed for 1 minute. Subsequently, 30-50µl RNase-free water was added to the column membrane and the sample centrifuged for 1 minute at 10,000 rpm to elute the RNA.

RNA quality and concentration were assessed using a microvolume spectrophotometer (NanoDrop, Wilmington, USA). Graphs were assessed for spectral quality, including the presence of a single peak and absorbance at 230, 260 and 280nm wavelengths. The ratios of 260/230 and 260/280 absorbance were determined. A 260/280 ratio of ~2 is considered 'pure' for RNA, whereas the 260/230 ratio would be expected to be within the range of 2.0-2.2 (2015). Samples were then stored at minus 80°C until cDNA synthesis.

2.3.2.1.2 cDNA synthesis

cDNA synthesis was done using the RT^2 First Strand Kit (Qiagen). The reagents of the RT^2 First Strand Kit were thawed and briefly centrifuged for 10–15 s) to bring the contents to the bottom of the tubes. 10µl of genomic DNA elimination mix for each RNA sample was prepared using composition as described in Table 2.8.

Table 2.8 Genomic DNA elimination mix composition

Component	Amount
RNA	0.5µg
Buffer GE	2µl
RNase-free water	Variable
Total Volume	10 µl

DNA elimination mix was then incubated at 42°C for 5 minutes and placed immediately on ice for at least 1 minute. 10µl of reverse transcription mix (4µl 5X Buffer BC3, 1µl Control P2, 2µl RE3 Reverse Transcriptase Mix and 3µl RNase-free water) was mixed gently by pipetting up and down to the DNA elimination mix and incubated at 42°C for exactly 15 minutes. The reaction was stopped immediately by incubating at 95°C for 5 minutes. 91µl of RNase-free water was added to each reaction. The samples were placed on ice and were stored at -80°C prior to PCR analysis.

cDNA quality and concentration were assessed using the microvolume spectrophotometer prior to PCR analysis. The quality assessment was done as described previously for RNA, with the exception that a 260/280 ratio of ~1.8 is deemed 'pure' for DNA. Examples of the absorbance spectra for each of the six cell lines are shown in Figure 2.1. All examples demonstrate a single peak and 260/230 and 260/280 within an acceptable range.

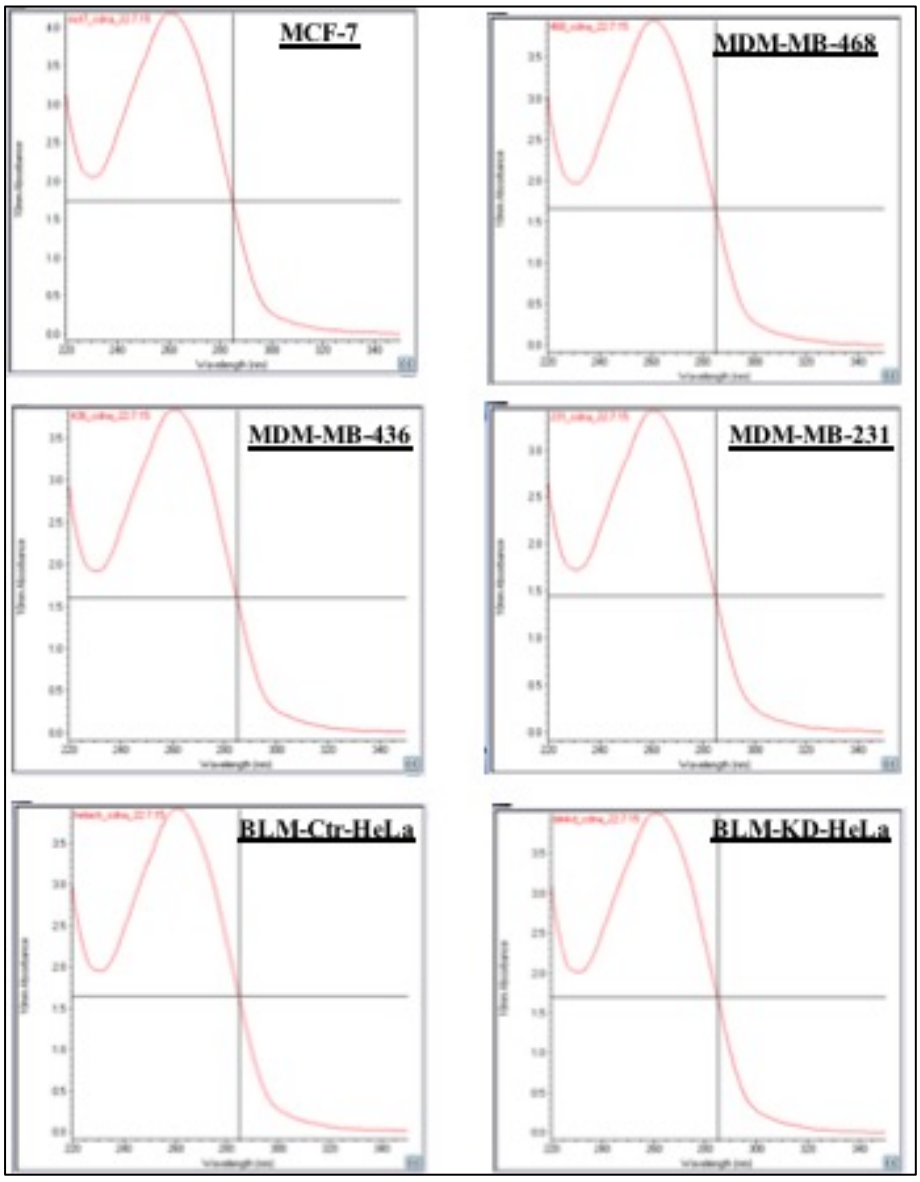


Figure 2.1 Spectrophotometer assessment of cDNA quality

Representative plots for assessment of cDNA quality for MCF 7, MDM-MB-231, MDM-MB-436, MDM-MB-468, BLM-Ctr (Control)-HeLa and BLM-KD (Knockdown)-HeLa breast cancer cell lines using a microvolume spectrophotometer. All cell lines demonstrate a single spectral peak.

2.3.2.1.3 No reverse transcriptase controls

It is virtually impossible to completely eliminate genomic DNA from RNA preparation hence we included no reverse transcriptase control to detect DNA contamination. No reverse transcriptase controls were made from RNA of each cell line to assess for genomic DNA contamination. The protocol was followed as per section 2.3.2.1.2 except RE3 Reverse Transcriptase Mix was replaced with 2µl RNase/DNase free water.

2.3.2.1.4 RT PCR Primer selection

RT PCR was performed using SYBR Green PCR Master mix (Applied Biosystems, Warrington, UK) with primer sets purchased from Qiagen (UK) and reconstituted in 1.1ml RNase/DNase free water. Primers were stored at -20°C. Table 2.9 shows details of all primers used. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeper gene. The real-time PCR for each RNA sample was performed in triplicate unless stated otherwise. NTC (No Template Control) was used to rule out cross-contamination of reagents and surfaces. For making NTC, all the RT-PCR reagents were used except the RNA template.

Table 2.9 List of primers used for RT PCR

Gene symbol/ Source	Catalogue number	Detected transcript (s)	Ensembl transcript ID	Entrez gene ID
RECQL1/ QIAGEN	QT00034503	NM_002907 (3795 bp) NM_032941 (3598 bp)	ENST00000341443	5965
RECQL4/ QIAGEN	QT00201740	NM_004260 (3840 bp)		9401
RECQL5/ QIAGEN	QT00084973	NM_001003715 (2444 bp) NM_001003716 (2911 bp)	ENST00000317905	9400
BLM/ QIAGEN	QT00027671	NM_000057 (4555 bp) NM_001287246 (4665 bp)	ENST00000355112	641
WRN/ QIAGEN	QT00074809	NM_000553 (5765 bp)	ENST00000298139	7486
GAPDH/ QIAGEN	QT00079247	NM_001256799 (1455 bp) NM_002046 (1421 bp)	ENST00000229239	2597

2.3.2.1.5 Real-time PCR using RT^2 Profiler PCR Array

10µl of PCR components mix (5µl 2x RT2 SYBR Green Mastermix, 2µl cDNA synthesis reaction, 1µl of primer and 2µl RNase-free water) was added to each well of the 96 well RT2 Profiler PCR array. In no template control wells, RNase-free water was used instead of cDNA. In no RT control wells, no RT control was used instead of cDNA. Plates were then sealed using optical adhesive film and centrifuged at 1000g prior to real-time PCR on the Applied Biosystems 7500

FAST cycler (Foster City, USA). All components for PCR analysis were purchased from Qiagen (UK).

2.3.2.1.6 Data analysis

Data analysis was performed using the Qiagen web-based data analysis centre (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php). Data was pre-prepared using a Microsoft Excel template prior to uploading to the data analysis centre. Variations in normalised mRNA levels were assessed for statistical significance by two-way ANOVA with multiple comparisons using GraphPad Prism 6 (GraphPad, LaJolla, USA).

GAPDH was selected as the housekeeper gene as this demonstrated the smallest change in Ct values across samples. Relative PCR quantitation allows direct comparison of gene expression levels for housekeeping genes and the target gene of interest (GOI) in the cell lines. Expression of all genes on the array was normalised against GAPDH. The difference between the Ct values (ΔCt) for each gene was calculated using the formula:

ΔCt (GOI Experimental cell line) = Ct (GOI Experimental) – Ct (Housekeeping gene)

ΔCt (control) = Ct (Control cell line) – Ct (Housekeeping gene).

Then, the difference in ΔCt values (ΔΔCt) was determined between the control cell line and the experimental cell line using the formula:

ΔΔCt = ΔCt (GOI Experimental cell line) - ΔCt (control cell line)

Since all calculations were in logarithm base 2, every time there is twice as much DNA, Ct values decrease by 1 and will not halve. So we calculated the value of 2^-ΔΔCt to get the expression fold change.

Differences in expression between two cell lines were expressed as a fold regulation, which has greater biological meaning. Fold change values greater than 1 represent a positive- or upregulation, and are equal to the fold regulation. Fold change values less than 1 represent a negative- or down-regulation and are equal to the negative inverse of the fold change.

The level of GOI expression in control HeLa SilenciX was set to 1 representing 100% GOI expression in the control HeLa SilenciX cell line. The level of silencing was expressed as a percentage of GOI silencing:

Percentage of GOI silencing = 100 – (fold change x 100)

2.3.2.2 Western blot analysis

Western blot analysis was used to evaluate the specificity of antibodies, before using them for IHC. Validation of BLM knock-down was also done by Western blot analysis in BLM HeLa SilenciX cells, in comparison with Control HeLa SilenciX cells.

2.3.2.2.1 Cell lysate preparation

Two million cells were harvested and washed with PBS. The cell pellet was resuspended in 100µl of lysate solution. The lysate solution was prepared by mixing 88µl of RIPA buffer (20mM Tris, 150mM sodium chloride, 1% Nonidet

P-40, 0.5% sodium deoxycholate, 1mM EDTA, 0.1% SDS) , protease cocktail inhibitor (10µL, 1:10 dilution) and phosphatase inhibitors 2 & 3 (1µL each). The cell suspension was incubated on ice for 1 hour, then was repeatedly aspirated through a fine-gauge needle to maximise lysis, before being incubated at -20ºC overnight. The following day, the suspension was centrifuged at 13,000 rpm for 20 minutes at 4ºC, and then the supernatant transferred to a clean Eppendorf. Lysates were stored at -20ºC. All lysate mix components were purchased from Sigma, UK.

2.3.2.2.2 Sample preparation

Sample preparation for Western blot analysis was done by the addition of 5µl cell lysate, 2.5µl sample buffer (NuPAGE® LDS Sample Buffer), 1µl reducing agent (NuPAGE® Reducing Agent) and 6.5µl dH20. Samples were heated at 100°C for 5 minutes. Samples were then centrifuged at 13,000rpm for 2 minutes and placed on ice. Gels (NuPAGE® Bis-Tris Mini Gels) were loaded in tanks and tanks were filled with 1X SDS Running Buffer (50ml MOPS SDS Running Buffer [Thermo Fisher Scientific] to 950ml dH20). At least one protein standard was included on each gel. Protein standard used was Precision Plus Protein™ All Blue Pre-stained Protein Standard (BioRad, UK). Samples and protein standard were loaded and electrophoresis was performed at 125V for 100 minutes. 500µl NuPAGE® Antioxidant was added to 200ml 1X running buffer in the upper buffer chamber.

The Gel was carefully removed from casing and stored in transfer buffer (100ml methanol, 50 ml NuPAGE® Transfer Buffer (20X), and 1ml NuPAGE®

Antioxidant made up to 1L with cold dH20). Filter paper, sponges and nitrocellulose membranes (GE Healthcare life sciences, Little Chalfont, UK) were soaked in transfer buffer. Cassettes were assembled containing the pre-soaked sponges, filter paper, membrane, and gel. The cassette was filled with transfer buffer and the outer tank with dH20 (two third). Proteins were transferred to nitrocellulose membrane by application of a 25V current for 90 minutes. Membranes were removed and placed in 5% milk solution (2.5g in 50ml PBS-T [1ml Tween 20 in 1L PBS]) for 60 minutes at room temperature. Membranes were rolled in 50 ml tube and primary antibody was added (diluted in 2ml 5% milk, blocking buffer) and incubated overnight at 4°C. The primary antibodies and their optimal concentrations used in this study are shown in Table 2.10. Unless otherwise stated, all equipment and reagents for western blot were purchased from Thermo Fisher Scientific.

Table 2.10 Primary antibodies used for Western blot analysis

Antigen	**Molecular weight**	**Antibody**	**Source**	**Catalog #**	**Dilution**
RECQL1	73 kDa	Rabbit pAb	Bethyl	A300-450A	1:1000
RECQL4	133 kDa	Rabbit pAb	Novus	25470002	1:750
RECQL5	108 kDa	Rabbit pAb	Sigma	HPA-029971	1:2000
BLM	159 kDa	Rabbit pAb	Novus	NBP1-89929	1:1500
WRN	162 kDa	Rabbit pAb	Novus	NBP1-87143	1: 1500
β-actin	42 kDa	Mouse mAb	Sigma	A2228 Clone AC-74	1:10000

Membranes were washed with PBS-T (x3 5 minutes) before incubation in a housekeeper gene (β-actin, 1:10,000) diluted in 5% milk solution for 1 hour at room temperature. β-actin was selected as a housekeeper gene as its molecular

weight was different from the target antigen. β-actin was raised in a different species to the primary antibody to aid detection.

Following further PBS-T washes (x3 5 minutes) membranes were incubated with 1:10000 infrared dye-labelled secondary antibody solution (0.5µl IRDye 800CW Donkey Anti-Rabbit IgG and 0.5µl IRDye 680CW Donkey Anti-Mouse IgG, Li-Cor, USA in 5ml 5% milk) in the dark for 1 hour. After washing (x2 5 minutes with PBS-T, x1 5 minutes with PBS) membranes were scanned at 700 and 800nm on a Li-Cor Odyssey scanner (Li-Cor, USA) to determine protein expression.

2.3.2.2.3 Statistical analysis

Protein expression quantification was performed using the Li-Cor Odyssey software (Image Studio Lite version 3.1). For each cell line, the signal intensity for the protein of interest was expressed relative to β-actin. The normalised expression value for BLM KD HeLa cell line was expressed relative to BLM proficient HeLa control cell line. Data analysis was performed using Microsoft Excel 2010 (Microsoft, Redmond, USA) and Graph Pad Prism 6 (GraphPad, La Jolla, USA). Variation in normalised protein levels was assessed for statistical significance using two-way ANOVA with multiple comparisons.

2.3.2.3 Cell survival assays

2.3.2.3.1 Growth Curve

100,000 BLM KD HeLa and BLM Ctr HeLa cells were seeded into 6 well plates and incubated overnight. After 24 hours cells were trypsinized, harvested and

counted using a haemocytometer. The number of cells per ml was calculated. This process was repeated every 24 hours to a maximum of 96 hours. GraphPad Prism 6 (GraphPad, La Jolla, USA) was used to visualize the data. This experiment was performed once in triplicate.

2.3.2.3.2 Plating efficiency

80% confluent BLM KD HeLa cells BLM Ctr HeLa cells were seeded into 6 well plates at densities of 100, 200, 300, 400, 500, and 600 cells per well. Plates were incubated at 37°C and 5% CO2 for 14 days. After 14 days, 6 well plates were removed, media discarded and wells washed with DPBS. Cells were then stained with 1ml of crystal violet (10% [w/v] in 70% ethanol) (Sigma) for 5 minutes. Crystal violet was removed; the plates were washed with tap water and left to dry overnight. Blue stained colonies with at least 50 cells were counted by eye using a colony counter (Stuart Scientific, UK). Plating efficiency (PE) was calculated using the following equation: PE = (no. of colonies formed/ no. cells seeded) x100. All experiments were performed in triplicate.

2.3.2.3.3 Clonogenic Survival assay

To evaluate the cytotoxicity of Cisplatin in BLM Ctr and BLM KD Hela cells, 500 cells/well were seeded in 6 well plates (total volume 2ml/well). The cells were allowed to adhere overnight. After 16 hours, increasing concentrations of the cytotoxic drug, Cisplatin, (1µM, 2µM, 3µM, 4µM, and 5µM) were added to the medium. These concentrations of Cisplatin were tested in previous cell line studies carried out by Prof. Madhusudan group (Abdel-Fatah et al, 2013a). 1µM

concentration of Cisplatin was used for functional studies as at this concentration some surviving fraction was seen in BLM-KD-HeLa cells on clonogenic assay. 0.1% DMSO was also included as a vehicle control. Plates were then incubated at 37 degree Celsius in an atmosphere of 5%CO2 and 95% air for 10-14 days. After 10-14 days, 6 well plates were removed from the incubator, media discarded and wells washed with DPBS. Cells were then stained with 1ml of crystal violet (10% [w/v] in 70% ethanol) (Sigma) for 5 minutes. Crystal violet was removed; the plates were washed with tap water and left to dry overnight. Blue stained colonies with at least 50 cells were counted. All experiments were performed in triplicate and two times. Data analysis was done using Microsoft Excel 2010 (Microsoft, Redmond, USA) and GraphPad Prism 6 (GraphPad, La Jolla, USA). Two way ANOVA was used to assess for statistically significant differences in survival between cell lines for the various drug concentrations.

2.3.2.3.4 MTS assay

CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS assay) (Promega) is a colorimetric quantification of the number of viable cells in proliferation and cytotoxicity assays. MTS reagent contains tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine methosulfate (PMS). The assay is based on the reduction of MTS tetrazolium compound by viable cells to produce a coloured formazan product that is soluble in cell culture media. This reduction process is accomplished by dehydrogenase enzymes found in metabolically active cells only. The formazan

dye produced by viable cells can then be quantified by measuring the absorbance at 490nm using a plate reader. The quantity of formazan as measured by the amount of absorbance at 490nm is directly proportional to the number of viable cells in the culture.

To evaluate the cytotoxicity of cytotoxic drug, Cisplatin, in BLM KD Hela cells and BLM Ctr Hela cells, the MTS assay was performed as per manufacturer recommendation. Cells were seeded at a density of 1000 cells per well (in 100μL volume) in 96 well plates. The cells were allowed to adhere overnight. After 16 hours, cytotoxic drugs were added at a range of concentrations in a volume of 20μL. For each cell line, DMSO vehicle control (0.1% final concentration) was also included. Plates were then incubated for a total of 7 days. On Day 7, 20μL of MTS Reagent (Promega) was added to each well and plates were incubated for 2 hours before reading at 490 nm using a FLUOstar OPTIMA plate reader (BMG LABTECH Ltd., Aylesbury, UK). The absorbance, relative to the DMSO control, was calculated for each drug concentration. All experiments were performed in triplicate at least three times unless otherwise stated. Data analysis was done using Microsoft Excel 2010 (Microsoft, Redmond, USA) and GraphPad Prism 6 (GraphPad, La Jolla, USA). Two way ANOVA was used to assess for statistically significant differences in survival between cell lines for the various drug concentrations.

2.3.2.4 Functional consequences of Cytotoxicity in BLM KD Hela cell lines

2.3.2.4.1 Flow cytometry

Flow cytometry was used to determine cell cycle progression and to assess the accumulation of γ-H2AX foci as a result of cisplatin cytotoxic chemotherapy using a dual staining protocol as described below. In addition, Annexin V and propidium iodide staining was used to do assessment of the apoptotic cells after treatment with cisplatin.

Flowcytometry is a high throughput method which allows the sequential analysis of individual cells. In this technique, cells are excited by laser followed by the collection of the emitted light. Flow cytometry technique helps rapid collection of data from large proportion of cells. Data can be collected about the size , granularity and fluorescence of cells. The first step is to label cells with fluorophore (chemicals that can fluoresce and absorb light at one wavelength and emit it at longer wavelength). The second step is processing of samples by the flow cytometer. Cells are submitted to hydrodynamic focusing which results in the alignment of single cells which then travel through a laser beam. For each cell, scattered light is collected of the same wavelength as the laser. Forward scatter provides information about the cell size. The forward scatter is directly proportional to the cell size. Side scatter gives information about the granularity and structure of the cells. If side scatter is higher, it means that the cell shape is less uniform. The emitted light is collected if fluorophore has bound to the cell. The intensity of fluorescent light is proportional to the amount of fluorophore

bound to the cell and wavelengths is dependent on the fluorophore used (Brown & Wittwer, 2000)

2.3.2.4.2 Gamma H2AX and cell cycle analysis by flow cytometry

H2AX is a member of the H2A family and accounts for 10 to 15% of the H2A of the core nucleosome structure. When DNA double-strand break (DSBs) occur, H2AX undergoes phosphorylation (performed by the members of PIK3 family including ATR, ATM, and DNA PKcs) at serine 139 on the carboxyl terminus. H2AX is then known as γ-H2AX. γ-H2AX (a marker of DNA DSBs) can be detected by various methods including flow cytometric assay. The advantage of using flow cytometry method is that it can be combined with cell cycle analysis, hence allowing the assessment of γ-H2AX foci in the various steps of cell cycle (Porcedda et al, 2008)

Cell cycle analysis can be performed by quantification of the DNA content of cells. Propidium iodide (PI) is a DNA binding stain which binds to DNA in a stoichiometric fashion. As a result of this, DNA binding is proportional to DNA content. Cells in S phase of the cell cycle bind more PI than cells in G1 phase of cell cycle, hence producing more fluorescence. The DNA content of cells in G2 phase is double as compared to G1, hence they should fluoresce twice intensely. PI can only bind to DNA if the cell membrane is permeable and this can be achieved by using 70% ethanol.

150,000 cells were seeded in 6 well plates and allowed to adhere overnight. Cisplatin (1μM) and MMS (100μM, positive control) were added after 16 hours

(or 0.1% DMSO vehicle control). After 24 hours of drug administration, the medium was discarded from wells and cells were washed with 1ml PBS twice. Cells were then harvested by trypsinisation and centrifuged at 1000 rpm for 5 minutes. Cell pellets were then fixed with 1ml 70% ice-cold ethanol and stored at 4°C until required. Cell suspensions were then centrifuged at 1000 rpm for 5 minutes and the supernatant discarded. Cell pellets were washed in PBS two times and then resuspended in 50µL 1X Permeabilization Solution. H2AX Phosphorylation assay kit (Merck Millipore, Watford, UK) was used for further processing of samples. Samples were then incubated with 3.5µL of fluorescein isothiocyanate (FITC) anti-phospho-Histone H2A.X (ser139) for 20 minutes. Excess FITC labelled antibody was removed by washing with 200µL 1X Wash Solution and samples centrifuged at 1000 rpm for 5 minutes. The supernatant was removed and the cell pellet re-suspended in 100µL of 100µg/ml RNase (Qiagen) in PBS. Cells were incubated for 15 minutes and then 400µL propidium iodide (2.5µg/ml, Merck Millipore) was added and samples incubated for a further 15 minutes in FACS tubes. An unstained control was included for each cell line, alongside PI only and FITC anti-phospho-Histone H2AX only samples, and a FITC IgG negative control. Samples were analysed on a Cytomics F500 flow cytometer (Beckman Coulter, USA) with emission data for PI collected using a 620nm bandpass filter (FL3) and for FITC-anti-phospho-Histone H2A.X with a 575nm bandpass filter (FL1). At the minimum, 10,000 cells were analysed from each sample. All experiments were done in duplicate two times.

Weasel (Victoria, Australia) flow cytometry analysis software was utilized for data analysis. Graphical visualisation and statistical analysis were carried out in GraphPad Prism 6 (GraphPad, La Jolla, USA). Two way ANOVA was used to determine whether statistically significant differences exist in the number of γH2AX foci between untreated and treated cells for each cell line. The percentage of cells in each phase of the cell cycle was assessed for each cell line in both the untreated and treated state. Two way ANOVA was used to determine whether statistically significant differences exist in the distribution of cells throughout the cell cycle after treatment with Cisplatin.

2.3.2.4.3 Apoptosis detection by FTIC-Annexin V flow cytometry

Cell death occur by two main mechanism: apoptosis and necrosis. Apoptosis is a programmed cell death which leads to cell dehydration, shrinkage, chromatin condensation, and nuclear fragmentation. During apoptosis, externalisation of phosphatidylserine residues occur and these residues flip to the cell surface. These residues can bind Annexin V in the presence of calcium and Annexin V can be used to detect cells expressing phosphatidylserine on their surface, hence undergoing apoptosis.

150,000 cells were seeded into 6 well plates and allowed to adhere overnight. Cisplatin (1μM) and MMS (100μM, positive control) were added after 16 hours (or 0.1% DMSO vehicle control). DMSO was only added in untreated, unstained, PI only and Annexin only wells. 24 hours after drug administration, media was removed from the well. Cells were washed with PBS twice and then harvested by trypsinization. The cell suspension was transferred to the media

removed from the flask. This suspension was spun at 1000 rpm for 5 minutes and the cell pellet washed in ice-cold PBS two times. Cell pellets were then resuspended in 100µl Annexin V binding buffer (FITC-Annexin V Apoptosis Detection Kit I, BD Pharmingen, San Jose, USA), transferred to FACS tubes and incubated for 10 minutes. 2.5µl propidium iodide (PI) (component no. 51-66211E, BD Pharmingen) and 2.5µl Annexin V (component no. 51-65874X, BD Pharmingen) were added to each tube and samples incubated in the dark for 15 minutes. 400µl of binding buffer was added and samples analysed on a Cytomics F500 flow cytometer (Beckman Coulter, Pasadena, USA) using a 488nm laser for excitation and emission data collected using a 575nm bandpass filter (FL1) for annexin V-FITC and a 620nm bandpass filter (FL3) for PI. At the minimum, 10,000 cells from each sample were analysed. Untreated controls were included for each cell line and each time point. An untreated/unstained, untreated/PI only and untreated/Annexin V only control was also included for each cell line. All experiments were done in duplicate two times.

Data analysis was done using Weasel (Victoria, Australia) flow cytometry analysis software. The percentage of early apoptotic cells (FITC-Annexin V positive, PI negative) and late apoptotic cells (FITC-Annexin V positive, PI positive) were determined and compared for untreated and treated samples by two way ANOVA. Graphical visualisation and statistical analysis were done in GraphPad Prism 6 (GraphPad, La Jolla, USA).

Chapter 3

RECQL1 helicase expression in sporadic breast cancers

3 RECQL1 helicase expression in sporadic breast cancers

3.1 Introduction

DNA helicases play a vital role in accomplishing the unwinding of DNA, which is an important process required during DNA replication and DNA repair. RECQL1 also known as RECQL or RECQ1 is one of the key member of RECQ family of DNA helicases. Other members of this family include RECQL4, RECQL5, BLM and WERNER (also called WRN) (Brosh, 2013b; Croteau et al, 2014c). RECQL1 is an ATP dependant DNA helicase which is located on chromosome 12p12 and it encodes a 649 amino acid protein (Sami & Sharma, 2013b; Sharma, 2014b; Sharma et al, 2012). Out of all RECQ helicases, RECQL1 is the smallest one and it is present in large amounts (Sharma & Brosh, 2008).

RECQL1 plays an integral role in the DNA replication. RECQL1 is a key component of the replication complex and perform vital role in the maintenance of replication fork progression (Lu et al, 2013; Popuri et al, 2012a; Thangavel et al, 2010). RECQL1 also plays a role in branch migration of holiday junctions. RECQL1 plays role in telomere maintenance (Popuri et al, 2017a). It has also shown to be involved in replicative stress survival and resistance to drugs in multiple myeloma cells (Viziteu et al, 2017). It also shows strand annealing activity (Pike et al, 2015; Sharma et al, 2005).

RECQL1 is also essential for genomic stability as it plays integral role in DNA repair pathway (mismatch repair, nucleotide excision repair and direct repair) (Sami & Sharma, 2013b; Wu & Brosh, 2010). There is evidence to suggest that RECQL1 depleted cells show increased frequency of spontaneous sister chromatid exchanges leading to chromosomal instability and accumulation of DNA damage (Sharma et al, 2007). RECQL1 depleted cells also demonstrate increased sensitivity to cytotoxic chemotherapeutic agents (Sharma et al, 2007). RECQL1 has also been shown to interact with various other DNA repair proteins such as PARP1, RAD51,Top3α, EXO1, MSH2/6, MLH1-PMS2 and Ku70/80 to fulfil its critical biological functions (Sami & Sharma, 2013a; Sharma, 2014a; Sharma et al, 2012; Wu & Brosh, 2010).

There is emerging data to suggest the role for RECQL1 in breast cancer pathogenesis. Importantly, two recent studies have shown that germ-line RECQL1 mutations are associated with increased breast cancer susceptibility (Cybulski et al, 2015a; Sun et al, 2015a). Cybulski found RECQL1 germ line mutation in 7 /1013 higher-risk breast cancer cases in Quebec and 30/13136 unselected breast cancer cases in Poland . Similarly Sun et al, identified pathogenic RECQL1 mutation in 9/448 Chinese BRCA negative familial breast cancer patients. Moreover, whole exome sequencing has identified distinct populations of breast cancer susceptibility mutations in RECQL1 (Banerjee & Brosh, 2015).

However the clinicopathological significance of RECQL1 in sporadic breast cancer remains largely unknown. In UK, Breast cancer is the second most common cause of cancer death in females in UK (Cancer research statistics, UK). Breast cancer is heterogeneous and there is a need to define predictive and prognostic biomarkers to develop personalised therapies and improve survival outcomes for these patients.

So our rationale of doing this study was to understand the prognostic and predictive significance of RECQL1 expression in sporadic breast cancers, hence we did comprehensive investigation of RECQL1 expression at the transcriptomic and protein level in large cohorts of breast cancer patients.

3.2 Methods

3.2.1 Patient selection for evaluation of RECQL1 expression

Detailed investigation of the protein expression of RECQL1 in sporadic breast cancer was done in two breast cancer patient cohorts. The first cohort was a series of 1650 patients with primary invasive breast carcinomas who were diagnosed with breast cancer between 1986 and 1999 and entered into the Nottingham tenovus series. The second cohort was an independent series of 252 ER-negative invasive breast tumours diagnosed and treated at Nottingham University Hospitals NHS trust between 1999 and 2007. Patient demographics of the Nottingham tenovus series (NTS) and ER-negative cohort are summarised in Table 2.4 and 2.5 respectively.

3.2.2 Tissue Microarray (TMA)

Breast tumours were arrayed in tissue microarrays (TMAs) constructed with two replicate 0.6 mm cores from the centre and periphery of the tumours. Details of TMA construction is detailed in section 2.1.1.

3.2.3 Determination of the optimal RECQL1 Antibody concentration for IHC analysis

Optimal concentration and conditions for staining were ascertained for RECQL1 antibody using standard IHC technique, as described in section 2.1.2. We tested each dilution on the same type of sample and also the same version of Breast TMA, to keep the experimental conditions standardized. We consulted on-line resource 'Human Protein Atlas' (www.proteinatlas.org) and identified that Breast tissue is known to express RECQL1 protein, hence, we decided to proceed with antibody optimisation on breast tissue. The RECQL1 antibody (Rabbit Antibody, polyclonal) was purchased from BETHYL Laboratory, catalogue number A300-450A. Recommended optimal dilutions for IHC by the manufacturer was 1:500 to 1:5000. We did epitope retrieval with Citrate buffer pH 6.0 as per manufacturer recommendation. Incubation time used was 60 minutes at room temperature. We used 1:500, 1:1000, 1:1500 and 1:2000 dilutions for optimisation.

All optimisation slides were then evaluated by an expert pathologist to decide if further optimisation was required or to identify the optimal concentration for staining the breast tumours.

After careful review by an expert pathologist, 1:1000 was found to be optimal dilution for staining (Table 3.1). At this concentration, a range of staining intensities was seen including both positive and negative cells. Moreover, at this concentration, minimum background / non-specific binding was seen (Figure 3.1).

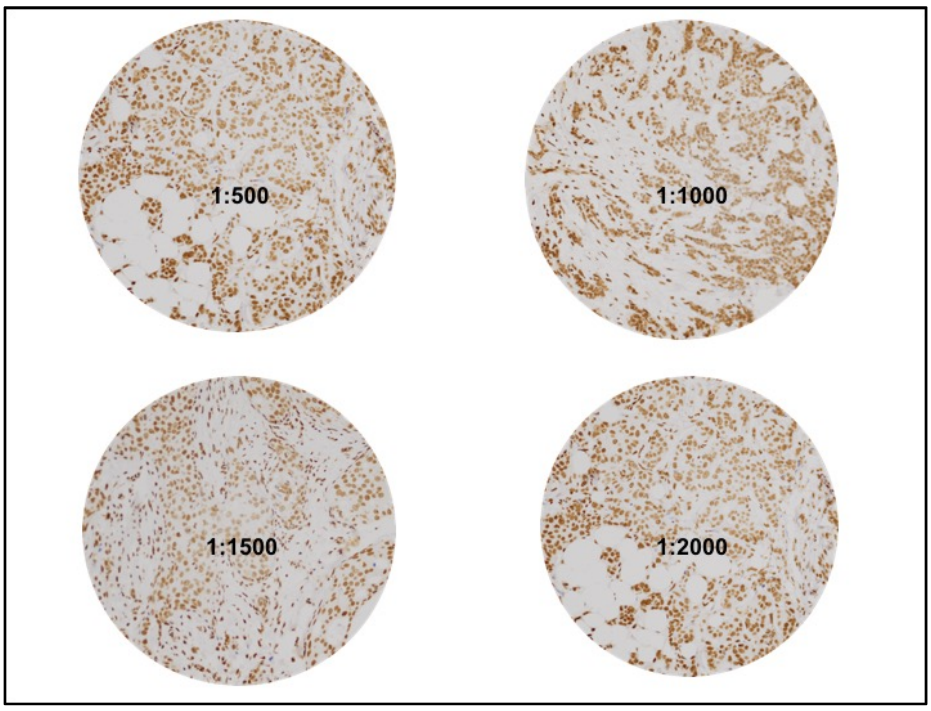


Figure 3.1 Range of RECQL1 antibody concentrations used for optimisation

1:500, 1:1000, 1:1500 and 1:2000 dilutions were used for optimisation. 1:1000 was selected as optimal dilution for staining as it showed best differential staining pattern and little or no background staining.

Table 3.1 Primary antibody details

Antibody	Clonality	Source	Catalogue No.	Optimal Dilution/ Incubation time
RECQL1	Rabbit Ab (Polyclonal)	BETHYL	A300-450A	1:1000 60 min (RT)

3.2.4 Inclusion of controls to ensure the quality of staining

A negative control was included by omitting primary antibody and exposing to antibody diluent only. This was to determine if the secondary antibody was binding non-specifically resulting in false positives. It will be ideal to use absorption control to test if the primary antibody binds specifically to the antigen of interest.

Beta 2 micro-globulin was used as a positive control to ensure that staining was successful. The same positive control section was included in each run to ensure consistency to be evaluated.

3.2.5 Cells lines and optimal antibody concentration used for Western Blot

Western blot analysis, as described in section 2.3.2.2, was used to evaluate the specificity of RECQL1 antibody, before using them for IHC. RECQL1 protein expression was assessed in four breast cancer cell lines MCF7, MDA-MB-231,

MDA-MB-436, and MDA-MB-468. The characteristics of these cell lines are explained in Table 2.9. The details of cell lysate preparation and sample preparation are also explained in section 2.3.2.2.1.

Figure 3.2 The Primary antibody and its optimal concentration used for western blot

Antibody	**Clonality**	**Source**	**Catalogue No.**	**Optimal Dilution/ Incubation time**
RECQL1	Rabbit Ab (Polyclonal)	BETHYL	A300-450A	1:1000 (Overnight at 4^0C)

3.2.6 IHC technique

Details of IHC technique used is described in detail in section 2.1.2.

3.2.7 Histopathological scoring of stained Breast TMAs

Scoring of all Breast TMAs was performed by me and an expert pathologist blinded to the clinicopathological characteristics of patients. H scoring was used to score breast tumours as described in detail in section 2.1.6. Not all cores within the TMA were suitable for IHC analysis as some cores were missing or lacked a tumour (<15% tumour). 890 breast tumours were suitable for analysis. As per discussion with my supervisor and pathologist, X-tile (Version 3.6.1, Yale University, USA) was used to dichotomize H-score expression of RECQL1 such that the resulting subgroups have significantly different survival courses. H

score of <215 was categorised as low RECQL1 protein expression and H score of ≥215 was categorised as high RECQL1 protein expression.

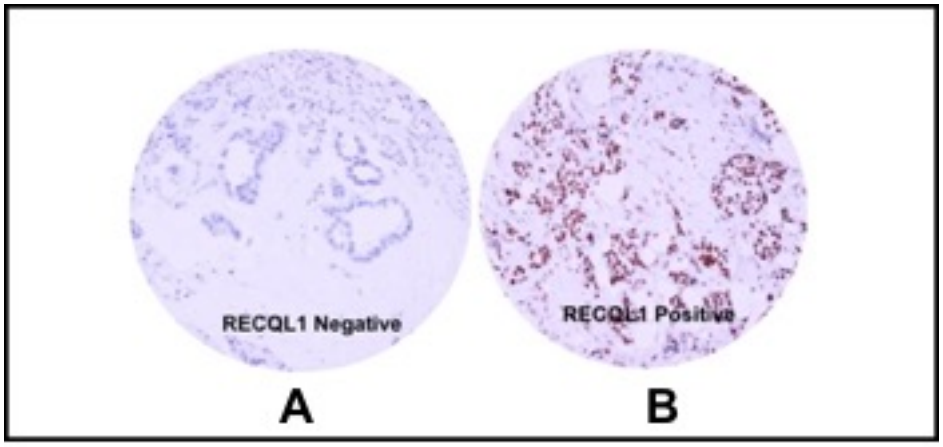


Figure 3.3 Photomicrographs of RECQL1 protein expression in breast cancers

A. RECQL1 negative tumour ; **B.** RECQL1 positive tumour

3.2.8 Quantitative real time PCR

Total RNA was extracted from MCF-7, MDA-MB-231, MDA-MB-468 and MDA-MB-436 cells using the RNeasy Mini kit (QIAGEN, UK). The RNA quantification was done using a NanoDrop 2000c Spectrophotometer (Thermo Scientific). The cDNA was synthesised from 0.5 µg of total RNA using RT^2 first strand kit (QIAGEN). The real-time qPCR was performed using SYBR Green PCR Master mix (Applied Biosystems) with a primer set (RECQL1 QuantiTect Primer Assay, catalog no. QT00034503, QIAGEN) targeting the RECQL1 gene. The glyceraldehyde-3-phosphate dehydrogenase housekeeper gene was used as an internal control (GAPDH QuantiTect Primer Assay, catalog no.

QT00079247, QIAGEN). The real-time PCR for each RNA sample was done in triplicate. To prevent cross-contamination of reagents and surfaces, NTC (No Template Control) was used. All the RT-PCR reagents were included in NTC except the RNA template. Minus reverse transcriptase (- RT) control was included to assess for genomic DNA contamination. Non-specific binding is a problem with SYBR green , hence we included a melting curve analysis to get information about the purity of the amplified product. A single peak was seen which indicated the presence of only one double stranded product.

3.2.9 Patient selection for RECQL1 mRNA expression in breast cancer

We investigated RECQL1 mRNA expression in METABRIC (Molecular Taxonomy of Breast Cancer International Consortium) cohort. The METABRIC study protocol and the molecular profiling methodology in a cohort of 1977 breast cancer samples are detailed by Curtis et al (Curtis et al, 2012b). Patient demographics, details of data acquisition and analysis are summarised in section 2.2.

RECQL1 probe (ILMN_1692705) was used because it was a perfect match and quality for its target, having a GC content of 58%, 0 SNPs and it does not possess a poly G tail at the end. The cut off for the high vs low RECQL1 gene expression was done using x-tile software (Version 3.6.1) so that the resultant subgroups had significant difference in survival outcomes.

3.2.10 Statistical analysis

Data analysis was performed using SPSS (SPSS, version 22 Chicago, IL) as described in detail in section 2.1.9.

3.2.11 Ethical approval

Ethical approval was obtained from the Nottingham Research Ethics Committee (Reference number C202313, Approval date : 21/10/2010).Tumour Marker Prognostics Studies (REMARK) criteria, recommended by McShane et al (McShane et al, 2005), were followed throughout this project.

3.3 Results

3.3.1 RECQL1 antibody specificity is confirmed by western blot analysis

In order to provide evidence of specificity of RECQL1 antibody used for IHC staining, western blot was used in breast cancer cell lines. As shown in Figure 3.4 A, Breast cancer cell lines (MCF-7, MDA-MB-231, MDA-MB-436, and MDA-MB-468) have good RECQL1 expression seen as a single band at the correct molecular weight 75kDa. Figure 3.4 B shows relative RECQL1 mRNA expression in breast cancer cell lines. All breast cancer cell lines showed a robust expression of RECQL1. MDA-MB-436 cell line showed maximum expression and MDA-MB-231 showed the least expression as shown in Figure 3.4 B.

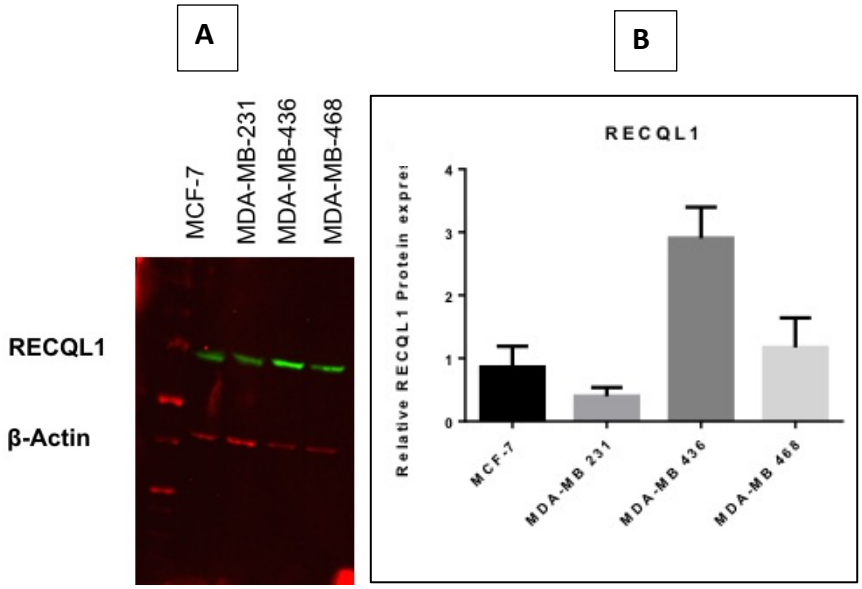


Figure 3.4 RECQL1 Expression in MCF7, MDA-MB-23, MDA-MB-436 & MDA-MB-468 cell lines

A. Western blot analysis to check the specificity of RECQL1 antibody **B.** RECQL1 protein expression in MCF7, MDA-MB-231, MDA-MB-436 & MDA-MB-468 breast cancer cell lines relative to β-Actin. Data presented as mean ± SEM of three independent experiments.

3.3.2 Normal breast tissue shows high nuclear RECQL1 expression but no cytoplasmic expression

We initially evaluated RECQL1 expression in normal breast tissues. 15 normal breast tissues were stained for RECQL1 and we observed high nuclear staining in the terminal duct lobular units. The mean H-score was 226, indicating differential expression of RECQL1 in normal breast tissues when compared with

breast cancer tissue. We did not see any cytoplasmic staining in any normal breast tissue.

3.3.3 RECQL1 is only localized to the nucleus in sporadic breast tumours

We proceeded to IHC analysis of primary breast series. Out of 1650 cases, a total of 897 breast cancer cases were deemed suitable for analysis. We did not observe any cytoplasmic staining of RECQL1. We saw the nuclear expression of RECQL1 only. 677 cases out of 897 (75.5%) demonstrated low RECQL1 nuclear expression level and 220 out of 897 (25.5%) showed high RECQL1 nuclear expression.

3.3.4 Low RECQL1 protein expression in the whole cohort is significantly associated with aggressive breast cancer phenotypes and low levels of other DNA repair proteins

As shown in Table 3.2 , in the whole cohort, low nuclear RECQL1 levels were significantly associated with larger tumour size (p=0.047) , lymph node positivity (p=0.048), higher tumour stage (p=0.03), high tumour grade (p=0.001), high mitotic index (p=0.001), tubule formation (p=0.01), pleomorphism (p=0.004) and tumour type (p=0.02).

Significantly low RECQL1 protein expression was seen in tumours which were ER- (p=0.03) , PR- (p=0.0007) and had HER-2 overexpression (p=0.04). High-risk tumours , Nottingham Prognostic Index (NPI) >3.4 were also significantly correlated with low RECQL1 level (p=0.0006).

We also observed that tumours with low RECQ1 protein expression also have statistically significant low XRCC1 (p=0.0005), BRCA1 negativity (p=0.03), low SMUG1 (0.007), low nuclear FEN1 (p=0.02), low PARP1 (p=0.048) , low DNA-PKc (p=0.004), low nuclear Chk1 (p=0.0005), low ATM (p=0.007) and low nuclear RAD51 (P=0.009).

Furthermore, low RECQL1 tumours were also significantly associated with low levels of other RECQ helicases including nuclear RECQL4 (p=0.001), low nuclear BLM (p=0.014) and low cytoplasmic WRN (p=0.002).

Table 3.2 RECQL1 protein levels and breast cancer

VARIABLE	RECQL1 protein level		P values	
	Low N (%)	High N (%)	Unadjusted	adjusted
A) <u>Pathological Parameters</u>				
Tumour Size				
<1cm	53 (7.8)	31 (14.1)	**0.031**	**0.0472**
>1-2cm	338 (49.9)	108 (49.1)		
>2-5cm	268 (39.6)	78 (35.5)		
>5cm	18 (2.7)	3 (1.4)		
Lymph Node Status				
Negative	351 (58.5)	143 (68.8)	**0.029**	**0.0483**
Positive (1-3)	199 (33.2)	54 (26.0)		
Positive (>3)	50 (8.3)	11 (5.3)		
Tumour Stage				
1	410 (60.5)	157 (70.7)	**0.017**	**0.0313**
2	210 (29.6)	52 (23.4)		
3	67(9.9)	13 (5.9)		

Tumour Grade				
G1	88 (13.0)	46 (20.9)	**0.0001**	**0.0018**
G2	230 (33.9)	89 (40.5)		
G3	360 (53.1)	85 (38.6)		
Mitotic Index				
M1 (low; mitoses < 10)	212 (32.2)	105 (48.2)	**0.0001**	**0.0012**
M2 (medium; mitoses 10-18)	131 (19.9)	34 (15.6)		
M3 (high; mitosis >18)	315 (47.9)	79 (36.2)		
Tubule Formation				
1 (>75% of definite tubule)	25 (3.8)	19 (8.7)	**0.006**	**0.0162**
2 (10%-75% definite tubule)	213 (32.4)	78 (35.8)		
3 (<10% definite tubule)	420 (63.8)	121 (55.5)		
Pleomorphism				
1 (small-regular uniform)	13 (2.0)	7 (3.2)	**0.001**	**0.0044**
2 (Moderate variation)	218 (33.2)	101 (46.5)		
3 (Marked variation)	425 (64.8)	109 (50.2)		
Tumour Type				
IDC-NST	421 (63.3)	107 (49.5)	**0.017**	**0.0298**
Tubular Carcinoma	122 (18.3)	59 (27.3)		
Medullary Carcinoma	17 (2.6)	6 (2.8)		
ILC	63 (9.5)	27 (12.5)		
Others	8 (1.2)	4 (1.9)		
Mixed NST/lobular/Special Type	34 (5.1)	13 (6.0)		
ER				
Negative	187 (28.1)	42 (19.7)	**0.015**	**0.0309**
Positive	478 (71.9)	171 (80.3)		
PR				
Negative	310 (48.0)	67 (31.5)	**0.0001**	**0.0007**

Positive	336 (52.0)	146 (68.5)		
Her2 overexpression				
No	559 (84.1)	197 (90.0)	**0.032**	**0.0431**
Yes	106 (15.9)	22 (10.0)		
Triple Negative Phenotype				
No	570 (83.8)	183 (81.7)	0.459	0.4725
Yes	110 (16.2)	41 (18.3)		
NPI				
≤ 3.4	117 (27.7)	88 (41.5)	**0.0001**	**0.0006**
>3.4	462 (72.3)	124 (58.5)		
B) <u>DNA Repair</u>				
XRCC1 (Nuclear)				
Low	98 (18.6)	9 (5.6)	**0.0001**	**0.0005**
High	430 (81.4)	151 (94.4)		
BRCA1 (Nuclear)				
Low	104 (18.9)	18 (10.7)	**0.014**	**0.0306**
High	447 (81.1)	150 (89.3)		
SMUG1 (Nuclear)				
Low	288 (58.2)	64 (45.1)	**0.002**	**0.007**
High	207 (41.8)	78 (54.9)		
FEN1 (Nuclear)				
Low	389 (75.4)	103 (65.2)	**0.012**	**0.0280**
High	127 (24.6)	55 (34.8)		
FEN1 (Cytoplasmic)				
Low	285 (55.2)	70 (44.3)	**0.016**	**0.0311**
High	231 (44.8)	88 (55.7)		
PARP1				
Low	270 (49.0)	67 (39.6)	**0.033**	**0.0481**
High	281 (51.0)	102 (60.4)		
TOPO2				

Low	237 (47.0)	64 (38.6)	0.057	0.0739
High	267 (53.0)	102 (61.4)		
KU 70/80				
Low	60 (13.0)	22 (17.7)	0.181	0.2112
High	400 (87.0)	102 (82.3)		
DNA-PKcs				
Low	87 (18.0)	7 (5.6)	**0.001**	**0.0044**
High	397 (82.0)	117 (94.4)		
ATR				
Low	110 (35.4)	38 (37.6)	0.682	0.87
High	201 (64.6)	63 (62.4)		
Chk1 (Nuclear)				
Low	605 (89.1)	164 (73.2)	**0.0001**	**0.0005**
High	74 (10.9)	60 (26.8)		
Chk1 (Cytoplasmic)				
Low	211 (31.1)	75 (33.5)	0.451	0.478
High	468 (68.9)	149 (66.5)		
ATM				
Low	238 (56.9)	54 (41.5)	**0.002**	**0.007**
High	180 (43.1)	76 (58.5)		
CHK2				
Low	112 (25.2)	19 (15.7)	**0.029**	0.327
High	333 (74.8)	102 (84.3)		
RAD51 (Nuclear)				
Low	234 (55.1)	43 (39.1)	**0.003**	**0.0095**
High	191 (44.9)	67 (60.9)		
C) <u>Other RECQ helicases</u>				
RECQL5(Nuclear)				
Low	273 (46.5)	68 (40.0)	0.133	0.1605
High	314 (53.5)	102 (60.0)		

RECQL4 (Nuclear)				
Low	245 (65.9)	173 (50.6)	**0.00004**	**0.0014**
High	127 (34.1)	169 (49.4)		
RECQL4 (Cytoplasmic)				
Low	297 (53.6)	73 (46.2)	0.100	0.125
High	257 (46.4)	85 (53.8)		
BLM (Nuclear)				
Low	163 (27.7)	28 (16.9)	**0.005**	**0.0146**
High	426 (72.3)	138 (83.1)		
BLM (Cytoplasmic)				
Low	438 (74.5)	114 (70.3)	0.292	0.3194
High	150 (25.5)	48 (29.6)		
WRN (Nuclear)				
Low	197 (48.8)	53 (42.1)	0.188	0.214
High	207 (51.2)	73 (57.9)		
WRN (Cytoplasmic)				
Low	221 (54.7)	52 (41.3)	**0.008**	**0.02**
High	183 (45.3)	74 (58.7)		

Bold = Statistically significant; HER2: human epidermal growth factor receptor 2; ER: oestrogen receptor; PR: progesterone receptor; Triple negative: ER-/PR-/HER2-

3.3.5 In ER-positive cohort, low RECQL1 protein expression remains significantly associated with aggressive tumour phenotypes and low levels of other DNA repair proteins

We evaluated RECQL1 protein expression in the ER+ cohort. In ER+ tumours (Table 3.3) low nuclear RECQL1 level was significantly associated with the higher mitotic index (p= 0.033). PR- and High-risk Nottingham Prognostic Index (NPI) >3.4 was also more common in tumours with a low RECQL1 level

(p values 0.003 and 0.03 respectively). Low XRCC1, low TOPO2A, and low nuclear Chk1 were also more likely in tumours with low nuclear RECQL1 protein level (p values 0.03, 0.02, and 0.003 respectively).

Table 3.3 RECQL1 protein levels and ER+ breast cancers

VARIABLE	**RECQL1 protein level**		**P- values**	
	Low N (%)	High N (%)	Un-adjusted	Adjusted
A) <u>Pathological Parameters</u>				
Tumour Size				
<1cm	44 (9.2)	24 (14.3)	0.157	0.3701
>1-2cm	247 (51.9)	88 (52.4)		
>2-5cm	175 (36.8)	55 (32.7)		
>5cm	10 (2.1)	1 (0.6)		
Tumour Stage				
1	285 (59.7)	120 (70.6)	**0.037**	0.184
2	146 (30.6)	40 (23.5)		
3	46 (9.6)	10 (5.9)		
Tumour Grade				
G1	82 (17.2)	42 (25.0)	**0.006**	0.066
G2	210 (44.0)	82 (48.8)		
G3	185 (38.8)	44 (26.2)		
Mitotic Index				
M1 (low; mitoses < 10)	198 (42.8)	97 (58.4)	**0.002**	**0.033**
	110 (23.8)	30 (18.1)		

M2 (medium; mitoses 10-18) M3 (high; mitosis >18)	155 (33.5)	39 (23.5)		
Tubule Formation				
1 (>75% of definite tubule)	23 (5.0)	16 (9.6)	0.063	0.1848
2 (10%-75% definite tubule)	182 (39.3)	69 (41.6)		
3 (<10% definite tubule)	258 (55.7)	81 (48.8)		
Pleomorphism				
1 (small-regular uniform)	12 (2.6)	5 (3.0)	**0.028**	0.231
2 (Moderate variation)	206 (44.7)	93 (56.4)		
3 (Marked variation)	243 (52.7)	67 (40.6)		
Tumour Type				
IDC-NST	253 (53.8)	69 (41.8)	0.142	0.249
Tubular Carcinoma	114 (24.3)	54 (32.7)		
Medullary Carcinoma	1 (0.2)	0 (0.0)		
ILC	63 (13.4)	26 (15.8)		
Others	7 (1.5)	4 (2.4)		
Mixed NST/lobular/Special Type	32 (6.8)	12 (7.3)		
Lymph Node Status				
Negative	249 (58.6)	110 (68.8)	0.067	0.231
Positive (1-3)	141 (33.2)	42 (26.3)		
Positive (>3)	35 (8.2)	8 (5.0)		
PR				
Negative	128 (28.0)	22 (13.4)	**0.0001**	**0.0033**
Positive	329 (72.0)	142 (86.6)		
Her2 overexpression				
No	419 (89.7)	158 (94.6)	0.058	0.239
Yes	48 (10.3)	9 (5.4)		
Triple Negative Phenotype				
No	401 (83.9)	146 (85.4)	0.646	0.8422
	77 (16.1)	25 (14.6)		

Yes				
NPI				
≤ 3.4	165 (36.6)	80 (49.7)	**0.002**	**0.033**
>3.4	286 (63.4)	81 (50.3)		
B) <u>DNA Repair</u>				
XRCC1 (Nuclear)				
Low	49 (13.1)	4 (3.1)	**0.002**	**0.0332**
High	326 (86.9)	123 (96.9)		
BRCA1 (Nuclear)				
Low	44 (11.2)	12 (8.8)	0.443	0.7056
High	350 (88.8)	124 (91.2)		
SMUG1 (Nuclear)				
Low	170 (50.1)	48 (43.6)	0.235	0.346
High	169 (49.9)	62 (56.4)		
FEN1 (Nuclear)				
Low	268 (73.2)	73 (60.2)	**0.007**	0.066
High	98 (26.8)	48 (39.7)		
FEN1 (Cytoplasmic)				
Low	203 (55.5)	54 (44.6)	**0.038**	0.7077
High	163 (44.5)	67 (55.4)		
PARP1				
Low	188 (49.3)	53 (40.5)	0.079	0.936
High	193 (50.7)	78 (59.5)		
TOPO2				
Low	171 (49.1)	49 (38.6)	**0.041**	**0.0253**
High	177 (50.9)	78 (61.4)		
KU 70/80				
Low	32 (10.3)	15 (16.5)	0.103	0.376
High	280 (89.7)	76 (83.5)		

DNA-PKc				
Low	56 (16.7)	6 (6.6)	**0.015**	0.1848
High	279 (83.3)	85 (93.4)		
ATR				
	106 (47.3)	39 (51.3)	0.547	0.834
Low	118 (52.7)	37 (48.7)		
High				
pChk1 (Nuclear)				
	419 (87.8)	125 (73.1)	**0.0001**	**0.0033**
Low	58 (12.2)	46 (26.9)		
High				
pChk1 (Cytoplasmic)				
	156 (32.7)	62 (36.3)	0.399	0.6534
Low	321 (67.3)	109 (63.7)		
High				
ATM				
	155 (53.4)	37 (36.6)	**0.004**	0.0610
Low	135 (46.6)	64 (63.4)		
High				
CHK2				
Low	64 (20.9)	14 (15.4)	0.244	0.245
High	242 (79.1)	77 (84.6)		
RAD51 (Nuclear)				
Low	144 (50.0)	31 (37.3)	**0.042**	0.705
High	144 (50.0)	52 (62.7)		
C) <u>Other RECQ helicases</u>				
RECQL5(Nuclear)				
Low	184 (44.8)	47 (35.9)	0.073	0.0660
High	227 (55.2)	84 (64.1)		
RECQL4 (Nuclear)				
Low	206 (53.5)	52 (50.8)	0.109	0.376

High	179 (46.5)	60 (49.2)		
RECQL4 (Cytoplasmic)				
Low	223 (60.1)	147 (43.1)	0.604	0.8422
High	148 (39.9)	194 (56.9)		
BLM (Nuclear)				
Low	89 (21.6)	22 (17.1)	0.264	0.3716
High	323 (78.4)	107 (82.9)		
BLM (Cytoplasmic)				
Low	298 (72.5)	93 (72.7)	0.973	0.968
High	113 (27.5)	35 (27.3)		
WRN (Nuclear)				
Low	140 (49.8)	40 (40.8)	0.124	0.376
High	141 (50.2)	58 (59.2)		
WRN (Cytoplasmic)				
Low	149 (53.0)	38 (38.8)	**0.015**	0.261
High	132 (47.0)	60 (61.2)		

Bold = Statistically significant;HER2: human epidermal growth factor receptor 2; ER: oestrogen receptor; PR: progesterone receptor; Triple negative: ER-/PR-/HER2- .

3.3.6 In the ER- cohort, no significant associations seen between RECQL1 expression and clinicopathological variables

In ER- tumours (Table 3.4), no significant clinicopathological associations were observed. However, there were significant associations seen between low RECQL1 protein expression and low SMUG1, low pChk1, low cytoplasmic RECQL4 and low nuclear BLM (p values <0.05).

Table 3.4 RECQL1 protein levels and ER- breast cancers

VARIABLE	RECQL1 protein level		Adjusted P- values
	Low N (%)	High N (%)	
A) <u>Pathological Parameters</u>			
Tumour Size			
<1cm	9 (4.8)	5 (11.9)	0.319
>1-2cm	83 (44.6)	15 (35.7)	
>2-5cm	87 (46.8)	20 (47.6)	
>5cm	7 (3.8)	2 (4.8)	
Tumour Stage			
1	115 (61.8)	29 (69.0)	0.640
2	51 (27.4)	10 (23.8)	
3	20 (10.8)	3 (7.1)	
Tumour Grade			
G1	4 (2.2)	1 (2.4)	0.645
G2	14 (7.5)	5 (11.9)	
G3	168 (90.3)	36 (85.7)	
Mitotic Index			
M1 (low; mitoses < 10)	11 (6.1)	3 (7.1)	0.955
M2 (medium; mitoses 10-18)	16 (8.8)	4 (9.5)	
M3 (high; mitosis >18)	154 (85.1)	35 (83.3)	
Tubule Formation			
1 (>75% of definite tubule)	2 (1.1)	1 (2.4)	0.753
2 (10%-75% definite tubule)	26 (14.4)	5 (11.9)	
3 (<10% definite tubule)	153 (84.5)	36 (85.7)	

Pleomorphism			
1 (small-regular uniform)	1 (0.6)	0 (0.0)	0.104
2 (Moderate variation)	7 (3.9)	5 (11.9)	
3 (Marked variation)	173 (95.6)	37 (88.1)	
Tumour Type			
IDC-NST	158 (87.8)	34 (82.9)	0.701
Tubular Carcinoma	5 (2.8)	1 (2.4)	
Medullary Carcinoma	15 (8.3)	5 (12.2)	
Others	1 (0.6)	0 (0.0)	
Mixed NST/lobular/Special Type	1 (0.6)	1 (2.4)	
Lymph Node Status			
Negative	96 (58.2)	27 (67.5)	0.556
Positive (1-3)	54 (32.7)	10 (25.0)	
Positive (>3)	15 (9.1)	3 (7.5)	
PR			
Negative	175 (99.4)	41 (100.0)	0.629
Positive	1 (0.6)	0 (0.0)	
Her2 overexpression			
No	128 (69.6)	32 (76.2)	0.394
Yes	56 (30.4)	10 (23.8)	
Triple Negative Phenotype			
No	157 (84.0)	30 (71.4)	0.058
Yes	30 (16.0)	12 (28.6)	
NPI			
≤ 3.4	7 (4.0)	4 (9.8)	0.137
>3.4	166 (96.0)	37 (90.2)	
B) <u>DNA Repair</u>			
XRCC1 (Nuclear)			
Low	45 (31.9)	5 (18.5)	0.163
High	96 (68.1)	22 (81.5)	

BRCA1 (Nuclear)			
Low	55 (37.2)	6 (20.7)	0.088
High	93 (62.8)	23 (79.3)	
SMUG1 (Nuclear)			
Low	113 (77.4)	14 (58.3)	**0.046**
High	33 (22.6)	10 (41.7)	
FEN1 (Nuclear)			
Low	109 (80.1)	25 (80.6)	0.950
High	27 (19.9)	6 (19.4)	
FEN1 (Cytoplasmic)			
Low	76 (55.9)	15 (48.4)	0.449
High	60 (44.1)	16 (51.6)	
PAPR1			
Low	76 (47.5)	14 (48.3)	0.939
High	84 (52.5)	15 (51.7)	
TOPO2			
Low	60 (42.0)	13 (40.6)	0.890
High	83 (58.0)	19 (59.4)	
KU 70/80			
Low	25 (18.1)	4 (15.4)	0.738
High	113 (81.9)	22 (84.6)	
DNA-PKc			
Low	26 (18.7)	1 (3.7)	0.053
High	113 (81.3)	26 (96.3)	
ATR			
	36 (43.9)	9 (50.0)	0.638
Low	46 (56.1)	9 (50.0)	
High			
pChk1 (Nuclear)			
	174 (93.0)	31 (73.8)	**0.0001**
Low	13 (7.0)	11 (26.2)	
High			

pChk1 (Cytoplasmic)			
Low	47 (25.1)	11 (26.2)	0.887
High	140 (74.9)	31 (73.8)	
ATM			
Low	78 (64.5)	15 (55.6)	0.386
High	43 (35.5)	12 (44.4)	
CHK2			
Low	43 (32.8)	4 (18.2)	0.168
High	88 (67.2)	18 (81.8)	
RAD51 (Nuclear)			
Low	83 (65.9)	11 (55.0)	0.346
High	43 (34.1)	9 (45.0)	
C) Other RECQ helicases			
RECQL5(Nuclear)			
Low	83 (50.9)	16 (53.3)	0.808
High	80 (49.1)	14 (46.7)	
RECQL4 (Nuclear)			
Low	118 (75.2)	16 (59.3)	0.086
High	39 (24.8)	11 (40.7)	
RECQL4 (Cytoplasmic)			
Low	87 (55.4)	8 (29.6)	**0.013**
High	70 (44.6)	19 (70.4)	
BLM (Nuclear)			
Low	69 (42.1)	5 (16.7)	**0.008**
High	95 (57.9)	25 (83.3)	
BLM (Cytoplasmic)			
Low	129 (78.7)	20 (71.4)	0.396
High	35 (21.3)	8 (28.6)	
WRN (Nuclear)			

Low	51 (45.9)	10 (47.6)	0.888
High	60 (54.1)	11 (52.4)	
WRN (Cytoplasmic)			
Low	65 (58.6)	11 (52.4)	0.599
High	46 (41.4)	10 (47.6)	

Bold = Statistically significant;HER2: human epidermal growth factor receptor 2; ER: oestrogen receptor; PR: progesterone receptor; Triple negative: ER-/PR-/HER2-

3.3.7 Low RECQL1 nuclear expression significantly associated with poor breast cancer specific survival in the whole and ER+ cohort of primary series (NTS)

We then progressed to survival analysis. In the whole cohort, patients whose tumours had low RECQL1 level had significantly shorter BCSS compared to patients with high RECQL1 level (p=0.001) (Figure 3.5 A). We observed a similar trend in ER+ cohort, low RECQL1 was associated with poor BCSS (p = 0.008) (Figure 3.5 B) in ER+ cohort including patients who received adjuvant endocrine therapy (p= 0.021) (Figure 3.5 C). In contrast, patients who received no endocrine therapy, RECQL1 level did not influence survival (p=0.485) (Figure 3.5 D). We also evaluated RECQL1 expression in triple negative breast cancers (TNBC). 82/147 TNBC cases had low and 65/147 cases had high RECQL1 protein expression. There was no significant association observed between RECQL1 expression and BCSS in triple negative patients (p=0.29).

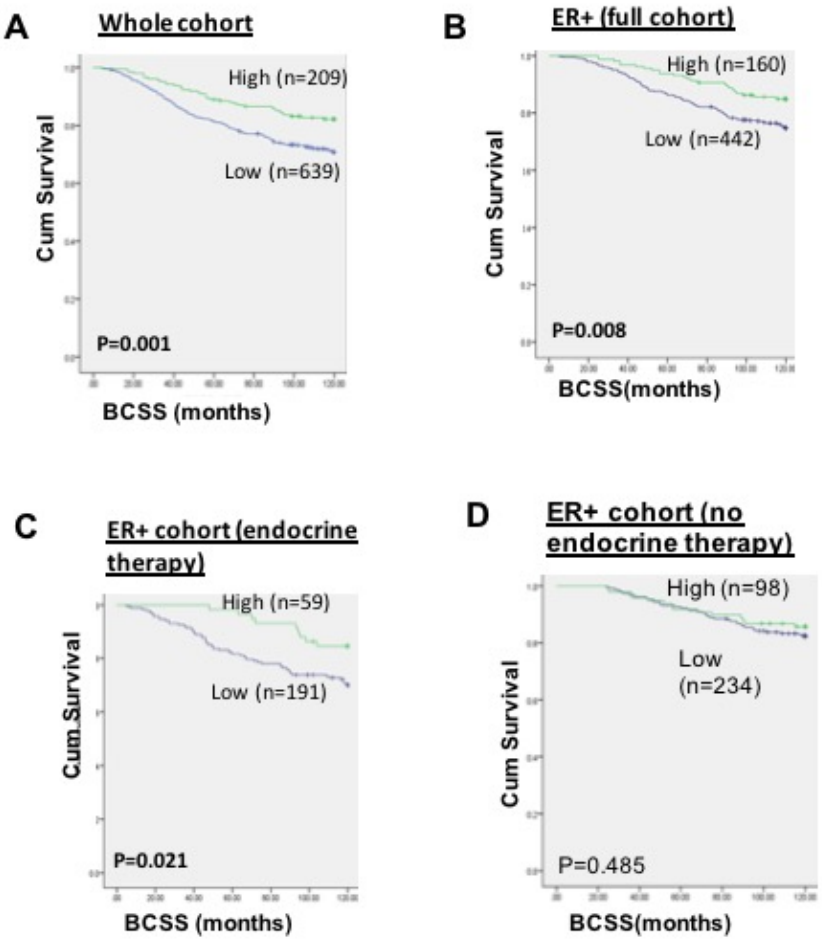


Figure 3.5 RECQL1 Expression and BCSS in the whole and ER+ Cohort

A. Whole cohort. **B.** ER+ (full cohort) **C.** ER+ tumours who received endocrine therapy. **D.** ER+ tumours who had no endocrine therapy [BCSS= breast cancer specific survival].

3.3.8 No survival benefit was seen in the ER- Cohort of primary series

In ER- tumours, RECQL1 expression did not influence survival including in patients who received CMF (cyclophosphamide+methotrexate+5-FU) chemotherapy (Figures 3.6 B,C, and D).

3.3.9 In the ER- cohort who received anthracycline chemotherapy, high RECQL1 expression is associated with breast cancer recurrence

In the primary series (Nottingham Tenovus Series), patients received CMF chemotherapy which is currently not the standard of care adjuvant treatment in breast cancer. Hence we proceeded to investigate RECQL1 level and survival in a further cohort of 252 ER- tumours (The characteristics of this cohort are summarised in Table 2.5) that received next generation anthracycline-based adjuvant chemotherapies.

We evaluated the effect of RECQL1 expression on disease-free survival at 5 years in patients who received adjuvant doxorubicin chemotherapy. We could not investigate long term effect as follow up data was not mature. At 5 years, 176/252 were alive, 73/252 were dead from breast cancer recurrence and 3/252 died from other causes.

Patients with high RECQL1 expression were more likely to suffer from disease recurrence as compared to patients with low RECQL1 expression ($p=0.048$) (Figure 3.6 A).

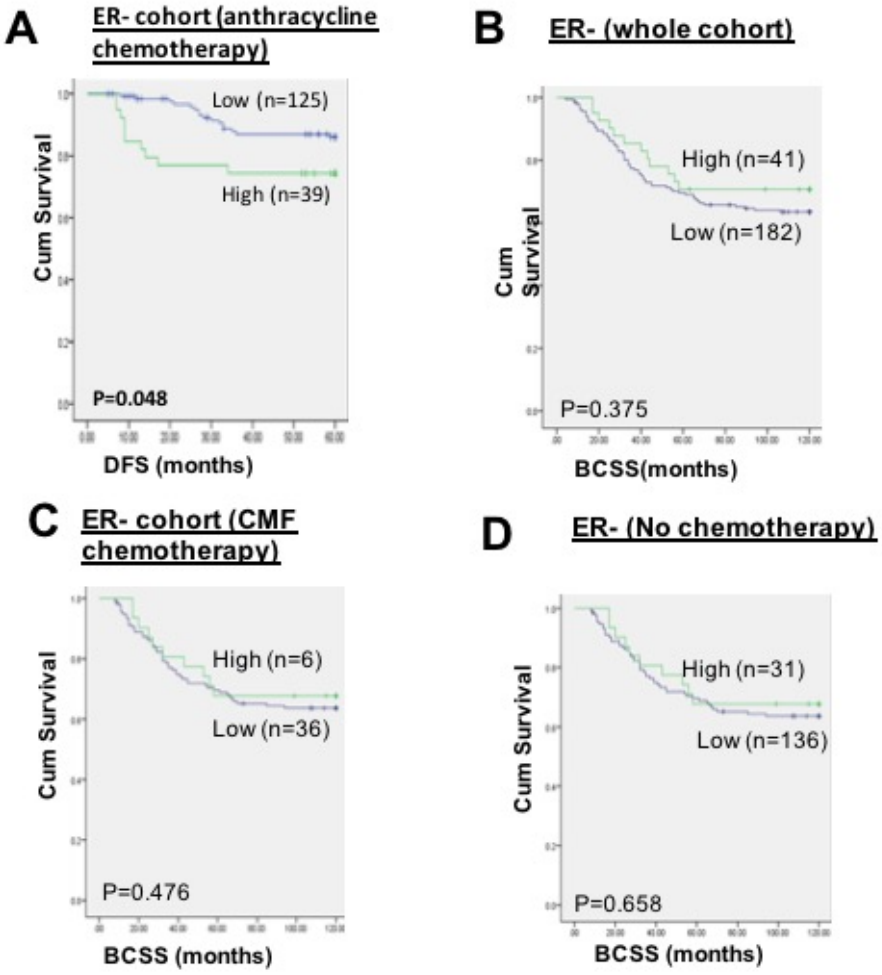


Figure 3.6 RECQL1 Expression in ER- Cohort

A. RECQL1 protein level and survival in patients with ER- tumours who received anthracycline chemotherapy. **B**. RECQL1 protein level and survival in the ER- cohort. **C.** RECQL1 protein level and survival in ER- tumours who received CMF chemotherapy. **D.** RECQL1 protein level and survival in patients with ER- tumours who received no chemotherapy . [BCSS= breast cancer specific survival, DFS = Disease free survival]

3.3.10 Summary of RECQL1 protein expression results

Together, the data suggest that low RECQL1 protein expression is associated with the aggressive phenotype and low expression of other DNA repair protein. Furthermore, low RECQL1 protein expression is associated with poor Breast cancer-specific survival in the whole cohort, ER + cohort, and ER- cohort who received anthracycline based chemotherapy.

3.3.11 Breast cancer cell lines show robust RECQL1 mRNA Expression

We initially profiled a panel of breast cancer cell lines. MDA-MB-231, MDA-MB-436, and MDA-MB-468 showed less expression of RECQL1 mRNA relative to expression of RECQL1 mRNA in MCF7 control cell line. (Figure 3.7)

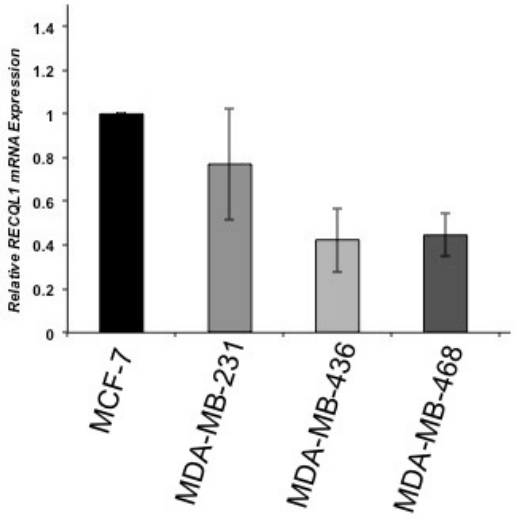


Figure 3.7 mRNA expression of RECQL1 in breast cancer cell lines

A graph of RECQL1 expression in MDA-MB-231, MDA-MB-436, and MDA-MB-468 breast cancer cell lines relative to MCF7 control breast cancer cell line. Data presented as mean ± SEM of two independent experiments, each performed in triplicate.

3.3.12 RECQL1 mRNA expression has clinicopathological significance in various subtypes of breast cancer

We investigated RECQL1 mRNA expression in the METABRIC cohort. 626 out of 1971 (31.7%) of breast tumours had low RECQL1 mRNA expression and 1345 out of 1971 (68.3%) of breast tumours had high RECQL1 mRNA expression. Clinicopathological associations are summarized in Table 3.5. The METABRIC study identified 10 novel biological subgroups, labelled integrative clusters (intClust) 1-10 by joint clustering of copy number and gene expression data (Curtis et al, 2012b). We evaluated associations between RECQL1 mRNA expression and these distinct biological subgroups (Table 3.5).

High RECQL1 mRNA was associated with low genomic instability intClust.3 ($p=0.026$) as characterised in the study (Curtis et al, 2012b). On the contrary, low RECQL1 mRNA expression was correlated to intClust.8 ($p=0.0455$) and intClust.9 ($p=0.0346$) phenotypes. Curtis et al. described intClust.8 as Luminal A ER+ sub-group and intClust.9 as luminal B ER+ sub-group (Curtis et al, 2012b).

Table 3.5 RECQL1 mRNA expression and breast cancer.

VARIABLE	RECQL1 mRNA Expression		P Values	
	Low	High	Un-adjusted	Adjusted
	N(%)	N (%)		
A) Pathological Parameters				
Lymph node stage				
Negative	345(55.1%)	690(51.3%)	0.283	0.3872
Positive (1-3)	93(14.9%)	221(16.4%)		
Positive (>3)	188(30.0%)	434(32.3%)		
Grade				
G1	52(8.8%)	117(9.0%)	**0.018**	0.0668
G2	268(45.4%)	502(38.6%)		
G3	270(54.8%)	680(52.3%)		
Tumour Size (cm)				
T 1a+b(1.0)	36(5.8%)	56(4.2%)	0.20	0.325
T 1c(>1.0-2.0)	228(36.8%)	538(40.2%)		
T2 (>2.0-5)	328(52.9%)	673(50.3%)		
T3 (>5)	28(4.5%)	70(5.2%)		
NPI				
≤ 3.4	132(22.5%)	259(20.1%)	0.236	0.3609
>3.4	454(77.5%)	1028(79.9%)		

Her2 overexpression (No)	550(87.9%)	1182(87.5%)	0.817	21.242
(Yes)	76(12.1%)	169(12.5%)		
Triple negative (No)	549(87.7)	1111(82.2)	**0.002**	0.052
(Yes)	77(12.3)	240(17.8)		
ER (Negative)	128(20.4%)	342(25.3%)	**0.018**	0.0585
(Positive)	498(79.6%)	1009(74.7%)		
PR (Negative)	287(45.8%)	649(48.0%)	0.364	0.4506
(Positive)	339(54.2%)	702(52.0%)		
Genefu subtype				
ER-/Her-2 negative	43(13.9%)	107(15.6%)	0.487	0.575
ER+/Her-2 negative/high proliferation	109(35.3%)	257(37.5%)	0.497	0.561
ER+/Her-2 negative/low proliferation	129(41.7%)	239(34.9%)	**0.038**	0.0898
Her-2 positive	28(9.1%)	82(12.0%)	0.176	0.3268
PAM50 subtype				
PAM50.Her2	87(15.6%)	151(12.4%)	0.067	0.145

PAM50.Basal	86(15.4%)	244(20.1%)	**0.020**	0.0563
PAM50.LumA	246(44.2%)	469(38.6%)	**0.027**	0.0702
PAM50.LumB	138(24.8%)	351(28.9%)	0.072	0.144
IntClust subgroups				
intClust.1	38(6.1%)	99(7.3%)	0.306	0.3978
intClust.2	28(4.5%)	44(3.3%)	0.179	0.31
intClust.3	69(11.0%)	221(16.4%)	**0.002**	**0.026**
intClust.4	100(16.0%)	243(18.0%)	0.272	0.39
intClust.5	58(9.3%)	131(9.7%)	0.762	0.825
intClust.6	26(4.2%)	60(4.4%)	0.770	0.8008
intClust.7	75(12.0%)	114(8.4%)	**0.013**	0.0563
intClust.8	115(18.4)	185(13.7%)	**0.007**	**0.0455**
intClust.9	62(9.9%)	84(6.2%)	**0.004**	**0.0346**
intClust.10	55(8.8%)	170(12.6%)	**0.013**	0.0676

Bold = Statistically significant;HER2: human epidermal growth factor receptor 2; ER: oestrogen receptor; PR: progesterone receptor; Triple negative: ER-/PR-/HER2-

3.3.13 RECQL1 mRNA expression has prognostic significance in various subtypes of breast cancer

We carried out survival analysis in the METABRIC cohort. Low RECQL1 mRNA expression was associated with poor BCSS (p=0.001) in the whole cohort [Figure 3.8 A]. In ER+ tumours, low RECQL1 mRNA expression remained associated with poor BCSS (p=0.001) [Figure 3.8 B], including in patients who received adjuvant endocrine therapy (p=0.003) [Figure 3.8 C]. However, in ER- tumours, RECQL1 mRNA expression did not show any significant impact on survival, including in patients who received adjuvant chemotherapy (p=0.071 and p=0.071 respectively) [Figures 3.8 D,E].

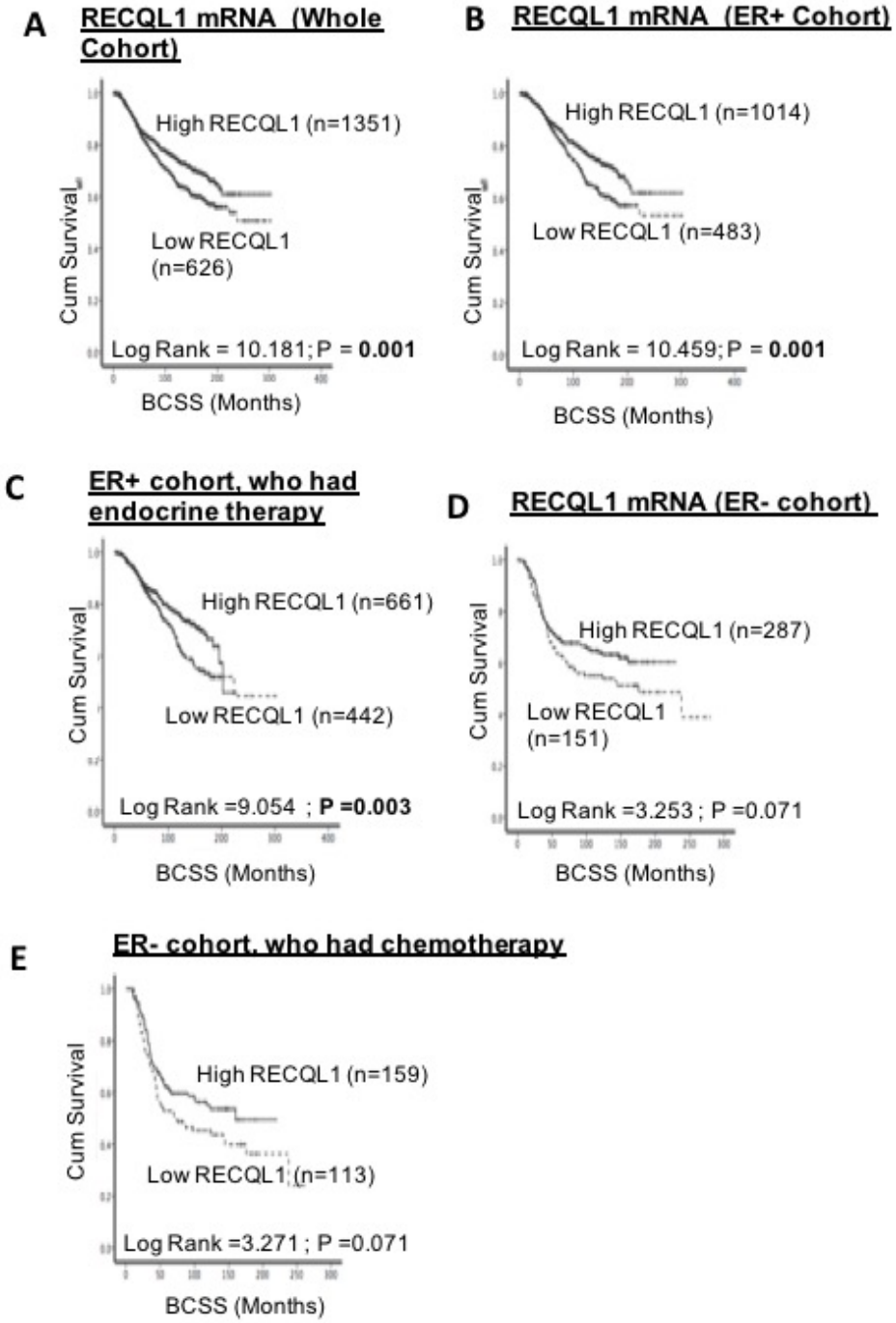


Figure 3.8 RECQL1 mRNA expression and breast cancer survival

RECQL1 mRNA expression and survival in **A.** the whole cohort. **B**. ER+ tumours. **C**. ER+ tumours who received endocrine therapy. **D.** ER- tumours. **E.** ER- tumours who received chemotherapy.

3.3.14 Summary of RECQL1 mRNA results

Taken together, this data provides evidence that RECQL1 mRNA level has clinicopathological and prognostic significance in various sub-types of breast cancers.

3.4 Discussion

RECQ DNA helicases have five members including RECQL1, RECQL5, RECQL4, WRN and BLM. RECQL1 is a key member of the RECQ family of DNA helicases. RECQL1 plays an integral role in the maintenance of replication fork progression, DNA repair and gene expression mechanisms (Sami & Sharma, 2013b; Sharma, 2014b; Sharma et al, 2012; Wu & Brosh, 2010).

However, the role of RECQL1 in cancer pathogenesis appears to be complex. RECQL1 has been shown to be overexpressed in glioblastoma (Mendoza-Maldonado et al, 2011b), hepatocellular carcinoma (Futami et al, 2010a), ovarian cancers (Sanada et al, 2013), melanoma (Jewell et al, 2010), non small cell lung cancer (Li et al, 2016), multiple myeloma (Viziteu et al, 2017) and head & neck cancer models (Zhang et al, 2006). The tumours may be reliant on RECQL1 to tolerate replication-induced DNA damage, a feature seen in proliferating cancer cells. On the other hand, in normal cells, RECQL1 may function as a 'caretaker of the genome' (Sami & Sharma, 2013b; Sharma, 2014b; Sharma et al, 2012; Wu & Brosh, 2010).

RECQL1 also might have a critical tumour suppressor function as recent studies have shown germ-line mutations of RECQL1 in breast cancer patients without hereditary BRCA mutation (Cybulski et al, 2015b; Sun et al, 2015b).

It remains unclear whether RECQL1 plays any role in sporadic breast cancer pathogenesis. Hence, we have conducted a comprehensive analysis of RECQL1 helicase and demonstrated the prognostic and predictive significance of RECQL1 in sporadic breast cancers.

In our study, we made an interesting observation that there was not any cytoplasmic staining for RECQL1. This is in contrast to the cytoplasmic staining observed for BLM, RECQL4, and WRN in breast cancers (Arora et al, 2015b; Arora et al, 2016; Shamanna et al, 2016). The data suggest differential regulation of localisation for different RECQ helicases.

At the protein level, tumours with low RECQL1 also showed aggressive phenotypes and poor survival including ER+ tumours. The mechanism of down-regulation of RECQL1 in sporadic breast cancer is currently unknown. It is a possibility that there might be epigenetic silencing of RECQL1 promoter similar to previously reported epigenetic silencing of the BRCA1 promoter in approximately 11%-14% of breast tumours (Turner et al, 2004a). Pre-clinical studies of RECQL1 depleted cells have shown increased genomic instability resulting in increased frequency of spontaneous sister chromatid exchanges, chromosomal instability, DNA damage accumulation and mutagenesis (Sami & Sharma, 2013b; Sharma, 2014b; Sharma et al, 2012; Wu & Brosh, 2010). We

anticipate that a ‘mutator phenotype’ (Loeb, 2001) due to RECQL1 deficiency may promote an aggressive phenotype in ER+ breast cancers.

Furthermore, we also noticed that low PARP1, BRCA1 negative, low RAD51, low ATM, low nuclear pChk1, low nuclear Chk2, low XRCC1, low FEN1, low SMUG1 and low DNA-PKcs were significantly more likely in tumours with low nuclear RECQL1 protein level. Hence, our data suggest that low RECQL1 may increase genomic instability which can then cause dysregulation of other DNA repair factors, hence promoting a ‘mutator phenotype’.

We also made a key observation that low RECQL1 might be predictive marker as in our study low RECQL1 influenced survival in ER+ cohorts that received endocrine therapy indicating its predictive significance.

In our ER- sub-group, RECQL1 failed to show any significant impact on survival either in the patient who received no chemotherapy or who received historical CMF chemotherapy. Excitingly, in ER-negative tumours that received the next generation anthracycline chemotherapy, we observed poor disease-free survival in tumours that demonstrated overexpression of RECQL1. Our data suggest that ER- tumours may be dependent on RECQL1 to tolerate replication-induced DNA damage induced by anthracycline chemotherapy.

Breast cancer represents a heterogeneous group of diseases with distinct prognostic outcomes as demonstrated by genomic analysis (Curtis et al, 2012b). We know that ER, PR, and HER-2 expression status, cause an impact on breast cancer pathogenesis and outcome. Similarly, markers of proliferation and

genomic stability appear to influence biological and clinical behaviour of breast cancers (Curtis et al, 2012b; Joh et al, 2011; Turner et al, 2004a). RECQL1 play various critical functions including its role in DNA replication and repair. We expected the differential roles of RECQL1 in various molecular subtypes of breast cancers. As anticipated, high RECQL1 mRNA was associated with intClust.3 which is characterised by low genomic instability (Curtis et al, 2012b). On the contrary, low RECQL1 mRNA was linked to intClust.8 luminal A ER+ sub-group (low proliferating) phenotype (Curtis et al, 2012b). Interestingly, low RECQL1 mRNA was also observed in intClust.9 phenotype which belongs to luminal B sub-group suggesting a more complex role for RECQL1 in this sub-group. In the METABRIC cohort, we observed poor survival outcomes in patients with low RECQL1 mRNA expression. In contrast to our results, Zhu et al. recently showed that high expression of RECQL1 mRNA expression was significantly associated with reduced relapse free survival and post progression survival in all breast cancers. However in this study high RECQL1 expressing patients who were basal-like and p53 mutant had better survival outcome (Zhu et al, 2018).

Taken together, the mRNA and protein expression data would suggest that differential helicase expressions lead to distinct molecular phenotypes. However, there was a limitation in our study, that mRNA expression and protein expression studies were conducted in two independent cohorts.

It is well-established practice now to use PARP inhibitors in BRCA deficient breast tumours (Tewari et al, 2015). Our data provide evidence to suggest that a

similar synthetic lethality approach could be exploited in RECQL1 deficient sporadic breast tumours to design personalised therapy for this group of patients.

In conclusion, in this study, we were able to provide evidence that RECQL1 DNA helicase has prognostic and predictive significance in breast cancer.

Chapter 4

RECQL5 helicase expression in sporadic breast cancers

4 RECQL5 helicase expression in sporadic breast cancers

4.1 Introduction

The five DNA RECQ helicases namely RECQL1, RECQL5, RECQL4,WRN and BLM are a highly conserved family of proteins which play essential roles in maintaining stability of human genome (Aygun & Svejstrup, 2010; Brosh, 2013b; Hanada & Hickson, 2007b; Popuri et al, 2013). RECQL5 is a key member of the mammalian RECQ helicase family (Popuri et al, 2013). RECQL5α (410aa), RECQL5β (435aa) and RECQL5γ (991aa) (Shimamoto et al, 2000) are the three isoforms of RECQL5 identified so far. RECQL5α and RECQL5γ have cytoplasmic localization, however, RECQL5β (RECQL5) isoform has nuclear localisation (Shimamoto et al, 2000). RECQL5 is located at chromosome 17 (cytoband q25.1).

RECQL5 is a 3'-5' helicase which has a weak Holiday junction unwinding activity (Popuri et al, 2013). RECQL5 preferentially unwinds the lagging strand of DNA at replication fork structures and can enhance strand exchange in vitro (Kanagaraj et al, 2006). RECQL5 also play a critical role in homologous recombination (HR), base excision repair (BER), DNA replication and transcription (Popuri et al, 2013). The evidence to support the RECQL5 role in HR come from various studies. It has been shown previously that RECQL5 deficient cells demonstrate an increased rate of sister chromatid exchange and

RECQL5 depleted cells accumulate H2Ax and RAD51 foci (Hu et al, 2007b). Furthermore, in vitro studies have shown that mechanistically RECQL5 physically interacts with RAD51, leading an ATPase dependent disruption of RAD51 mediated presynaptic filament formation and hence showing anti-recombinase activity (Schwendener et al, 2010) like seen in Bloom syndrome helicase (BLM) (Hu et al, 2005).In addition, RECQL5 associates with the MRN complex and block MRE11 exonuclease activity (Zheng et al, 2009), which may also contribute to the HR function of RECQL5. Interestingly while the MRN complex is required to bring RECQL5 to sites of DSBs it's recruitment is independent of the helicase activity (Popuri et al, 2012b).

RECQL5 might be playing a role in base excision repair (BER) pathway but its precise role remains unclear. FEN1 activity is stimulated by RECQL5 interaction with the long patch BER proteins PCNA and FEN1. RECQL5 also co-localises with FEN1 after oxidative damage of DNA (Speina et al, 2010). In addition, the short patch BER protein XRCC1 is retained at sites of oxidative damage in the absence of RECQL5 and expression of PARP1 and XRCC1 may be regulated by RECQL5 (Tadokoro et al, 2012).

RECQL5 has also been shown to protect the cells from DNA replication stress. After replication stress, RECQL5 foci increase and overexpression of RECQL5 can overcome thymidine induced replication stress (Blundred et al, 2010). RECQL5 can also halt spontaneous replication fork collapse and RECQL5 depleted cells show hypersensitivity to Camptothecin, a DNA replication inhibitor (Hu et al, 2009; Wang et al, 2011). RECQL5 has also been involved in

regulation of transcription elongation and suppression of genomic instability associated with transcriptional stress (Aygun & Svejstrup, 2010; Izumikawa et al, 2008; Saponaro et al, 2014).

To accomplish its various biological functions, RECQL5 interacts with various other key DNA repair proteins including RAD51, RAD50, WRN, BLM, PARP1, FEN1, RNA Polymerase II, MRE11, NBS1, PCNA, Top IIa and Top IIIa/b (Popuri et al, 2013). RECQL5 has been shown to play complementary role with WRN syndrome helicase. It works with WRN at stalled replication forks and relocate to nucleus after replicative stress (Popuri et al, 2017b).

Germline mutation of three of the RECQ helicases (BLM, WRN, and RECQL4) has been associated to cancer predisposition syndromes namely Bloom syndrome, Werner syndrome and Rothmund-Thomson syndrome (Brosh, 2013b; Hanada & Hickson, 2007b). So far, RECQL5 helicase has not been shown to be associated with any disease phenotype. In preclinical studies, RECQL5 deficient mice cells show high levels of spontaneous double-strand breaks, are prone to gross chromosomal rearrangements and are susceptible to get lymphomas and various solid tumours including breast cancer (Hu et al, 2007b) (Lao et al, 2013).

Patterson et al. has recently shown that high expression of RECQL5 is seen in bladder cancer and it is associated with increased proliferation and poor survival (Patterson et al, 2016). It remains unclear whether RECQL5 play any role in non-familial breast cancer pathogenesis. Our hypothesis was that RECQL5 may

have an influence on sporadic breast cancer pathogenesis and prognosis. Therefore, in this study, we have evaluated RECQL5 mRNA expression in 1950 breast cancers, and RECQL5 protein level in 1902 breast cancers.

4.2 Methods

4.2.1 Patient selection for RECQL5 protein expression study

We performed a comprehensive evaluation of the protein expression of RECQL5 in two breast cancer patient cohorts. The first cohort consisted of 1650 primary invasive breast carcinomas patients who were diagnosed between 1986 and 1999 and entered into the Nottingham tenovus series. The second cohort was an independent series of 252 ER-negative invasive breast cancers diagnosed and treated at Nottingham University Hospitals between 1999 and 2007. Patient demographics of the Nottingham tenovus series (NTS) and ER-negative cohort is summarised in Table 2.4 and 2.5 respectively.

4.2.2 Tissue Microarray (TMA)

Breast tumours were arrayed in tissue microarrays (TMAs) constructed with two replicate 0.6 mm cores from centre and periphery of the tumours. Details of TMA construction is detailed in section 2.1.1.

4.2.3 Determination of optimal RECQL5 antibody concentration for IHC

Standard IHC technique, as described in the section 2.1.2, was used to ascertain the optimal concentration and conditions for RECQL5 antibody staining. Each

dilution was tested on the same type of sample and also on the same version of TMA, to keep the experimental conditions standardized. The online resource 'Human Protein Atlas' (www.proteinatlas.org) was consulted to select the best tissue for optimisation, but we could not find any RECQL5 protein expression data. However, we identified that RECQL5 RNA expression was seen in various tissues including breast tissue. We proceeded with antibody optimisation on breast tissue. The RECQL5 antibody (Rabbit Antibody, polyclonal) was purchased from Sigma Laboratory, catalogue number HPA-029971. Manufacturer recommended optimal dilutions for IHC between 1:50 and 1:200. Antigen retrieval method used was HIER (Citrate buffer pH 6.0) as per manufacturer recommendation. Incubation time used was 60 minutes at room temperature. We tried 1:50, 1:100, 1:150, and 1:200 dilutions for optimisation.

All optimisation slides were then evaluated by site specialist expert pathologist (TAF and EAR) to decide if further optimisation was required or to identify the optimal concentration for staining the breast tumours. After a thorough review by an expert pathologist, 1:100 was selected as optimal concentration for IHC staining because it showed a differential pattern of staining with minimum background / non-specific binding (Figure 4.1).

Table 4.1 Primary antibody details

Antibody	**Clonality**	**Source**	**Catalogue No.**	**Antigen Retrieval Method**	**Optimal Dilution/ Incubation time**
RECQL5	Rabbit Ab Polyclonal	Sigma	HPA-029971	Citrate Buffer pH 6.0	1:100 (60 min at RT)

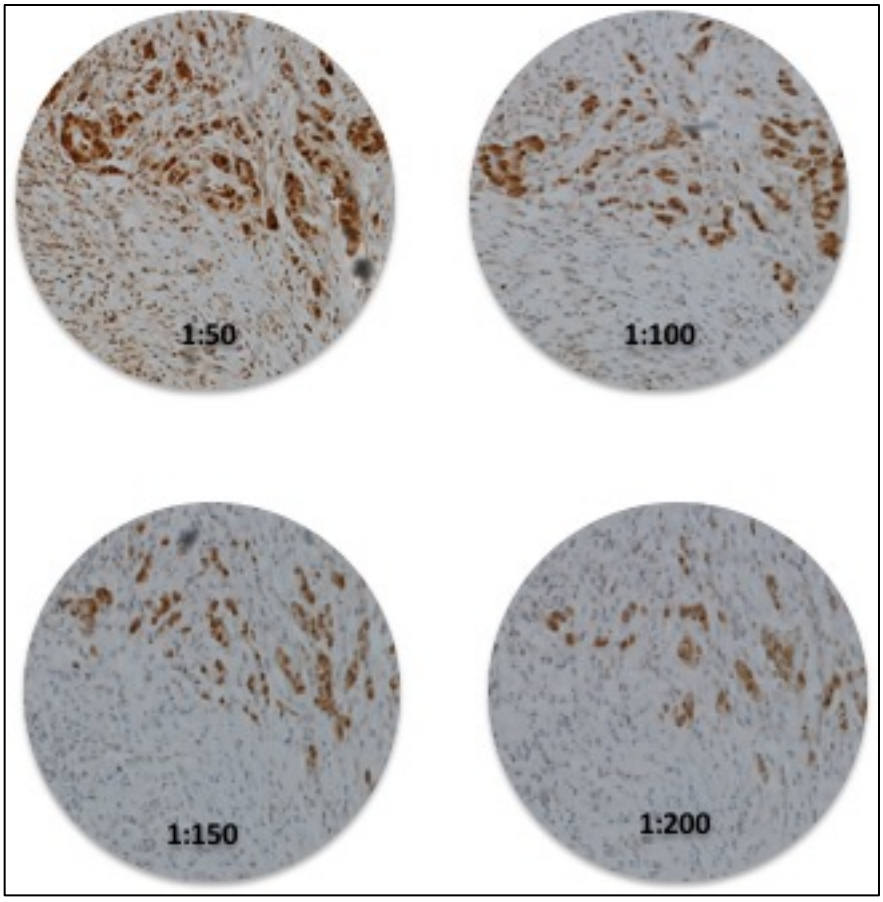


Figure 4.1 Photomicrographs of RECQL5 protein expression in Breast TMA with different optimisation dilutions

1:50, 1:100, 1:150 and 1:200 dilutions were used for optimisation. 1:100 was selected as optimal dilution for staining as it showed the best differential staining pattern and little or no background staining.

4.2.4 Inclusion of controls to ensure the quality of staining

Both positive and negative controls were used to ensure the quality of IHC staining. A negative control was included by omitting primary antibody and exposing to antibody diluent only. This was to determine if the secondary antibody was binding non-specifically resulting in false positives. It will be ideal to use absorption control to test if the primary antibody binds specifically to the antigen of interest. As described previously (Li et al., 2012), Beta 2 microglobulin (B2M) is highly expressed in breast cancer, hence this was used as positive control. The same positive control section was included in each run to ensure consistency to be evaluated.

4.2.5 Cell lines and optimal antibody concentration used for Western Blot analysis

Western blot analysis as described in the section 2.3.2.2 was used to determine the specificity of anti RECQL5 antibody, before using it for IHC. We assessed RECQL5 protein expression in four breast cancer cell lines MCF7, MDA-MB-231, MDA-MB-436, and MDA-MB-468. The characteristics of these cell lines are explained in detail in section 2.9. The details of cell lysate preparation and sample preparation are also detailed in section 2.3.2.2.1.

Table 4.2 The Primary antibody and its optimal concentration used for western blot

Antibody	Clonality	Source	Catalogue No.	Optimal Dilution/ Incubation time
RECQL5	Rabbit Ab (Polyclonal)	Sigma	HPA-029971	1:1000 (Overnight at 4^0C)

4.2.6 IHC technique

Details of the IHC technique used is described in detail in section 2.1.2.

4.2.7 Histopathological scoring of stained Breast TMAs

Scoring of all Breast TMAs was performed by me and an expert histopathologist blinded to the clinicopathological characteristics of patients. Not all cores within the TMA were suitable for IHC analysis as some cores were missing or lacked a tumour (<15% tumour). 1200 breast tumours were suitable for analysis. H scoring was used to score breast tumours as described in detail in section 2.1.6. H-score (range 0-300) was calculated by multiplying the intensity of staining and percentage staining. RECQL5 protein expression was categorised based on the frequency histogram distributions. As our data was non-parametric, median H Score was used to dichotomize our data into low and high expression. A median H score of ≥10 was taken as the cut-off for high RECQL5. To get IHC data on RAD51 expression in Breast cancer, we collaborated with Prof. Emad Rakha's group. RAD51 antibody was previously optimised, stained and scored

by his group (Alshareeda et al, 2016). The details of RAD51 antibody used for IHC is detailed in Table 2.2. RAD51 protein expression was also categorised based on the frequency histogram distributions. A median H score of ≥8 was taken as the cut-off for high RAD51 nuclear expression.

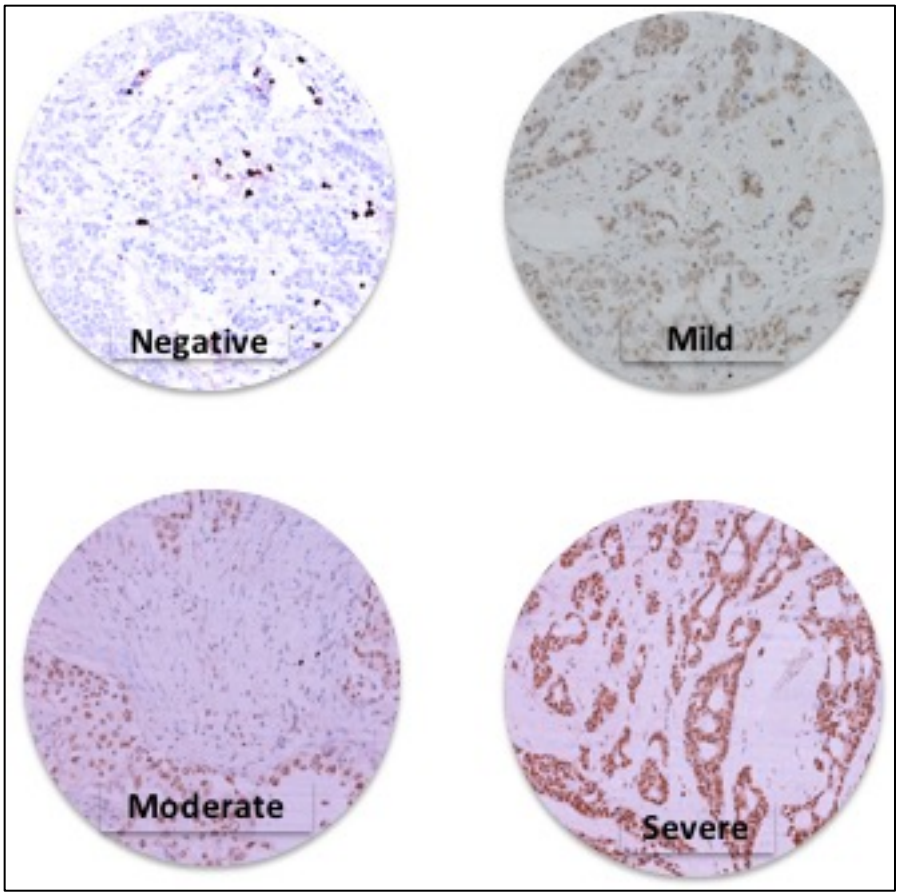


Figure 4.2 Photomicrographs of RECQL5 protein expression in breast cancers

4.2.8 Quantitative real-time PCR

Total RNA was extracted from MCF-7, MDA-MB-231, MDA-MB-468 and MDA-MB-436 cells using RNeasy Mini kit (QIAGEN, UK). The RNA quantification was done using a NanoDrop 2000c Spectrophotometer (Thermo Scientific). The cDNA was synthesised from 0.5 μg of total RNA using RT^2 first strand kit (QIAGEN). The real-time qPCR was performed using SYBR Green PCR Master mix (Applied Biosystems) with primer set (RECQL5 QuantiTect Primer Assay, catalogue no. QT00084973, QIAGEN) targeting RECQL5 gene. The glyceraldehyde-3-phosphate dehydrogenase was used as a housekeeper gene (GAPDH QuantiTect Primer Assay, catalog no. QT00079247, QIAGEN). The real-time PCR for each RNA sample was done in triplicate. NTC (No Template Control) was used to rule out cross contamination of reagents and surfaces. To constitute NTC, all the RT-PCR reagents were included except the RNA template. Minus reverse transcriptase (- RT) control was used to make the assessment of genomic DNA contamination. Non-specific binding is a problem with SYBR green , hence we included a melting curve analysis to get information about the purity of the amplified product. A single peak was seen which indicated the presence of only one double stranded product.

4.2.9 Patient selection for RECQL5 mRNA expression in Breast Cancer

We investigated RECQL5 mRNA expression in METABRIC (Molecular Taxonomy of Breast Cancer International Consortium) cohort. The METABRIC study protocol and the molecular profiling methodology in a cohort of 1977 breast cancer samples are detailed by Curtis et al (Curtis et al, 2012b). Patient demographics, details of data acquisition and analysis are summarised in Table 2.7 .

RECQL5 probe (ID: ILMN_1697682) was used because it was a perfect match and quality for its target, having a GC content of 58%, 0 SNPs and it does not possess a poly G tail at the end. The cut off for the high vs low RECQL5 gene expression was done using x-tile software (Version 3.6.1) so that the resultant subgroups had a significant difference in survival outcomes.

4.2.10 Statistical analysis

Data analysis was performed using SPSS (SPSS, version 22 Chicago, IL) as described in detail in section 2.1.9.

4.2.11 Ethical approval

Ethical approval was obtained from the Nottingham Research Ethics Committee (Reference number C202313, Approval date : 21/10/2010). Tumour Marker Prognostics Studies (REMARK) criteria, recommended by McShane et al (McShane et al, 2005), were followed throughout this project.

4.3 Results

4.3.1 RECQL5 antibody specificity is confirmed by Western blot analysis

We initially investigated RECQL5 protein expression in the Breast cancer cell lines MCF-7, MDA-MB-231, MDA-MB-436, and MDA-MB-468. As shown in Figure 4.3B, MCF-7, MDA-MB-231, MDA-MB-436, and MDA-MB-468 have a robust expression of RECQL5 relative to β-Actin. To provide evidence of specificity of anti RECQL5 antibody used for IHC study, we did western blot in breast cancer cell lines. As shown in Figure 4.3A, Breast cancer cell lines (MCF-7, MDA-MB-231, MDA-MB-436, and MDA-MB-468) have a robust RECQL5 expression seen as a single band at 108kDa.

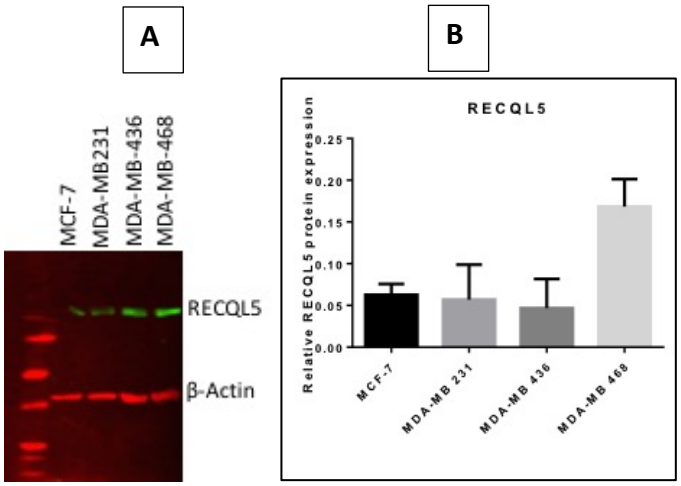


Figure 4.3 Relative RECQL5 protein expression in Breast cancer cell lines

A. Western Blot of RECQL5 Expression in MCF7, MDA-MB-231, MDA-MB-436, & MDA-MB-468 cell lines **B.** RECQL5 protein expression in MCF7, MDA-MB-231, MDA-MB-436 & MDA-MB-468 breast cancer cell lines relative to β-Actin. Data presented as mean ± SEM of three independent experiments.

4.3.2 RECQL5 is localized to the nucleus in sporadic breast tumours

We then proceeded to IHC analysis of primary breast series. We initially did the analysis for RECQL5 expression only. We did not observe any cytoplasmic staining of RECQL5. We observed that RECQL5 was localized to the nucleus only. 556 out of 1200 (46.3%) of tumours revealed low nuclear RECQL5 expression and 644 out of 1200 (53.7%) of tumours showed high nuclear RECQL5 expression.

4.3.3 Low RECQL5 protein expression is significantly associated with aggressive breast cancer phenotypes and low levels of other DNA repair proteins

As shown in Table 4.3, tumours with low nuclear RECQL5 levels were significantly associated with high grade (p=0.008) , tubule formation (p=0.005), tumour type (p=0.009), HER2 overexpression (p=0.036) and ER- (p=0.008).

Furthermore, low RECQL 5 expression was also found to be associated with low levels of other DNA repair proteins such as BRCA1, XRCC1, FEN1, SMUG1, APE1, Polβ, ATM, ATR, cytoplasmic Chk1, Chk2, RAD51, and DNA-PKc (p values < 0.01) [Table 4.3]

Low RECQL5 was also significantly associated with low nuclear and cytoplasmic RECQL4 and BLM (p values<0.01) [Table 4.3].

Table 4.3 RECQL5 protein levels and breast cancer

VARIABLE	RECQL5 (Nuclear) Protein Expression		P- value Un-adjusted	P –Value Adjusted
	Low N (%)	High N (%)		
A) Pathological Parameters				
Tumour Size				
<1cm	38 (6.9)	69 (10.7)	0.061	0.09
>1-2cm	271 (49.0)	325 (50.6)		
>2-5cm	230 (41.6)	235 (36.6)		
>5cm	14 (2.5)	13 (2.0)		
Tumour Stage				
1	326 (58.8)	400 (62.2)	0.161	0.1982
2	183 (33.0)	181 (28.1)		
3	45 (8.1)	62 (9.6)		
Tumour Grade				
G1	67 (12.1)	122 (19.0)	**0.003**	**0.008**
G2	182 (32.9)	209 (32.6)		
G3	304 (55.0)	311 (48.4)		
Mitotic Index				
M1 (low; mitoses < 10)	172 (31.3)	233 (36.5)	**0.050**	0.075
M2 (medium; mitoses 10-18)	97 (17.6)	124 (19.4)		
M3 (high; mitosis >18)	281 (51.5)	281 (44.0)		
Tubule Formation				
1 (>75% of definite tubule)	27 (4.9)	35 (5.5)	**0.002**	**0.005**
2 (10%-75% definite tubule)	155 (28.2)	239 (37.5)		
3 (<10% definite tubule)	368 (66.9)	364 (57.1)		
Pleomorphism				
1 (small-regular uniform)	12 (2.2)	18 (2.8)	0.377	0.431
2 (Moderate variation)	194 (35.3)	245 (38.4)		
3 (Marked variation)	344 (62.5)	375 (58.8)		
Tumour Type				
IDC-NST	309 (63.4)	322 (59.9)	**0.004**	**0.009**
Tubular Carcinoma	73 (15.0)	51 (22.1)		
Medullary Carcinoma	16 (3.3)	6 (1.1)		
ILC	38 (7.8)	13 (9.1)		
Others	51 (10.5)	26 (7.8)		

Lymphovascular Invasion No Yes	361 (65.9) 187 (34.1)	421 (66.2) 215 (33.8)	0.908	43.58
B) Aggressive phenotype				
Her2 overexpression No Yes	466 (86.3) 74 (13.7)	567 (90.6) 59 (9.4)	**0.022**	**0.036**
Triple Negative Phenotype No Yes	420 (78.1) 118 (21.9)	516 (82.8) 107 (17.2)	**0.041**	0.065
Basal Like Phenotype No Yes	446 (85.3) 77 (14.7)	532 (89.3) 64 (10.7)	**0.045**	0.069
Cytokeratin 6 (CK6) Negative Positive	391 (82.1) 85 (17.9)	440 (84.1) 83 (15.9)	0.402	0.448
Cytokeratin 14 (CK14) Negative Positive	410 (87.2) 60 (12.8)	451 (86.7) 69 (13.3)	0.814	0.831
Cytokeratin 18 (CK18) Negative Positive	54 (12.2) 388 (87.8)	44 (9.1) 442 (90.9)	0.117	0.161
Cytokeratin 19 (CK19) Negative Positive	36 (7.6) 440 (92.4)	27 (5.2) 495 (94.8)	0.121	0.161
C) Hormone receptors				
ER Negative Positive	171 (31.3) 375 (68.7)	150 (23.8) 480 (76.2)	**0.004**	**0.008**
PR Negative Positive	245 (46.9) 483 (53.1)	249 (42.0) 344 (58.0)	0.097	0.137

AR Negative Positive	191 (42.4) 259 (57.6)	166 (33.7) 327 (66.3)	**0.006**	**0.011**
D) DNA Repair				
BRCA1 Absent Normal	103 (26.0) 293 (74.0)	67 (15.1) 377 (84.9)	**$8.4x10^{-6}$**	**0.00001**
XRCC1 Low High	91 (22.1) 320 (77.9)	49 (10.7) 408 (89.3)	**$5.0x10^{-6}$**	**0.00001**
FEN1 Low High	292 (78.5) 80 (21.5)	295 (69.1) 132 (30.9)	**0.003**	**0.007**
SMUG1 Low High	176 (48.4) 188 (51.6)	141 (33.6) 279 (66.4)	**$2.6x10^{-5}$**	**0.0001**
APE1 Low High	292 (63.8) 166 (36.2)	213 (39.7) 324 (60.3)	**$1.0x10^{-6}$**	**0.00001**
Pol β Low High	221 (45.9) 261 (54.1)	189 (33.3) 378 (66.7)	**$3.5x10^{-5}$**	**0.0001**
ATR Low High	344 (74.1) 120 (25.9)	315 (60.7) 204 (39.3)	**$8.0x10^{-6}$**	**0.00001**
ATM Low High	195 (57.4) 145 (42.6)	187 (48.4) 199 (51.6)	**0.016**	**0.027**
DNA-PKcs Low High	200 (45.9) 236 (54.1)	133 (26.7) 365 (73.3)	**$1.0x10^{-6}$**	**0.00001**
TOP2A Low Overexpression	204 (53.1) 180 (46.9)	164 (37.6) 272 (62.4)	**$8.0x10^{-6}$**	**0.00001**
pCHK1 (Cytoplasmic) Low High	214 (38.6) 341 (61.4)	188 (29.2) 456 (70.8)	**0.001**	**0.003**
CHK2 Low High	230 (57.2) 172 (42.8)	168 (38.4) 270 (61.6)	**$1.0x10^{-6}$**	**0.00001**

RECQL4 (Nuclear) Low High	 279 (71.2) 113 (28.8)	 209 (47.3) 233 (52.7)	**$2.7x10^{-12}$**	**0.00001**
RECQL4 (Cytoplasmic) Low High	 230 (58.8) 161 (41.2)	 204 (46.4) 236 (53.6)	**0.00033**	**0.001**
BLM (Nuclear) Low High	 170 (37.0) 290 (63.0)	 85 (16.5) 429 (83.5)	**$4.5x10^{-13}$**	**0.00001**
BLM (Cytoplasmic) Low High	 355 (77.2) 105 (22.8)	 355 (16.5) 155 (30.4)	**0.008**	**0.014**
RAD51 (Nuclear) Low High	 176 (57.3) 131 (42.7)	 156 (45.6) 186 (54.4)	**0.003**	**0.007**
RAD51 (Cytoplasmic) Low High	 15 (4.9) 292 (95.1)	 25 (7.4) 311 (92.6)	0.180	0.211

Bold = Statistically significant; HER2: human epidermal growth factor receptor 2; ER: oestrogen receptor; PR: progesterone receptor; Triple negative: ER-/PR-/HER2-

4.3.4 RECQL5 protein expression is not significantly associated with breast cancer specific survival

We then proceeded to the univariate survival analysis. In the whole cohort, RECQL5 protein expression failed to show any statistically significant correlation with breast-cancer specific survival [Figure 4.4 A]. We then carried out subgroup analysis and observed no statistically significant association with survival in ER+ [Figure 4.4 B] and ER- subgroups [Figure 4.4 C].

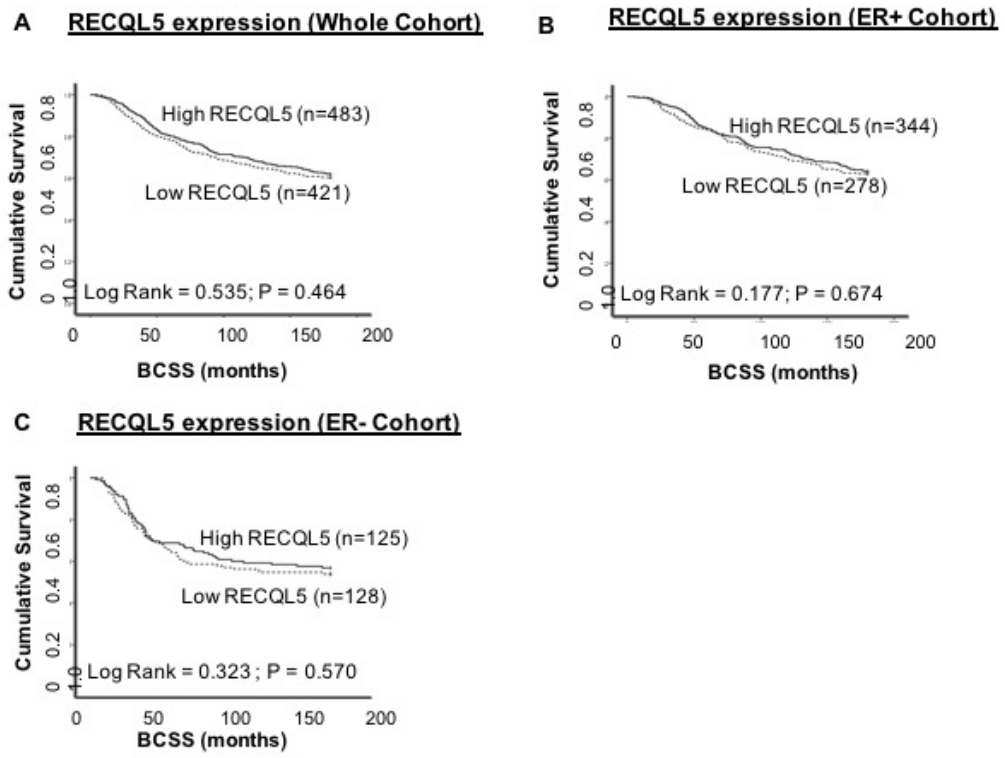


Figure 4.4 RECQL5 Expression in Whole, ER+ & ER- Cohort

A. RECQL5 protein level and survival in the whole cohort. **B.** RECQL5 protein level and survival in ER+ tumours. **C.** RECQL5 protein level and survival in ER- tumours [BCSS= breast cancer-specific survival].

4.3.5 RECQL5/RAD51 co-expression is significantly associated with aggressive breast cancer phenotypes

Despite having clinicopathological associations with aggressive phenotypes, RECQL5 failed to show any prognostic significance, so we hypothesised that RECQL5 might be interacting with RAD51 to influence clinical outcomes. We therefore, proceeded to co-expression analysis.

RECQL5 has been shown to interact with RAD51 causing disruption of RAD51 mediated presynaptic filament formation (Popuri et al, 2013; Schwendener et al, 2010). Our RECQL5/RAD51 co-expression analysis show that tumours with high RECQL5-low RAD51 nuclear protein levels were significantly associated with high grade ($p=0.0005$), high mitosis ($p=0.017$) , and pleomorphism ($p=0.043$) [Table 4.4]. Tumours with low RECQL5-low RAD51 nuclear protein levels were significantly associated with tubule formation, NPI>3.4 and ER/PR negativity ($p <0.005$) [Table 4.4].

Table 4.4 RECQL5 and RAD51 protein Co-expression and breast cancer

VARIABLE	RECQL5 and RAD51 protein co-expression				P -Value	
	RQ5n-/RAD51n- N (%)	RQ5n+/ RAD51n- N (%)	RQ5n-/ RAD51n+ N (%)	RQ5n+/ RAD51n+ N%	Unadjusted	Adjusted
A) Pathological Parameters						
Tumour Size						
≤1cm	12 (6.8)	9 (5.8)	10 (7.6)	16 (8.6)	**0.031**	**0.045**
>1-2cm	77 (43.8)	65 (41.9)	73 (55.7)	107 (57.5)		
>2-5cm	84 (47.7)	78 (50.3)	45 (34.4)	62 (33.3)		
>5cm	3 (1.7)	3 (1.9)	3 (2.3)	1 (0.5)		
Tumour Stage						
1	96 (54.5)	83 (53.2)	79 (60.3)	112 (60.2)	0.370	0.401
2	64 (36.4)	57 (36.5)	44 (33.6)	52 (28.0)		
3	16 (9.1)	16 (10.3)	8 (6.1)	22 (11.8)		
Tumour Grade						
G1	13 (7.4)	18 (11.5)	14 (10.7)	37 (19.9)	**$4.1x10^{-5}$**	**0.0005**
G2	59 (33.5)	38 (24.4)	53 (40.5)	73 (39.2)		
G3	104 (59.1)	100 (64.1)	64 (48.9)	76 (40.9)		

Mitotic Index M1 (low; mitoses < 10) M2 (medium; mitoses 10-18) M3 (high; mitosis >18)	 45 (26.0) 38 (22.0) 90 (52.0)	 34 (22.5) 28 (18.5) 89 (58.9)	 41 (31.5) 24 (18.5) 65 (50.0)	 72 (40.0) 38 (21.1) 70 (38.9)	**0.008**	**0.017**
Tubule Formation 1 (>75% definite tubule) 2 (10%-75% definite tubule) 3 (<10% definite tubule)	 5 (2.9) 45 (26.0) 123 (71.1)	 2 (1.3) 49 (32.5) 100 (66.2)	 7 (5.4) 43 (33.1) 80 (61.5)	 7 (3.9) 75 (41.7) 98 (54.4)	**0.025**	**0.046**
Pleomorphism 1 (small-regular uniform) 2 (Moderate variation) 3 (Marked variation)	 0 (0.0) 61 (35.5) 111 (64.5)	 1 (0.7) 42 (27.8) 108 (71.5)	 2 (1.6) 48 (37.2) 79 (61.2)	 2 (1.1) 82 (45.6) 96 (53.3)	**0.027**	**0.043**
Tumour Type IDC-NST Tubular Medullary ILC Others	 121 (69.9) 21 (12.1) 7 (4.0) 14 (8.1) 3 (1.7)	 113 (72.4) 22 (14.1) 2 (1.3) 8 (5.1) 1 (0.6)	 75 (58.1) 27 (20.9) 3 (2.3) 13 (10.1) 0 (0.0)	 102 (55.1) 50 (27.0) 0 (0.0) 20 (10.8) 1 (0.5)	**0.002**	**0.005**
Lymph Node Status Negative Positive (1-3) Positive (>3)	 81 (54.4) 56 (37.6) 12 (8.1)	 69 (51.1) 54 (40.0) 12 (8.9)	 72 (60.0) 44 (36.7) 4 (3.3)	 97 (58.4) 53 (31.9) 16 (9.6)	0.353	0.417
<u>B) Aggressive Phenotype</u>						
Her2 overexpression No Yes	 153 (87.4) 22 (12.6)	 123 (80.4) 30 (19.6)	 105 (81.4) 24 (18.6)	 159 (88.3) 21 (11.7)	0.105	0.136

Triple Negative Phenotype No Yes	149 (84.7) 27 (15.3)	130 (83.3) 26 (16.7)	115 (87.8) 16 (12.2)	151 (81.2) 35 (18.8)	0.457	5.94
NPI ≤3.4 >3.4	31 (18.3) 138 (81.7)	31 (21.2) 115 (78.8)	34 (27.4) 90 (72.6)	68 (38.4) 109 (61.6)	**$1.1x10^{-4}$**	**0.0005**
C) Hormone Receptors						
ER Negative Positive	60 (35.1) 111 (64.9)	53 (34.9) 99 (65.1)	32 (25.4) 94 (74.6)	28 (15.7) 150 (84.3)	**$7.6x10^{-5}$**	**0.0005**
PR Negative Positive	91 (54.5) 76 (45.5)	77 (52.0) 71 (48.0)	49 (39.5) 75 (60.5)	63 (35.6) 114 (64.4)	**0.001**	**0.003**

Bold = Statistically significant; HER2: human epidermal growth factor receptor 2; ER: oestrogen receptor; PR: progesterone receptor; Triple negative: ER-/PR-/HER2- . Adjusted p values were calculated using Benjamini-Hochberg false discovery rate method to adjust for multiple testing.

4.3.6 High RECQL5 and low RAD51 expression is significantly associated with poor breast cancer-specific survival in whole and ER+ cohort

In univariate analysis, tumours with high RECQL5-low RAD51 nuclear protein levels were associated with poor breast cancer-specific survival in the whole cohort (p=0.003) [Figure 4.5 A]. In sub-group analysis, ER+ tumours with high RECQL5-low RAD51 nuclear protein levels were associated with poor breast cancer specific survival (p=0.010) [Figure 4.6 B]. In ER- cohort, RECQL5-RAD51 co-expression did not influence survival (p=0.628) [Figure 4.6 C].

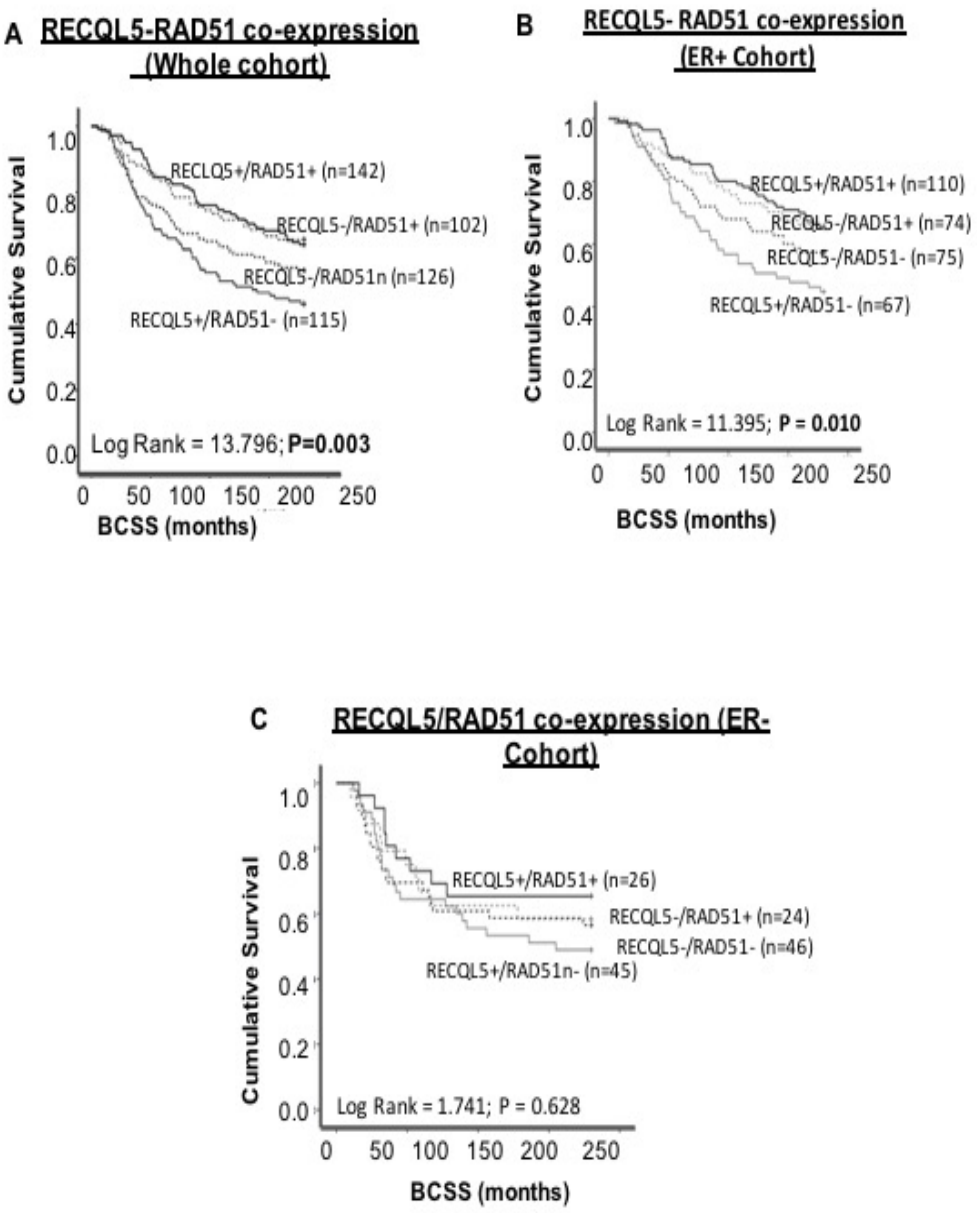


Figure 4.5 RECQL5/RAD51 Co-expression in Whole, ER+ & ER- Cohort

A. RECQL5/RAD51 protein co-expression and survival in the whole cohort. **B.** RECQL5/RAD51 protein co-expression and survival in ER+ tumours. **C.** RECQL5/RAD51 protein co-expression and survival in ER- tumours [BCSS= breast cancer specific survival].

4.3.7 RECQL5-RAD51 co-expression is an independent prognostic marker of breast cancer-specific survival on multivariate analysis

In multivariate analysis (Table 4.5), RECQL5-RAD51 co-expression remained a statistically significant independent marker of prognosis (p=0.022) in the whole cohort. NPI and HER-2 expression were other factors independently associated with breast cancer-specific survival (p values 0.001 and 0.0001 respectively). In the ER+ sub-group, RECQL5-RAD51 co-expression was of borderline significance (p=0.07), but not in the ER- sub-group (p=0.172).

Table 4.5 Multivariate analysis of RECQL5-RAD51 protein co-expression in breast cancer

Variable	**B**	**P value**	**Exp (B)**	**95.0% CI for Exp (B)**	
				Lower	Upper
Breast Cancer Specific Survival in the whole cohort					
RECQL5-RAD51 co-expression	-0.069	**0.022**	0.934	0.880	0.990
NPI	0.769	**0.001**	2.158	1.378	3.379
ER Status	0.293	0.097	1.340	.949	1.893
HER2 Status	0.728	**0.0001**	2.071	1.432	2.994
Breast Cancer Specific Survival in the ER+ cohort					
RECQL5-RAD51 co- expression	-0.063	0.073	0.939	0.876	1.006
NPI	0.784	**0.001**	2.190	1.376	3.484
HER2 Status	0.848	**0.001**	2.335	1.448	3.767
Breast Cancer Specific Survival in the ER- cohort					

RECQL5-RAD51 co-expression	-0.081	0.172	0.923	0.822	1.036
NPI	0.651	0.519	1.917	0.265	13.882
HER2 Status	0.551	0.057	1.735	0.984	3.062

4.3.8 Breast cancer cell lines have a robust expression of RECQL5 mRNA

We initially profiled a panel of breast cancer cell lines. MDA-MB-231, MDA-MB-436, and MDA-MB-468 showed lower expression of RECQL5 mRNA as compared to MCF7 control cell line. (Figure 4.6).

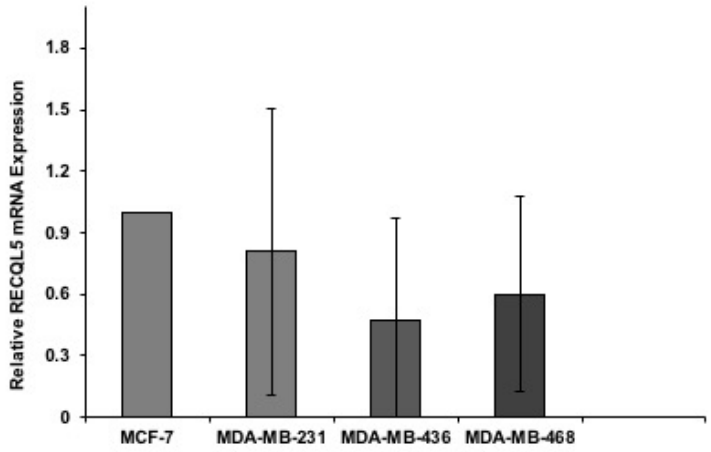


Figure 4.6 Relative mRNA expression of RECQL5 in Breast cancer cells lines

A graph of mRNA expression of RECQL5 in MDA-MB-231, MDA-MB-436, and MDA-MB-468 breast cancer cell lines relative to MCF7 control breast cancer cell line. Data presented as mean ± SEM of two independent experiments, each performed in triplicate.

4.3.9 High RECQL5 mRNA expression is significantly associated with aggressive clinicopathological features and molecular phenotypes

We then proceeded to investigate RECQL5 mRNA expression in the METABRIC cohort. 1306 out of 1977 (66%) of breast tumours had low RECQL5 mRNA expression and 671 out of 1977 (34%) of breast tumours had high RECQL5mRNA expression. High RECQL5 mRNA expression was significantly associated with aggressive clinicopathological features including high histological grade (adjusted p=0.023**)**, HER2 overexpression, and triple negative phenotypes (unadjusted p values <0.05) [Table 4.6]. High RECQL5 mRNA expression was also found to be significantly correlated to previously described breast cancer molecular phenotypes; PAM50.Her2 (p=0.0008) [Table 4.6]. High RECQL5 mRNA expression was also more likely in ER+/HER2 -/high proliferation Geneufu molecular sub-types (p value = 0.018) [Table 4.6]. On the other hand, PAM50.LumA was associated with low levels of RECQL5 mRNA (p value <0.05) [Table 4.6]. Similarly, low *RECQL5*mRNA expression was associated with intClust.3 (p<0.00001) and intClust.4 (ps<0.001) molecular phenotypes that have a good prognosis (Curtis et al, 2012b). However**,** high RECQL5 mRNA expression was significantly associated with intClust.1 (p<0.00001), and intClust.9 (p<0.00001) that have intermediate to poor clinical outcome (Curtis et al, 2012b).High RECQL5 mRNA was also associated with intClust.10 which was described as highly genomic instability group (Curtis et al, 2012b)

Table 4.6 Association between RECQL5 mRNA expression and clinicopathologic variables in METABRIC cohort

VARIABLE	RECQL5 mRNA Expression		P Values	
	Low	High	*Unadjusted*	*Adjusted*
	N(%)	N (%)		
A) Pathological Parameters				
Lymph node stage				
Negative	684(52.6%)	351(52.5%)	0.990	25.74
Positive (1-3)	207(15.9%)	107(15.9%)		
Positive (>3)	409(31.5%)	213(31.7%)		
Grade				
G1	129(10.3%)	40(6.2%)	**0.007**	**0.023**
G2	512(41.0%)	258(40.2%)		
G3	607(48.6%)	343(53.5%)		
Tumour Size (cm)				
T 1a+b(1.0)	61(4.7%)	31(4.7%)	0.097	0.157
T 1c(>1.0-2.0)	532(41.0%)	234(35.6%)		
T2 (>2.0-5)	639(49.2%)	362(55.0%)		
T3 (>5)	67(68.4%)	31(31.6%)		
NPI				
≤ 3.4	461(35.3%)	219(32.6%)	0.238	0.269
>3.4	137(64.7%)	1160(67.4%)		
Her2 overexpression (No)	1159(88.7%)	573(85.4%)	**0.032**	0.075
(Yes)	147(11.3%)	98(14.6%)		
Triple negative (No)	1079(82.6)	581 (88.6)	**0.023**	0.066
(Yes)	227(17.4)	90(13.4)		
ER (Negative)	322(24.7%)	148(22.1%)	0.199	0.246
(Positive)	984(75.3%)	523(77.9%)		
PR (Negative)	603(46.2%)	333(49.6%)	0.145	0.188
(Positive)	703(53.8%)	338(50.4%)		

Genefu subtype				
ER-/Her-2 negative	110(16.3%)	40(12.6%)	0.129	0.186
ER+/Her-2 negative/high proliferation	229(33.9%)	137(43.1%)	**0.005**	**0.018**
ER+/Her-2 negative/low proliferation	265(39.1%)	103(32.4%)	**0.038**	0.082
Her-2 positive	72(10.7%)	38(11.9%)	0.543	0.564
PAM50 subtype				
PAM50.Her2	130(11.2%)	108(17.6%)	**$1.5X10^{-4}$**	**0.0008**
PAM50.Basal	233(20.1%)	97(15.8%)	**0.029**	0.075
PAM50.LumA	488(42.1%)	227(37.1%)	**0.042**	0.084
PAM50.LumB	309(26.6%)	180(29.4%)	0.214	0.252
IntClust subgroups				
intClust.1	49(3.8%)	88(13.1%)	**$8.3X10^{-15}$**	**0.00001**
intClust.2	55(4.2%)	17(2.5%)	0.059	0.102
intClust.3	221(16.9%)	69(10.3%)	**$7.8X10^{-5}$**	**0.0005**
intClust.4	267(20.4%)	76(11.3%)	**$3.9X10^{-7}$**	**0.00001**
intClust.5	120(63.5%)	69(36.5%)	0.433	0.469
intClust.6	50(3.8%)	36(5.4%)	0.113	0.172
intClust.7	113(8.7%)	40.2(11.3%)	0.056	0.104
intClust.8	187(14.3)	113(16.8%)	0.139	0.191
intClust.9	74(5.7%)	72(10.7%)	**$4.6X10^{-5}$**	**0.0004**
intClust.10	170(13.0%)	55(8.2%)	**0.001**	**0.0043**

Bold = Statistically significant;HER2: human epidermal growth factor receptor 2; ER: oestrogen receptor; PR: progesterone receptor; Triple negative: ER-/PR-/HER2- . Adjusted p values were calculated using Benjamini-Hochberg false discovery rate method to adjust for multiple testing.

4.3.10 High RECQL5 mRNA expression is significantly associated with poor breast cancer-specific survival in the whole cohort and ER+ group

We then proceeded to survival analysis in METABRIC cohort. High RECQL5 mRNA expression was associated with poor breast cancer specific survival (BCSS) ($p<0.0001$) in the whole cohort [Figure 4.7 A]. In the ER+ sub-group,

high RECQL5 mRNA expression was found to be associated with poor BCSS ($p<0.0001$) [Figure 4.7 B]. In the ER+ sub-group that received adjuvant endocrine therapy, high RECQL5 mRNA expression remained associated with poor BCSS ($p<0.001$) [Figure 4.8 C]. However, in ER- sub-group, RECQL5 mRNA expression did not significantly influence the outcome in the ER- cohort, including ER- patients who received chemotherapy ($p=0.116$ and $p=0.213$ respectively) [Figure 4.8 D,E].

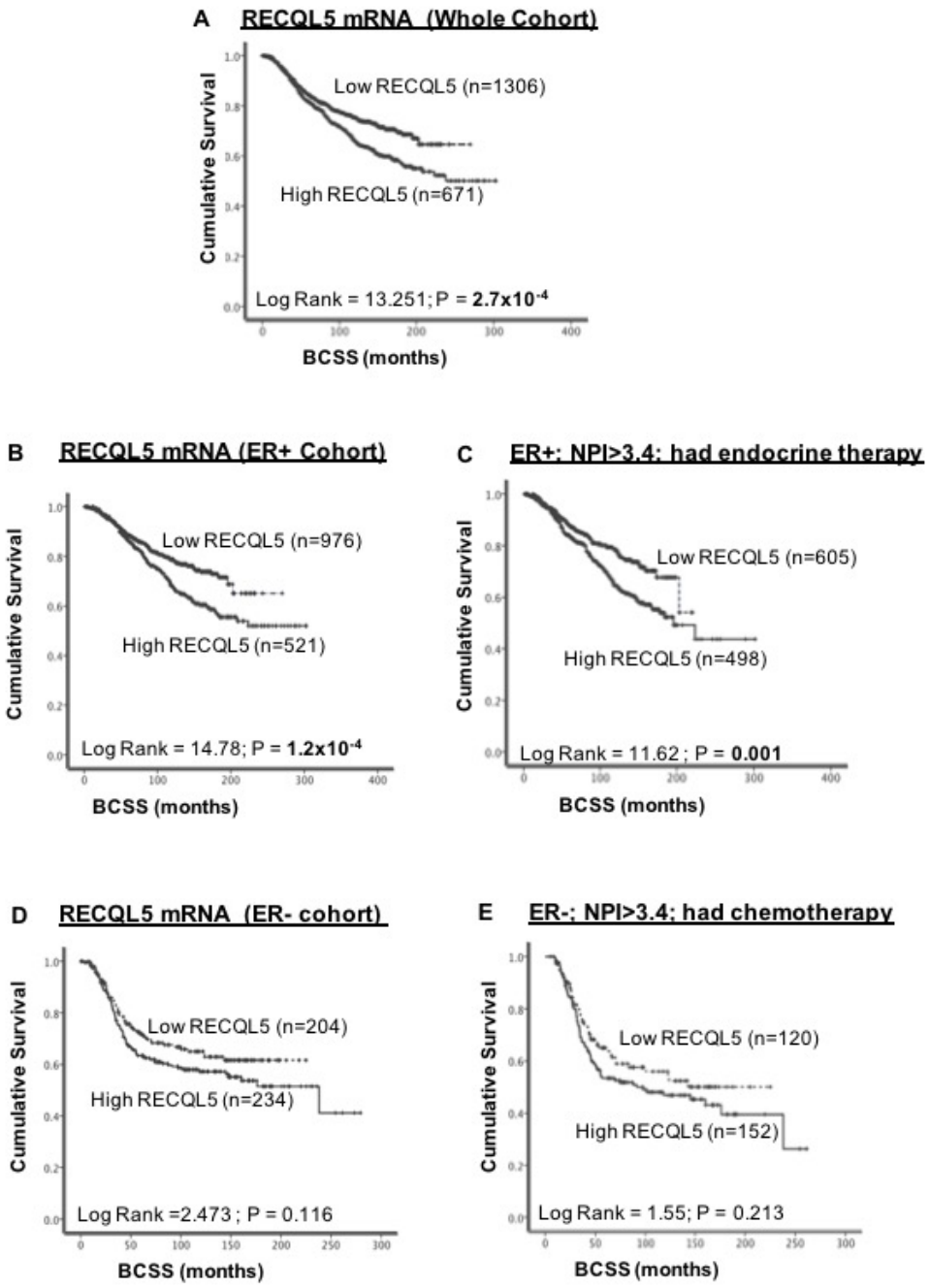


Figure 4.7 RECQL5 mRNA expression and breast cancer survival

A. Whole cohort **B**. ER+ full cohort **C**. ER+ cohort, who received endocrine therapy

D. ER- full cohort **E.** ER- cohort who received chemotherapy

4.3.11 RECQL5 mRNA expression is an independent marker of breast cancer survival on multivariate analysis

Multivariate Cox regression analysis was carried out, which included other validated prognostic factors (Grade, size and Lymph node status), RECQL5 mRNA expression remained a powerful independent predictor for breast cancer-specific survival in the whole cohort (p=0.023), ER+ sub-group (p=0.045) but not in the ER- sub-group (p=0.635) (Table 4.7).

Table 4.7 Multivariate analysis of RECQL5 mRNA expression in breast cancer

	P-Value	**Risk Ratio**	**95% CI for Risk Ratio**	
			Lower	**Upper**
Breast Cancer Specific Survival (Whole Cohort)				
Grade	**0.000004**	1.447429	1.237421	1.693079
Size (Cm)	**0.000001**	1.121003	1.076755	1.167069
LN Status	**0.000001**	1.875371	1.666800	2.110040
RECQL5 mRNA expression	**0.023530**	1.223718	1.027549	1.457337
Breast Cancer Specific Survival (ER+ Cohort)				
Grade	**0.0002**	1.411263	1.176981	1.692178
Size (Cm)	**0.000001**	1.180525	1.123389	1.240566
LN Status	**0.000001**	1.822326	1.578493	2.103825
RECQL5 mRNA expression	**0.045**	1.242008	1.004707	1.535359
Breast Cancer Specific Survival (ER- Cohort)				
Grade	0.968564	1.008054	0.676448	1.502217
Size (Cm)	0.239188	1.046124	0.970452	1.127695
LN Status	**0.000001**	1.908038	1.539252	2.365181

RECQL5 mRNA expression	0.635591	1.078023	0.790092	1.470886

In summary, this data provides evidence that RECQL5 mRNA expression has an impact on prognosis and clinicopathological significance, particularly in ER+ breast cancers.

4.4 Discussion

RECQL5 is a key member of the RECQ family of DNA helicases (Aygun & Svejstrup, 2010; Brosh, 2013b; Hanada & Hickson, 2007b; Popuri et al, 2013). RECQL5 protein performs various key roles in DNA replication, chromosomal segregation, transcription, and DNA repair (including homologous recombination, single-strand break repair and base excision repair). Role of RECQL5 as a tumour suppressor has been demonstrated in pre-clinical studies. Mice who are deficient in RECQL5 show predisposition to lymphomas and solid tumours including breast cancer (Aygun & Svejstrup, 2010; Brosh, 2013b; Hanada & Hickson, 2007b; Popuri et al, 2013). In humans, RECQL5 gene polymorphisms may predispose an individual to various cancer including: osteosarcomas, laryngeal carcinomas and breast cancers (Dong et al, 2015; He et al, 2014; Ramamoorthy et al, 2012b; Zhi et al, 2014) . The low RECQL5 expression has been observed at the mRNA and protein levels in colorectal cancers (Lao et al, 2013). This study by Lao et al concluded that RECQL5 deficiency may predispose to colorectal cancer. However, the major limitations

of this study was a small sample size and unclear evidence of clinicopathological correlations or prognosis (Lao et al, 2013).

The clinicopathological significance of RECQL5 in sporadic breast cancer remains largely unknown. Zhu et al. has recently shown that increased expression of RECQL5 mRNA expression was significantly associated with improved relapse free survival in all breast cancer patients, but poor overall survival in HER2 negative patients (Zhu et al, 2018). In the current study, we have detailed evaluation of RECQL5 DNA helicase, and elucidated its complex role in breast cancers. At the mRNA level, we observed a high expression of RECQL5 in 34% of breast tumours. High RECQL5 mRNA expression shown a statistically significant association with aggressive phenotypes and poor survival. The prognostic significance was mainly observed in ER+ sub-group, supporting the evidence that high RECQL5 mRNA expression was more likely in ER+/HER2 -/high proliferation Genefu molecular sub-types.

At the protein level, we observed high RECQL5 protein expression in 53.7% of breast tumours. Low RECQL5 protein expression was associated with aggressive phenotype but RECQL5 protein expression did not influence survival outcomes in this group of patients. Our observation indicates that RECQL5 may be subjected to complex post-transcriptional regulation.

RECQL5 has previously been shown to directly interact with RAD51 (Schwendener et al, 2010). So it is possible that RECQL5 might be interacting with RAD51. As shown previously, the RECQL5 may be performing its anti-

recombinogenic role by disrupting RAD51 mediated presynaptic filament formation during Homologous recombination (Qi & Zhou, 2014). Hence, we investigation RECQL5-RAD51 co-expression in this study and made an interesting observation that breast tumours with high RECQL5/low RAD51 expression had aggressive phenotypes and they were also associated with poor survival outcomes. In further sub-group analysis, similar to mRNA data, the prognostic significance was more obvious in ER+ breast cancer but not in ER- tumours.

In conclusion, this study provides evidence that RECQL5 may influence breast cancer pathogenesis and could be a promising biomarker in breast cancer. So RECQL5 should be investigated further as a potential drug target.

Chapter 5

RECQL4 helicase expression in sporadic breast tumours

5 RECQL4 helicase expression in sporadic breast cancers

5.1 Introduction

RECQL4 is a key member of the RECQ family of DNA helicases and essential for the maintenance of genomic stability. RECQL4 is a DNA helicase involved in unwinding a variety of DNA substrates that can arise during DNA replication and repair. RECQL4 has also been implicated in DNA DSB (double-strand breaks) repair via homologous recombination (HR) repair pathway of DSB repair. Lu et al. has shown that RECQL4 plays an important role in DNA end resection which is the initial step of HR repair pathway (Lu et al, 2016). The primary fibroblast cells derived from Rothmund-Thomson's syndrome (RTS) patients show accumulation of higher levels of 53BP1 foci compared to normal fibroblasts, suggesting ineffective DSB repair pathway (Croteau et al, 2012b; Dietschy et al, 2009; Larizza et al, 2006).

Preclinical studies have shown that RECQL4 is a very important element of human replication complex and play a vital role in the initiation of DNA replication, the progression of stalled replication forks and telomere maintenance. RECQL4 has been shown to interact with several other proteins including Rad51, PARP1, FEN1, APE1, Polβ, BLM, RECQL4, WRN, TRF1, TRF2, p53, TOM20, TFAM, URB1/2, XPA, p300 and RPA to accomplish its biological role.

Rothmund-Thomson's syndrome (RTS), RAPADILINO & Baller-Gerold Syndrome are rare autosomal recessive disorders caused by the germ-line mutation in the RECQL4 gene. Mutations in the RECQL4 gene cause about two-thirds of all cases of Rothmund-Thomson syndrome (RTS). RTS is characterised by growth deficiency, photosensitivity, early greying and hair loss, cataracts, and cancer predisposition, especially osteosarcomas. RTS patients are prone to develop cancers such as lymphomas and bone sarcomas (Siitonen et al, 2009; Wang et al, 2003). RECQL4 plays a critical role in genomic stability (Mo et al, 2018). Cells from RECQL4 mutant mice have high frequencies of premature centromere separation and aneuploidy suggesting that the chromosomal instability may be the underlying cause of cancer predisposition in these mutant mice (Mann et al, 2005). In preclinical models, overexpression of RECQL4 has been seen in prostate cancer cell lines and depletion of RECQL4 by siRNA or shRNA vectors significantly reduced the growth and survival of metastatic prostate cancer cells indicating that RECQL4 is an important factor for prostate cancer pathogenesis (Su et al, 2010b). To further support this hypothesis, a study has demonstrated that the depletion of RECQL4 by shRNA dramatically increased the apoptotic potential of breast cancer cells in vitro (Fang et al, 2013). Moreover, RECQL4-deficient fibroblasts isolated from RTS patients have been shown to be hypersensitive to genotoxic agents in vitro studies (Jin et al, 2008). RECQL4 gene is localized to chromosome 8q24 and this site has been shown to be frequently amplified in sporadic breast cancers (Thomassen et al, 2009; Yokota et al, 1999). We

hypothesised that RECQL4 may be dysregulated in sporadic breast cancers and have clinicopathological significance. Hence the rationale of our study was to do a comprehensive evaluation of RECQL4 gene copy number changes, mRNA expression, and protein levels, aiming to understand the prognostic and predictive significance of RECQL4 expression in sporadic breast cancer patients.

5.2 Methods

5.2.1 Patient selection for RECQL4 protein expression analysis

Comprehensive evaluation of the protein expression of RECQL4 in breast cancer was performed in two breast cancer patient cohorts. The first cohort consisted of 1650 primary invasive breast carcinomas patients who were diagnosed between 1986 and 1999 and entered into the Nottingham tenovus series. The second cohort was an independent series of 252 ER-negative invasive breast cancers patients diagnosed and treated at Nottingham University Hospitals between 1999 and 2007. Patient demographics of Nottingham tenovus series (NTS) and ER-negative cohort are summarised in Table 2.4 and 2.5 respectively.

5.2.2 Tissue Microarray (TMA)

Breast tumours were arrayed in tissue microarrays (TMAs) constructed with two replicate 0.6 mm cores from the centre and periphery of the tumours. Details of TMA construction is detailed in section 2.1.1.

5.2.3 Identification of optimal dilution of RECQL4 antibody for IHC staining

Optimal concentration and conditions for staining were identified for RECQL4 antibody using standard IHC technique, as described in section 2.1.2. Each dilution was tested on the same type of sample and also the same version of TMA, to keep the experimental conditions standardized. After consulting online resource 'Human Protein Atlas' (www.proteinatlas.org), we identified that RECQL4 protein expression is seen in breast tissue, hence, we decided to proceed with antibody optimisation on breast tissue. The RECQL4 antibody (Rabbit Antibody, polyclonal) was purchased from Novus Biological, catalogue number25470002. The recommended optimal dilution for IHC by the manufacturer was 1:10 - 1:500. Antigen retrieval method used was Citrate buffer pH 6.0 as per manufacturer recommendation. Incubation time used was 60 minutes at room temperature. We used 1:150, 1:175, 1:200, 1:225, 1:250 dilutions for optimisation.

All optimisation slides were then evaluated by an expert pathologist (MA and EAR) to decide if further optimisation was required or to identify the optimal concentration for staining the breast tumours. After careful review by site specialist pathologist, we selected 1:175 as optimal dilution because it gave the best differential pattern of staining with minimum background / non-specific binding as shown in Figure 5.1.

Table 5.1 Primary antibody details

Antibody	Clonality	Source	Catalogue No.	Optimal Dilution/ Incubation time
RECQL4	Rabbit Ab (Polyclonal)	Novus Biological	25470002	1:175 60 min (RT)

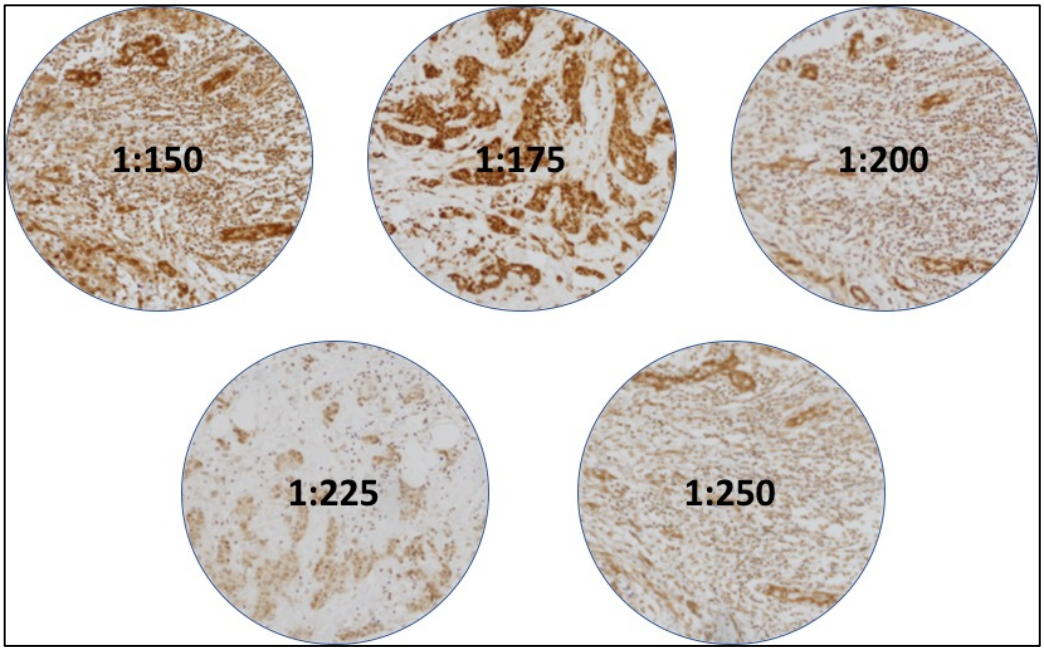


Figure 5.1 Photomicrographs of RECQL4 protein expression in Breast TMA with different optimisation dilutions

1:150, 1:175, 1:200, 1:225 and 1:250 dilutions were used for optimisation. 1:175 was selected as optimal dilution for staining as it showed the best differential staining pattern and little or no background staining.

5.2.4 Controls included ensuring staining quality

A negative control was included by omitting primary antibody and exposing to antibody diluent only. This was to determine if the secondary antibody was binding non-specifically resulting in false positives. It will be ideal to use absorption control to test if the primary antibody binds specifically to the antigen of interest. Because of the high expression of Beta 2 microglobulin (B2M) in breast cancer (Li et al., 2012), this was used as positive control. The same positive control section was included in each run to ensure consistency to be evaluated.

5.2.5 Cell lines and optimal antibody dilution used for Western Blot analysis

The primary antibody and its optimal concentration used for western blot is shown in Table 5.2. Western blot analysis as described in section 2.3.2.2 was used to evaluate the specificity of RECQL4 antibody, before using them for IHC. RECQL4 protein expression was assessed in four breast cancer cell lines MCF7, MDA-MB-231, MDA-MB-436, and MDA-MB-468. The characteristics of these cell lines are explained in Table 2.9. The details of cell lysate preparation and sample preparation are also detailed in section 2.3.2.2.1.

Table 5.2 The Primary antibody and its optimal concentration used for western blot

Antibody	Clonality	Source	Catalogue No.	Optimal Dilution/ Incubation time
RECQL4	Rabbit Ab (Polyclonal)	Novus Biological	25470002	1:750 (Overnight at 4^0C)

5.2.6 IHC technique

Details of the IHC technique used is described in detail in section 2.1.2.

5.2.7 Histopathological scoring of stained Breast TMAs

Scoring of all Breast TMAs was performed by me and an expert pathologist blinded to the clinicopathological characteristics of patients. H scoring was used to score breast tumours as described in detail in section 2.1.6. 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 RECQL4 into low and high expression. For nuclear RECQL4, H-Score of <30 was low RECQL4 nuclear expression and H-Score ≥30 was high nuclear RECQL4 expression. For cytoplasmic RECQL4, tumours with H Score of <100 were categorised as low cytoplasmic RECQL4 expression and tumours with H-Score of ≥100 were categorised as high cytoplasmic RECQL4 expression.

5.2.8 Quantitative real-time PCR

Total RNA was extracted from MCF-7, MDA-MB-231, MDA-MB-468, and MDA-MB-436 cells using RNeasy Mini kit (QIAGEN, UK). The RNA quantification was done using a NanoDrop 2000c Spectrophotometer (Thermo Scientific). The cDNA was synthesised from 0.5 μg of total RNA using RT^2 first strand kit (QIAGEN). The real-time qPCR was performed using SYBR Green PCR Master mix (Applied Biosystems) with a primer set (RECQL4 QuantiTect Primer Assay, catalogue no. QT00201740, QIAGEN) targeting the RECQL4 gene. The housekeeper gene used as an internal control was GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (GAPDH QuantiTect Primer Assay, catalog no. QT00079247, QIAGEN). The real-time PCR for each RNA sample was done in triplicate. NTC (No Template Control) was used to assess for cross-contamination of reagents and surfaces. To make NTC, all the RT-PCR reagents were included except the RNA template. Minus reverse transcriptase (-RT) control was used to do an assessment for genomic DNA contamination. A melting curve analysis was included to get information about the purity of the amplified product. A single peak was seen which indicated the presence of only one double stranded product.

5.2.9 Methodology for RECQL4 mRNA expression in Breast Cancer

RECQL4 gene copy number changes and mRNA expression were investigated in METABRIC (Molecular Taxonomy of Breast Cancer International Consortium) cohort. The METABRIC study protocol and the molecular

profiling methodology in a cohort of 1977 breast cancer samples are detailed by Curtis et al (Curtis et al, 2012b). Patient demographics, details of data acquisition and analysis are summarised in Table 2.7.

RECQL4 probe (ID: ILMN_1703092) was used because it was a perfect match and quality for its target, having a GC content of 58%, 0 SNPs and it does not possess a poly G tail at the end. The cut off for the high vs low RECQL4 gene expression was done using x-tile software (Version 3.6.1) so that the resultant subgroups had significant difference in survival outcomes. Real-time PCR RT-qPCR was performed on the ABI Prism 7900HT sequence detection system (Applied Biosystems) using SYBR1 Green reporter. All the samples were analysed as triplicates.

For this cohort, genes copy number was assayed on the Affymetrix SNP 6.0 platform (data available through the European Genotype Archive (http://www.ebi.ac.uk/ega/page.php) under accession Number: EGAS00000000082).

The Chi-square test was used for testing association between categorical variables and a multivariate Cox model was fitted to the data using as endpoint breast cancer-specific death. Recursive partitioning (Hothorn et al, 2006) was used to identify a cut-off in gene expression values such that the resulting subgroups have significantly different survival courses. Jonckheere's trend test was performed to evaluate the correlation between RECQL4 gene copy number and RECQL4 m RNA expression in the METABRIC cohort.

5.2.10 Statistical analysis of immunohistochemistry data

Data analysis was performed using SPSS (SPSS, version 22 Chicago, IL) as described in detail in section 2.1.9.

5.2.11 Ethical approval

Ethical approval was obtained from the Nottingham Research Ethics Committee (Reference number C202313, Approval date : 21/10/2010). Tumour Marker Prognostics Studies (REMARK) criteria, recommended by McShane et al (McShane et al, 2005), were followed throughout this project.

5.2.12 RTS Patient demographics

RTS patient demographics are summarised in Table 5.3

Table 5.3 RTS Patients

Rothmund-Thomson Syndrome (Type II)			
	All n=58	Female n=24	Male n=34
Age, median(range)	17.5 yrs (10 mos – 51 yrs)	13 yrs (10 mos – 40 yrs)	18.5 yrs (10 mos – 51 yrs)
<40 years old, n(%)	55 (94.8%)	23 (95.8%)	32 (94.1%)
Follow-up, median(range)	12 yrs (10 mos – 42 yrs)	10 yrs (10 mos – 37 yrs)	16 yrs (10 mos – 42 yrs)

5.3 Results

5.3.1 RECQL4 antibody specificity is confirmed by Western blot analysis

We checked the specificity of RECQL4 antibody before using it for IHC staining. We saw a single band at 133 kDa. We profiled a panel of breast cancer cell lines (MCF-7, MDA-MB231, MDA-MB-436, and MDA-MB-468). All breast cancer cell lines have shown a robust expression of RECQL4 protein (Figure 5.2).

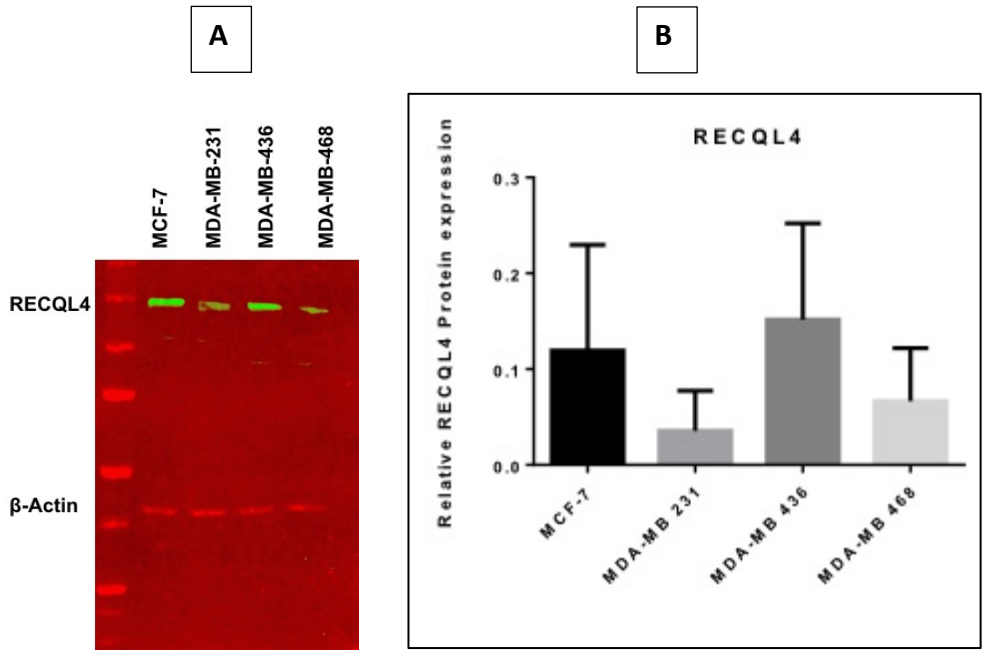


Figure 5.2 Western Blot and relative RECQL4 protein expression in the breast cancer cell lines

A. Western Blot of RECQL4 Expression in MCF7, MDA-MB-231, MDA-MB-436, & MDA-MB-468 cell lines **B.** RECQL4 protein expression in MCF7, MDA-MB-231, MDA-MB-436 & MDA-MB-468 breast cancer cell lines relative to β-Actin. Data presented as mean ± SEM of three independent experiments.

5.3.2 Breast cancer shows complex subcellular localisation of RECQL4

On immunohistochemical evaluation in the Nottingham Tenovus Series, complex sub-cellular localization of RECQL4 protein was observed in breast cancers. 17.6% of tumours showed nuclear staining only and 23.4% showed cytoplasmic staining only. 24.8% tumours demonstrated nuclear-cytoplasmic co-expression and 34.2% revealed negative staining.

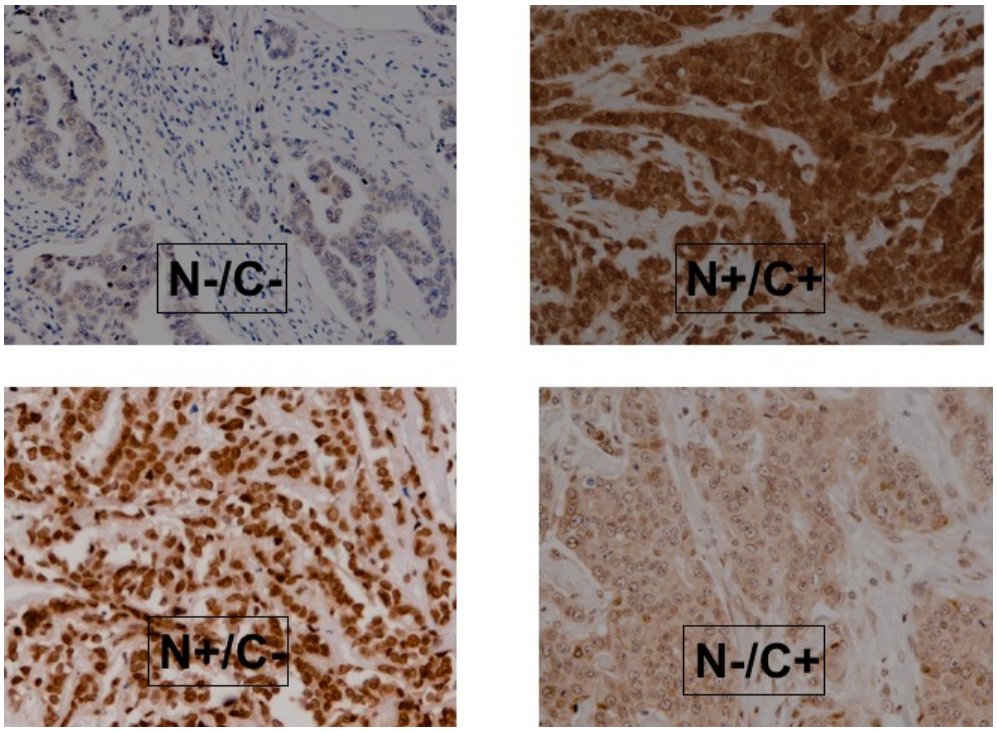


Figure 5.3 Photomicrographs of RECQL4 protein expression in breast cancers

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

5.3.3 Normal breast tissue shows strong nuclear RECQL4 expression but no cytoplasmic expression

We evaluated normal breast tissue from five BRCA1 or 2 carrier patients who underwent prophylactic mastectomy and observed strong nuclear staining in all patients and did not observe any cytoplasmic staining Taken together, the data suggest that altered sub-cellular localisation is a feature of breast tumours but not normal breast tissue.

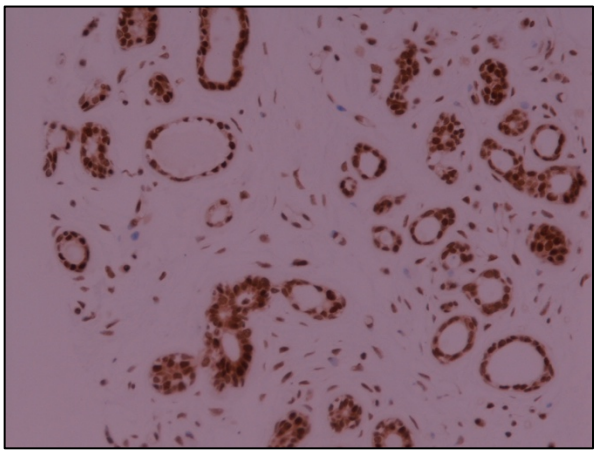


Figure 5.4 Photomicrograph of RECQL4 protein expression in BRCA mutated carrier normal breast tissue

5.3.4 Low RECQL4 nuclear protein expression is significantly associated with aggressive breast cancer phenotypes and low levels of other DNA repair proteins

682/1253 (57.7%) of tumours showed a low nuclear RECQL4 level and 571/1253 (42.3%) of tumours showed a high nuclear RECQL4 level. Low nuclear RECQL4 protein level demonstrated significant association with high tumour grade, higher mitotic index and tumour type ($p<0.05$). Low nuclear RECQL4 protein level was also showed a significant correlation with high-risk NPI>3.4 tumours, ER-, PR- tumours ($p<0.01$). Low FEN1, low nuclear RAD51, low nuclear RECQL5, low Polβ, low Chk1, low Chk2, low cytoplasmic CDK18, and low nuclear BLM were also significantly associated with tumours that had low nuclear RECQL4 protein level ($p<0.05$) (Table 5.4).

Table 5.4 RECQL4 (Nuclear) Expression and breast cancer

VARIABLE	RECQL4 (Nuclear) Protein Expression		Unadjusted P- values	Adjusted P - Values
	Low N (%)	High N (%)		
A) Pathological Parameters				
Tumour Size <1cm >1-2cm >2-5cm >5cm	 56 (9.6) 283 (48.5) 231 (39.6) 13 (2.2)	 42 (9.8) 227 (53.0) 150 (35.0) 9 (2.1)	0.496	0.54
Tumour Stage 1 2 3	 354 (60.6) 176 (30.1) 54 (9.2)	 275 (64.1)	0.437	0.52

		122 (28.4) 32 (7.5)		
Tumour Grade G1 G2 G3	 91 (15.6) 164 (28.1) 329 (56.3)	 69 (16.1) 165 (38.6) 194 (45.3)	**0.001**	**0.003**
Mitotic Index M1 (low; mitoses < 10) M2 (medium; mitoses 10-18) M3 (high; mitosis >18)	 160 (28.2) 103 (18.2) 304 (53.6)	 158 (37.5) 94 (22.3) 169 (40.1)	**$1.4X10^{-4}$**	**0.0005**
Tubule Formation 1 (>75% of definite tubule) 2 (10%-75% definite tubule) 3 (<10% definite tubule)	 30 (5.3) 182 (32.1) 355 (62.6)	 24 (5.7) 157 (37.3) 240 (57.0)	0.199	0.27
Pleomorphism 1 (small-regular uniform) 2 (Moderate variation) 3 (Marked variation)	 12 (2.1) 196 (34.7) 357 (63.2)	 8 (1.9) 170 (40.5) 242 (57.6)	0.178	0.27
Tumour Type IDC-NST Tubular Carcinoma Medullary Carcinoma ILC Others Mixed NST/lobular/Special Type	 367 (64.3) 112 (19.6) 20 (3.5) 29 (5.1) 8 (1.4) 35 (6.1)	 242 (57.5) 94 (22.3) 6 (1.4) 47 (11.2) 5 (1.2) 27 (6.4)	**0.002**	**0.005**
Lymph Node Status Negative Positive (1-3) Positive (>3)	 297 (58.7) 169 (33.4) 40 (7.9)	 248 (62.5) 128 (32.2) 21 (5.3)	0.238	0.309
B) Aggressive phenotype				
Her2 overexpression No Yes	 480 (83.5) 95 (16.5)	 355 (84.7) 64 (15.3)	0.596	0.619
Triple Negative Phenotype No Yes	 483 (82.4) 103 (17.6)	 361 (84.1) 68 (15.9)	0.468	0.529
NPI ≤ 3.4 >3.4	 153 (27.6) 402 (72.4)	 143 (34.8) 268 (65.2)	**0.016**	**0.032**

C) Hormone receptors				
ER Negative Positive	181 (31.9) 387 (68.1)	84 (20.1) 333 (79.9)	**$4.1X10^{-5}$**	**0.0002**
PR Negative Positive	271 (48.2) 291 (51.8)	149 (37.1) 253 (62.9)	**0.001**	**0.003**
D) DNA Repair				
FEN1 (Nuclear) Low High	328 (77.0) 98 (23.0)	194 (66.4) 98 (33.6)	**0.002**	**0.004**
PolB Low High	111 (20.9) 419 (79.1)	25 (6.7) 348 (93.3)	**$3.8X10^{-9}$**	**$1.0X10^{-5}$**
ATR Low High	123 (49.6) 125 (50.4)	113 (50.4) 111 (49.6)	0.854	22.20
Chk1 (Nuclear) Low High	519 (89.0) 64 (11.0)	273 (63.6) 156 (36.4)	**$3.8x10^{-22}$**	**$1.0X10^{-5}$**
Chk1 (Cytoplasmic) Low High	346 (59.2) 238 (40.8)	225 (52.4) 204 (47.6)	**0.031**	**0.05**
ATM Low High	201 (54.6) 167 (45.4)	123 (50.0) 123 (50.0)	0.261	0.32
CHK2 Low High	48 (12.8) 326 (87.2)	18 (6.8) 246 (93.2)	**0.014**	**0.03**
RAD51 (Cytoplasmic) Low High	14 (4.3) 311 (95.7)	16 (6.9) 215 (93.1)	0.178	0.25
RAD51 (Nuclear) Low High	188 (57.3) 140 (42.7)	81 (34.8) 152 (65.2)	**$1.3X10^{-7}$**	**$1.0X10^{-5}$**

CDK18 (Cytoplasmic) Low High	 265 (53.1) 234 (46.9)	 157 (45.2) 190 (54.8)	**0.024**	**0.04**
RECQL5(Nuclear) Low High	 279 (57.1) 210 (42.9)	 113 (32.7) 233 (67.3)	**$3.4X10^{-12}$**	**$1.0X10^{-5}$**
BLM (Nuclear) Low High	 167 (32.8) 342 (67.2)	 58 (16.6) 291 (83.4)	**$1.2X10^{-7}$**	**$1.0X10^{-5}$**
BLM (Cytoplasmic) Low High	 373 (73.9) 132 (26.1)	 239 (69.1) 107 (30.9)	0.127	0.206

Bold = Statistically significant; HER2: human epidermal growth factor receptor 2; ER: oestrogen receptor; PR: progesterone receptor; Triple negative: ER-/PR-/HER2- . Adjusted p values were calculated using Benjamini-Hochberg false discovery rate method to adjust for multiple testing. *Fischer test was used to obtain p values where one or more of cells has an expected frequency of five or less.

5.3.5 High RECQL4 cytoplasmic protein expression is significantly associated with high levels of other DNA repair proteins

642/1212 (51.8%) of tumours had high cytoplasmic RECQL4 protein expression and 570/1212 (48.2%) and low cytoplasmic RECQL4 level. High cytoplasmic RECQL4 level was significantly associated with high mitotic index, triple negativity, tumour type (p values <0.01) [Table 5.5]. High cytoplasmic RECQL4 tumours were also associated with high FEN1, high POLB, high Chk1, high Chk2, high RAD51 (nuclear & cytoplasmic), high CDK18, high nuclear RECQL5 and high cytoplasmic BLM and high nuclear RECQL4 (p values<0.05) [Table 5.5].

Table 5.5 RECQL4 (Cytoplasmic) Expression and breast cancer

VARIABLE	RECQL4 (Cytoplasmic) Protein Expression		Unadjusted P- values	Adjusted P - Values
	Low N (%)	High N (%)		
A) Pathological Parameters				
Tumour Size				
<1cm	48 (9.2)	50 (10.3)	0.893	24.11
>1-2cm	265 (51.0)	242 (49.9)		
>2-5cm	195 (37.5)	184 (37.9)		
>5cm	12 (2.3)	9 (1.9)		
Tumour Stage				
1	318 (60.9)	307 (63.3)	0.498	0.54
2	155 (29.7)	142 (29.3)		
3	49 (9.4)	36 (7.4)		
Tumour Grade				
G1	81 (15.5)	77 (15.9)	0.148	0.19
G2	184 (35.3)	144 (29.7)		
G3	256 (49.1)	264 (54.4)		
Mitotic Index				
M1 (low; mitoses < 10)	188 (36.6)	127 (27.1)	**0.006**	**0.014**
M2 (medium; mitoses 10-18)	96 (18.7)	100 (21.3)		
M3 (high; mitosis >18)	229 (44.6)	242 (51.6)		
Tubule Formation				
1 (>75% of definite tubule)	28 (5.5)	26 (5.5)	0.177	0.227
2 (10%-75% definite tubule)	162 (31.6)	174 (37.1)		
3 (<10% definite tubule)	323 (63.0)	269 (57.4)		
Pleomorphism				
1 (small-regular uniform)	13 (2.5)	6 (1.3)	0.061	0.096
2 (Moderate variation)	203 (39.7)	161 (34.4)		
3 (Marked variation)	295 (57.7)	301 (64.3)		
Tumour Type				
IDC-NST	311 (60.6)	295 (62.4)	**0.006**	**0.013**
Tubular Carcinoma	97 (18.9)	108 (22.8)		
Medullary Carcinoma	11 (2.1)	15 (3.2)		
ILC	54 (10.5)	22 (4.7)		
Others	9 (1.8)	3 (0.6)		
	31 (6.0)	30 (6.3)		

Mixed NST/lobular/Special Type				
Lymph Node Status Negative Positive (1-3) Positive (>3)	 282 (59.9) 152 (32.3) 37 (7.9)	 259 (60.8) 144 (33.8) 23 (5.4)	0.331	0.3886
B) Aggressive phenotype				
Her2 overexpression No Yes	 435 (85.0) 77 (15.0)	 394 (82.8) 82 (17.2)	0.350	0.393
Triple Negative Phenotype No Yes	 456 (87.2) 67 (12.8)	 382 (78.6) 104 (21.4)	**$2.8X10^{-4}$**	**0.001**
NPI ≤ 3.4 >3.4	 164 (33.1) 331 (66.9)	 129 (27.7) 337 (72.3)	0.067	0.101
C) Hormone receptors				
ER Negative Positive	 135 (26.5) 375 (73.5)	 129 (27.5) 340 (72.5)	0.716	0.743
PR Negative Positive	 227 (45.7) 270 (54.3)	 192 (41.6) 269 (58.4)	0.209	0.256
D) DNA Repair				
FEN1 (Nuclear) Low High	 291 (77.4) 85 (22.6)	 229 (67.4) 111 (32.6)	**0.003**	**0.008**
PolB Low High	 94 (20.0) 375 (80.0)	 40 (9.3) 391 (90.7)	**$1.0X10^{-6}$**	**$1.0X10^{-5}$**

ATR (Nuclear)				
	118 (55.4)	118 (45.7)	**0.037**	0.06
Low	95 (44.6)	140 (54.3)		
High				
Chk1 (Nuclear)				
	439 (84.1)	348 (71.8)	**$2.0x10^{-6}$**	**$1.0X10^{-5}$**
Low	83 (15.9)	137 (28.2)		
High				
Chk1 (Cytoplasmic)				
	351 (67.2)	215 (44.2)	**$1.8x10^{-13}$**	**$1.0X10^{-5}$**
Low	171 (32.8)	271 (55.8)		
High				
ATM				
	174 (56.7)	149 (48.9)	**0.053**	0.089
Low	133 (43.3)	156 (51.1)		
High				
CHK2				
Low	42 (13.6)	24 (7.3)	**0.009**	**0.018**
High	266 (86.4)	304 (92.7)		
RAD51 (Cytoplasmic)				
Low	21 (7.4)	9 (3.3)	**0.031**	**0.05**
High	261 (92.6)	263 (96.7)		
RAD51 (Nuclear)				
Low	156 (54.7)	112 (40.9)	**0.001**	**0.003**
High	129 (45.3)	162 (59.1)		
CDK18 (Cytoplasmic)				
Low	264 (60.6)	155 (38.1)	**$7.1X10^{-11}$**	**$1.0X10^{-5}$**
High	172 (39.4)	252 (61.9)		
RECQL5(Nuclear)				
Low	230 (53.0)	161 (40.6)	**$3.3X10^{-4}$**	**0.001**
High	204 (47.0)	236 (59.4)		
RECQL4 (Nuclear)				
Low	345 (66.0)	236 (48.6)	**$2.2X10^{-8}$**	**$1.0X10^{-5}$**
High	178 (34.0)	250 (51.4)		
BLM (Nuclear)				
Low	126 (28.5)	99 (23.9)	0.122	0.173
High	316 (71.5)	316 (76.1)		
BLM (Cytoplasmic)				
Low	366 (83.4)	245 (59.6)	**$1.3X10^{-14}$**	**$1.0X10^{-5}$**
High	73 (16.6)	166 (40.4)		

Bold = Statistically significant;HER2: human epidermal growth factor receptor 2; ER: oestrogen receptor; PR: progesterone receptor; Triple negative: ER-/PR-/HER2- . Adjusted p values were calculated using Benjamini-Hochberg false discovery rate method to adjust for multiple testing.

5.3.6 Low nuclear and high cytoplasmic RECQL4 protein expression is significantly associated with aggressive breast cancer phenotypes

We then proceed to combined nuclear and cytoplasmic co-expression analysis. 23.4% were low nuclear/high cytoplasmic, 34.2% were low nuclear/low cytoplasmic, 24.8% were high nuclear/high cytoplasmic and 17.6% were high nuclear/low cytoplasmic. Tumours with high cytoplasmic/low nuclear RECQL4 levels were more likely to have high grade, high mitotic index, pleomorphism, NPI>3.4, ER-, and triple negative ($p<0.01$) [Table 5.6]. Interestingly, low cytoplasmic/low nuclear RECQL4 tumours were more likely to manifest low DNA repair levels including low Chk1, low Chk2, low FEN1, low RECQL5, low nuclear BLM, low CDK18, and low polβ expression [Table 5.6].

Table 5.6 RECQL4 Nuclear & Cytoplasmic expression and breast cancer

VARIABLE	RECQL4(Nuclear & Cytoplasmic) Protein Co-expression				P- value Unadjusted	P -Value Adjusted
	RECQL4n-/RECQL4c- N (%)	RECQL4n+/ RECQL4c- N (%)	RECQL4n-/RECQL4c+ N (%)	RECQL4n+/ RECQL4c+		
A) Pathological Parameters						
Tumour Size						
≤1cm	31 (9.1)	17 (9.6)	25 (10.6)	25 (10.0)	0.927	25.9
>1-2cm	171 (50.0)	94 (52.8)	110 (46.6)	132 (53.0)		
>2-5cm	133 (38.9)	62 (34.8)	96 (40.7)	88 (35.3)		
>5cm	7 (2.0)	5 (2.8)	5 (2.1)	4 (1.6)		
Tumour Stage						
1	209 (60.8)	109 (61.2)	142 (60.4)	165 (66.0)	0.785	0.81
2	102 (29.7)	53 (29.8)	73 (31.1)	69 (27.6)		
3	33 (9.6)	16 (9.0)	20 (8.5)	16 (6.4)		
Tumour Grade						
G1	55 (16.0)	26 (14.6)	35 (14.8)	42 (16.9)	**0.001**	**0.002**
G2	101 (29.4)	83 (46.6)	62 (26.3)	82 (32.9)		
G3	187 (54.5)	69 (38.8)	139 (58.9)	125 (50.2)		

Mitotic Index M1 (low; mitoses < 10) M2 (medium; mitoses 10-18) M3 (high; mitosis >18)	 100 (29.9) 69 (20.6) 166 (49.6)	 88 (49.4) 27 (15.2) 63 (35.4)	 58 (25.6) 33 (14.5) 136 (59.9)	 315 (32.1) 196 (20.0) 471 (48.0)	**$1.4X10^{-8}$**	**$1.0x10^{-5}$**
Tubule Formation 1 (>75% definite tubule) 2 (10%-75% definite tubule) 3 (<10% definite tubule)	 20(6.0) 102 (30.4) 213 (63.6)	 8 (4.5) 60 (33.7) 110 (61.8)	 10 (4.4) 78 (34.4) 139 (61.2)	 54 (5.5) 336 (34.2) 592 (60.3)	0.282	0.329
Pleomorphism 1 (small-regular uniform) 2 (Moderate variation) 3 (Marked variation)	 11 (3.3) 120 (35.9) 203 (60.8)	 2 (1.1) 83 (46.9) 92 (52.0)	 1 (0.4) 74 (32.7) 151 (66.8)	 5 (2.1) 87 (36.0) 150 (62.0)	**0.014**	**0.020**
Tumour Type IDC-NST Tubular Carcinoma Medullary Carcinoma ILC Others Mixed NST/Lobular/Special Type	 221 (65.8) 62 (18.5) 9 (2.7) 19 (5.7) 6(61.8) 19 (5.7)	 90 (50.8) 35 (19.8) 2 (1.1) 35 (19.8) 3 (1.7) 12 (6.8)	 143 (62.2) 49 (21.3) 11 (4.8) 10 (4.3) 1 (0.4) 16 (7.0)	 152 (62.6) 59 (24.3) 4 (1.6) 12 (4.9) 2 (0.8) 14 (5.8)	**$6.7x10^{-7}$**	**$1.0x10^{-5}$**
Lymph Node Status Negative Positive (1-3) Positive (>3)	 180 (59.6) 97 (32.1) 25 (8.3)	 102 (60.4) 55 (32.5) 12(7.1)	 114 (57.3) 71 (35.7) 14 (7.0)	 145 (63.9) 73 (32.2) 9 (4.0)	0.530	0.593
B) Aggressive Phenotype						

Her2 overexpression No Yes	 284 (84.0) 54 (16.0)	 151 (86.8) 23 (13.2)	 191 (82.3) 41 (17.7)	 203 (83.2) 41(16.8)	 0.662	 0.71
Triple Negative Phenotype No Yes	 294 (85.2) 51 (14.8)	 162 (91.0) 16 (9.0)	 184 (78.0) 52 (22.0)	 198 (79.2) 52 (20.8)	 **0.001**	 **0.001**
NPI ≤3.4 >3.4	 97 (29.8) 229 (70.2)	 67 (39.6) 102 (60.4)	 54 (24.0) 171 (76.0)	 75 (31.1) 166 (68.9)	 **0.010**	 **0.015**
C) Hormone Receptors						
ER Negative Positive	 105 (31.4) 229 (68.6)	 30 (17.0) 146 (83.0)	 75 (32.8) 154 (67.2)	 54 (22.5) 186 (77.5)	 **$3.4X10^{-4}$**	 **0.0007**
PR Negative Positive	 162 (49.4) 166 (50.6)	 65 (38.5) 104 (61.5)	 108 (47.2) 121 (52.8)	 84(36.2) 148 (63.8)	 **0.006**	 **0.009**
D. DNA Repair						
FEN1 (Nuclear) Low High	 199 (81.2) 46 (18.8)	 92 (70.2) 39 (29.8)	 127 (70.9) 52 (29.1)	 102(63.4) 59 (36.6)	 **0.001**	 **0.002**
FEN1 (cytoplasmic) Low High	 147 (60.0) 98 (40.0)	 71 (54.2) 60 (45.8)	 88 (49.2) 91 (50.8)	 75 (46.6) 86 (53.4)	 **0.035**	 **0.049**

POLB						
Low	81 (25.9)	13 (8.3)	28 (13.1)	12(5.5)	**$5.7X10^{-11}$**	**$1.0x10^{-5}$**
High	232 (74.1)	143 (91.7)	186 (86.9)	205 (94.5)		
ATR						
Low	68 (54.4)	50 (56.8)	55 (45.1)	63(46.3)	0.211	0.256
High	57 (45.6)	38 (43.2)	67 (54.9)	73 (53.7)		
Chk1 (Nu)						
Low	240 (69.8)	89 (50.0)	140 (59.6)	100(40.0)	**$3.6x10^{-12}$**	**$1.0x10^{-5}$**
High	104 (30.2)	89 (50.0)	95 (40.4)	150 (60.0)		
Chk1 (Cyto)						
Low	158 (45.9)	68 (38.2)	71 (30.1)	68(27.2)	**6.0x10-6**	**$1.0x10^{-5}$**
High	186 (54.1)	110 (61.8)	165 (69.9)	182 (72.8)		
ATM						
Low	121 (57.1)	53 (55.8)	79 (51.3)	70(46.4)	0.208	0.264
High	91 (42.9)	42 (44.2)	75 (48.7)	81 (53.6)		
CHK2						
Low	113 (54.6)	39 (38.6)	68 (41.2)	52(31.9)	**$1.3x10^{-4}$**	**0.0003**
High	94 (45.4)	62 (61.4)	97 (58.8)	111 (68.1)		
RAD51 Cytoplasmic						
Low	10 (5.5)	11 (10.9)	4 (2.8)	5(3.9)	**0.038**	**0.05**
High	171 (94.5)	90 (89.1)	139 (97.2)	124 (96.1)		
RAD51 Nuclear						
Low	116 (63.4)	40 (39.2)	71 (49.3)	41(31.5)	**$1.3X10^{-7}$**	**$1.0x10^{-5}$**
High	67 (36.6)	62 (60.8)	73 (50.7)	89 (68.5)		
CDK18 (Cytoplasmic)						
Low	192 (64.9)	72 (51.4)	70 (35.0)	85(41.1)	**$5.2X10^{-11}$**	**$1.0x10^{-5}$**
High	104 (35.1)	68 (48.6)	130 (65.0)	122 (58.9)		
RECQL5(Nuclear)						
Negative	103 (34.6)	23(16.0)	64 (30.5)	35(17.1)	**$3.6X10^{-13}$**	**$1.0x10^{-5}$**
Positive	195 (65.4)	121 (84.0)	146 (69.5)	170 (82.9)		

BLM (Nuclear) Low High	 103 (34.6) 195 (65.4)	 23 (16.0) 121 (84.0)	 64 (30.5) 146 (69.5)	 35(17.1) 170 (82.9)	**$2.0X10^{-6}$**	**$1.0x10^{-5}$**
BLM (Cytoplasmic) Low High	 246 (83.1) 50 (16.9)	 120 (83.9) 23 (16.1)	 126 (60.6) 82 (39.4)	 119(58.6) 84 (41.4)	**$7.4X10^{-13}$**	**$1.0x10^{-5}$**

5.3.7 Low nuclear RECQL4 expression was not associated with poor breast cancer specific survival

In univariate analysis, patients whose tumours had low nuclear RECQL4 expression had poor breast cancer-specific survival ($p = 0.05$) (Figure 5.5 A) , however significance was borderline. Moreover, in the ER+ cohort (patients who had endocrine therapy), patients with low nuclear RECQL4 expression had trend towards poor survival (Figure 5.5 D), but it was not statistically significant ($p=0.058$). Furthermore, in the ER- cohort, we did not see any significant effect of RECQL4 nuclear expression on survival (Figure 5.6).

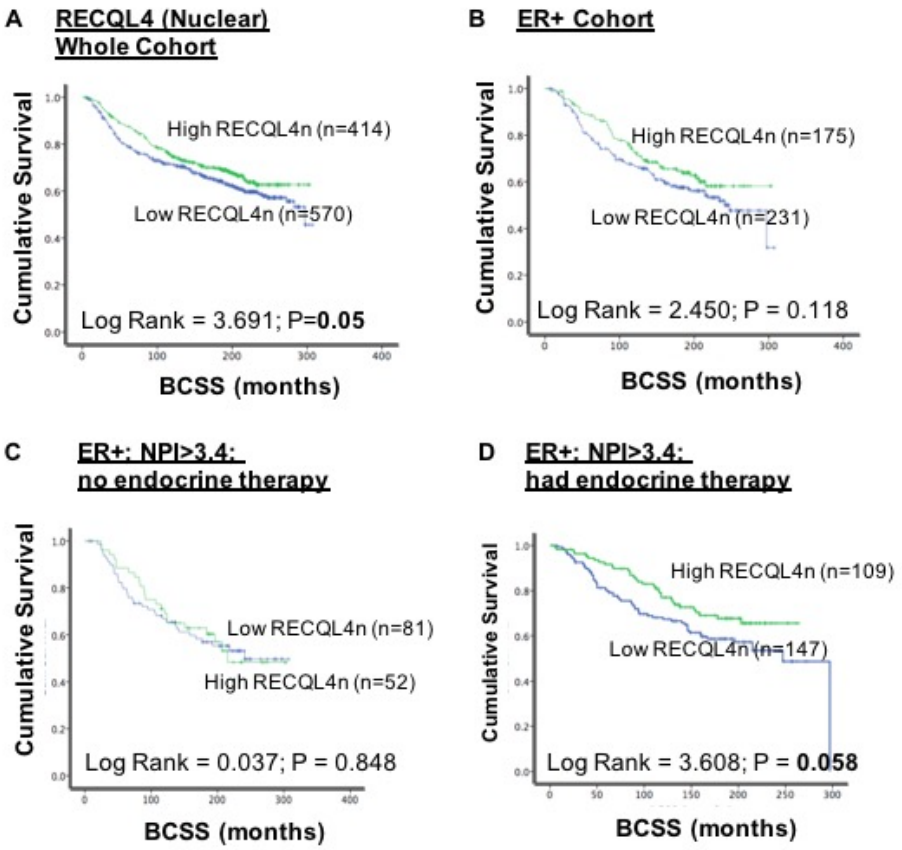


Figure 5.5 RECQL4 nuclear protein expression and breast cancer survival

A. Whole cohort **B.** ER+ cohort **C.** ER+ cohort who had no endocrine therapy **D.** ER+ cohort who had endocrine therapy

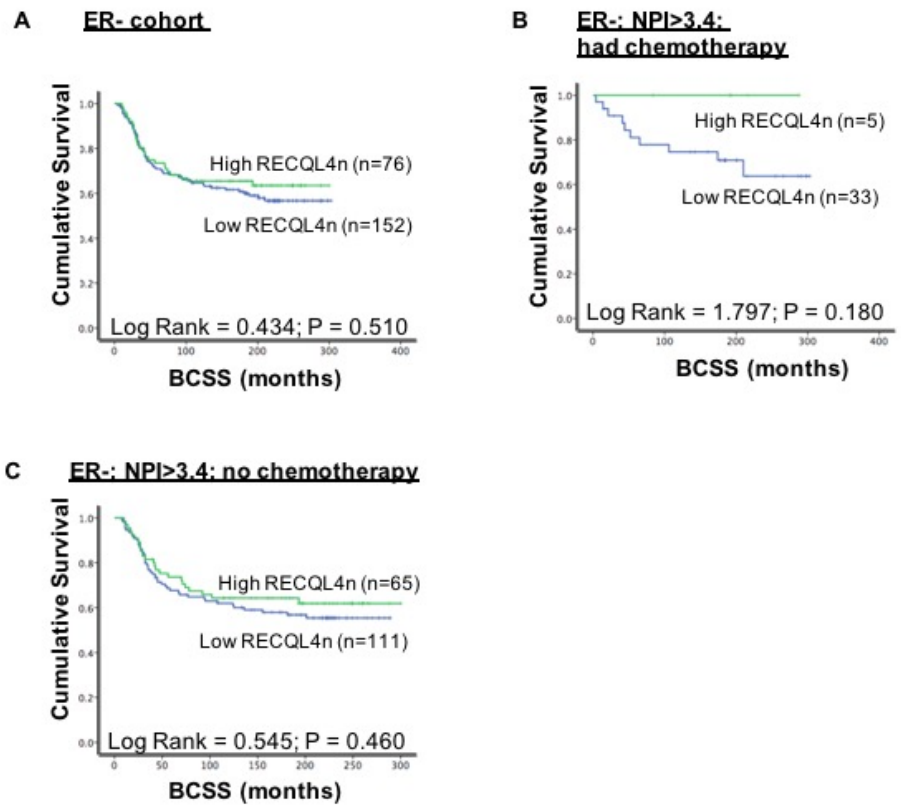


Figure 5.6 RECQL4 nuclear protein expression and breast cancer survival in ER- Cohort

5.3.8 RECQL4 cytoplasmic expression is not associated with breast cancer survival

We did not see any significant impact of RECQL4 cytoplasmic expression on patient survival in whole cohort, ER+ or ER- cohort (Figure 5.7).

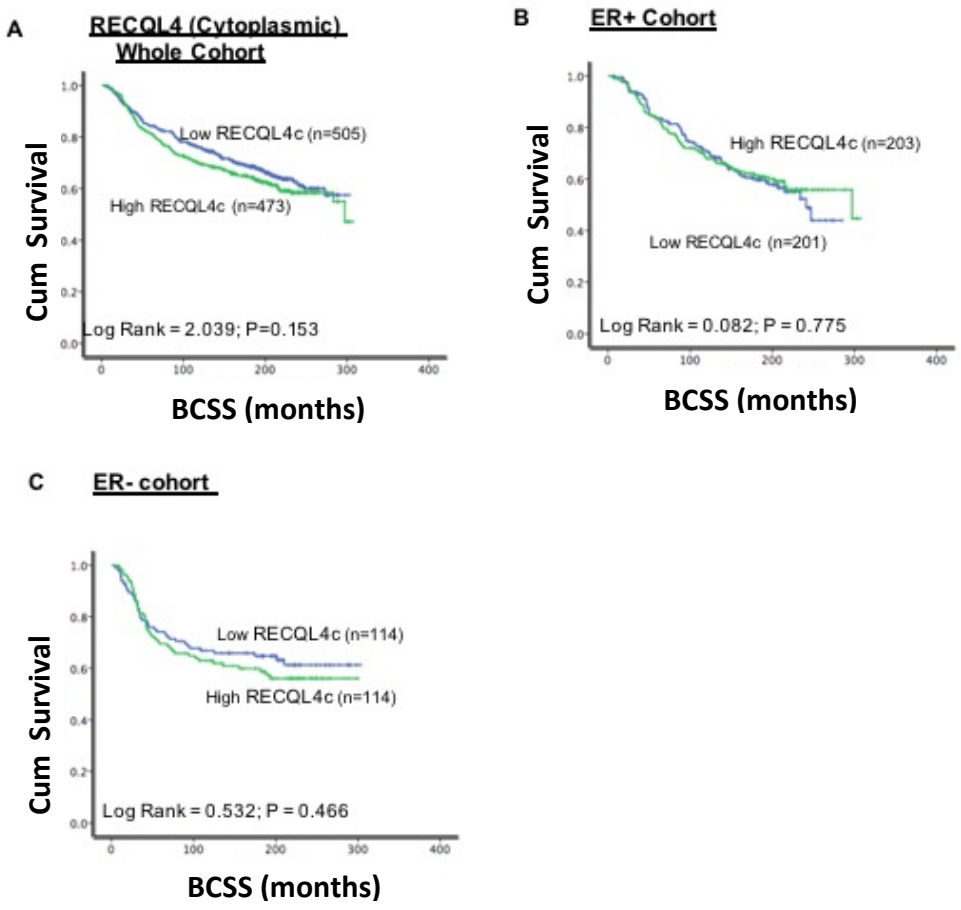


Figure 5.7 RECQL4 cytoplasmic protein expression and breast cancer survival

A. Whole cohort **B.** ER+ cohort **C.** ER- cohort

5.3.9 RECQL4 nuclear and cytoplasmic co-expression is significantly associated with poor survival

On combined RECQL4 nuclear and cytoplasmic expression analysis, we found statistically significant (p=0.04) poor survival outcome in patients with low nuclear and high cytoplasmic RECQL4 expression (Figure 5.8 A). In the ER- sub-group, interestingly, tumours that had high nuclear/low cytoplasmic

RECQL4 expression had poor survival (p=0.019) (Figure 5.8 B) including in patients who received adjuvant chemotherapy where the significance is more pronounced (p=0.005) (Figure 5.8 C). In ER-negative tumours that received no chemotherapy or in the ER+ sub-groups no significant difference in survival was observed (Figure 5.8 D,E,F, & G).

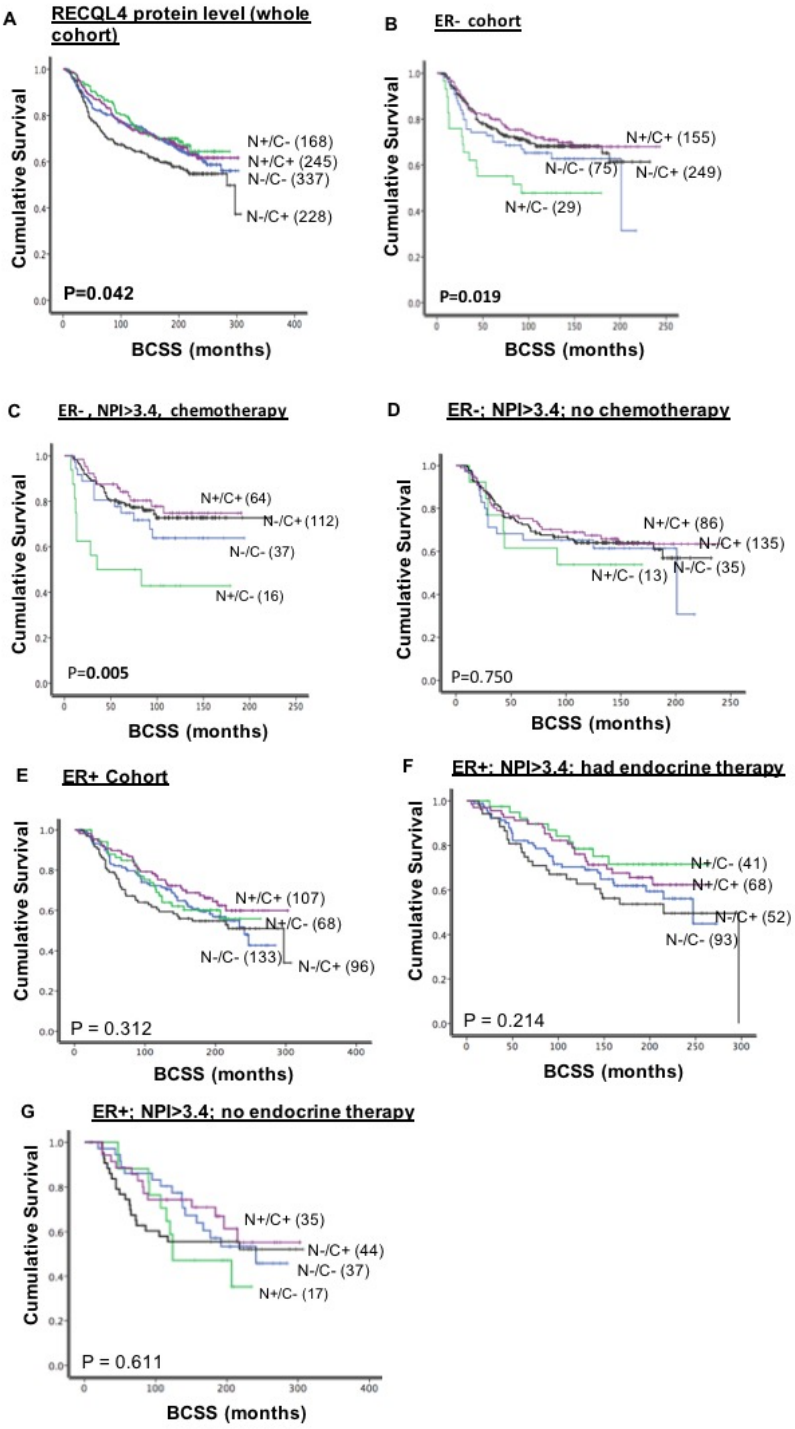


Figure 5.8 RECQL4 nuclear and cytoplasmic co-expression and breast cancer survival

A. Whole cohort **B.** ER- cohort **C.** ER- cohort, who had chemotherapy **D.** ER- cohort, who had no chemotherapy **E.** ER+ cohort **F.** ER+ cohort, who had endocrine therapy **G.** ER+ cohort, who had no endocrine therapy

5.3.10 High nuclear/high cytoplasmic RECQL4 expression is associated with significantly shorter time to disease recurrence

We also looked at local recurrence data which was available in 1755 tumours. 208/1755 (11.9%) of tumours had a local recurrence after adjuvant radiotherapy. In this subgroup of patients who had local recurrence, high nuclear/high cytoplasmic RECQL4 expression was associated with significantly shorter time to disease recurrence (p=0.002) (Figure 5.9 A). Our observation suggests that RECQL4 expression may be helpful in predicting resistance to adjuvant radiotherapy. Similarly, time to distant metastasis was also significantly shorter in tumours with high nuclear/high cytoplasmic RECQL4 expression (p=0.027) (Figure 5.9 B) suggesting an aggressive phenotype.

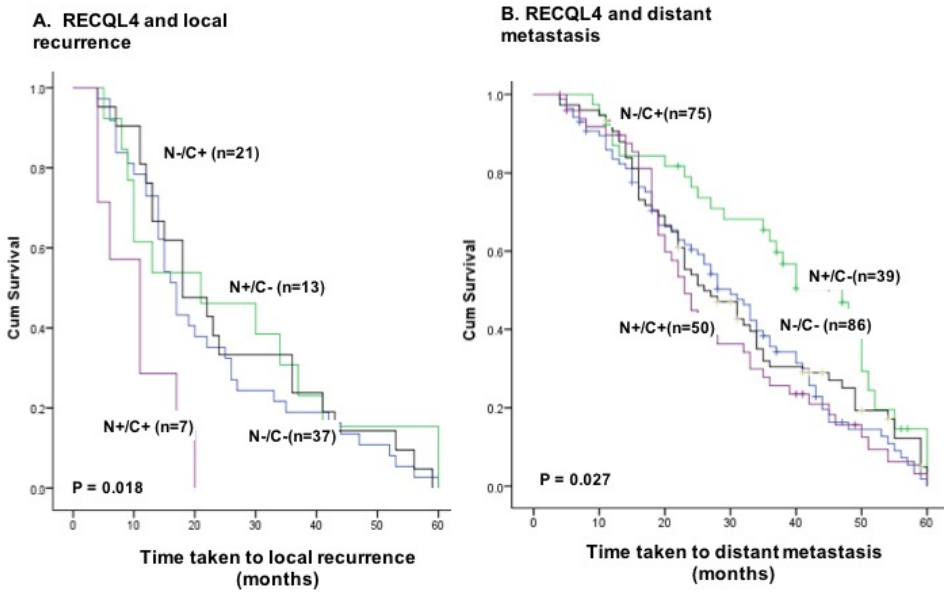


Figure 5.9 **A.** RECQL4 expression and time to local recurrence **B.** RECQL4 expression and time to distance metastasis

5.3.11 RECQL4 nuclear protein expression remains an independent predictor of survival on multivariate analysis

In multivariate analysis nuclear RECQL4 level independently influenced survival (p=0.032) (Table 5.7). Tumour grade, lymph node status and Her-2 status were other factors which were independently associated with breast cancer-specific survival.

Table 5.7 RECQL4 protein expression and survival – Multivariate Analysis

	P value	Exp (B)	95% CI of Exp (B)	
			Lower	Upper
Breast cancer specific Survival				
RECQL4 (Nuclear)	**0.032**	0.753	0.581	0.976
RECQL4 (Cyto)	0.352	1.126	0.877	1.446
Tumour Grade	**3.0×10^{-6}**	1.718	1.370	2.155
Lymph Node Status	**5.6×10^{-12}**	1.980	1.630	2.404
Tumour Size	0.061	1.211	0.991	1.478
ER Status	0.698	1.061	0.788	1.428
HER2 Status	**0.001**	1.686	1.246	2.280
Endocrine Therapy	0.072	1.239	0.981	1.564
Chemotherapy	0.410	1.153	0.821	1.620

5.3.12 c-MYC and RECQL4 co-expression shows clinicopathological association with aggressive phenotypes

As discussed previously, chromosome 8q24 has been shown to be frequently amplified in sporadic breast cancers. As both RECQL4 and cMYC gene are located to chromosome 8q24.21, we hypothesised that there might be a clinicopathological association between RECQL4 and c-MYC. A total of 1098 tumours were suitable for c-MYC-RECQL4 analysis. When c-MYC and RECQL4 were investigated together, we observed a significant clinicopathological association with aggressive phenotypes (ps<0.05) (Table 5.8).

Table 5.8 RECQL4 & c-MYC expression and breast cancer

VARIABLE	RECQL4 & C-MYC (Nuclear) Protein Co-expression				P- value Adjusted
	Rn-/cMYC- N (%)	Rn-/cMYCn+ N (%)	Rn+/ cMYCn- N (%)	Rn+/ cMYCn+ N (%)	
Mitotic Index					
M1 (low; mitoses < 10)	61(32.3)	52(27.4)	44(39.3)	35(26.1)	**0.035**
M2 (medium; mitoses 10-18)	29(15.3)	29(15.3)	21(18.8)	34(25.4)	
M3 (high; mitosis >18)	99(52.4)	109(57.4)	47(42.0)	65(48.5)	
Tubules					
1	10(5.3)	5(2.6)	4(3.6)	4(3.0)	**0.047**
2	69(36.5)	62(32.6)	34(30.4)	64(47.8)	
3	110(58.2)	123(64.7)	74(66.1)	66(49.3)	
Tumour Type					
IDC-NST	121 (64.4)	122(62.9)	65(57.5)	86(63.7)	**0.001**
Tubular Carcinoma	38(20.2)	38(19.6)	18(15.9)	36(26.7)	
Medullary Carcinoma	4(2.1)	14(7.2)	1(0.9)	4(3.0)	
ILC	12(6.4)	9(4.6)	19(16.8)	5(3.7)	
Others	1(0.5)	1(0.5)	0(0.0)	0(0.0)	
Mixed NST/Lobular/Special Type	12(6.4)	10(5.2)	10(8.8)	4(3.0)	

ER Negative Positive	57(30.2) 132(69.8)	66(34.0) 128(66.0)	26(23.4) 85(76.6)	27(20.9) 102(79.1)	**0.042**
VARIABLE	**RECQL4 & C-MYC (Cytoplasmic) Protein Co-expression**				
	Rc-/CMYCc- N (%)	Rc-/CMYCc+ N (%)	Rc+/CMYCc- N (%)	Rc+/CMYCc+ N (%)	**P- value Adjusted**
Triple Negative Non Triple Negative Triple Negative	153 (86.9) 23 (13.1)	128 (90.1) 14 (9.9)	106 (81.5) 24 (18.5)	148 (77.5) 43 (22.5)	**0.009**
Tumour Type IDC-NST Tubular Carcinoma Medullary Carcinoma ILC Others Mixed NST/Lobular/Special Type	103(59.9) 34(19.8) 4(2.3) 21(12.2) 0(0.0) 10(5.8)	94(66.7) 26(18.4) 6(4.3) 7(5.0) 1(0.7) 7(5.0)	83(65.4) 21(16.5) 1(0.8) 10(7.9) 1(0.8) 11(8.7)	113(60.4) 48(25.7) 12(6.4) 7(3.7) 0(0.0) 7(3.7)	**0.018**
Lymph Node Status Negative LN Postive (1-3) LN Positive (>3)	102(65.4) 47(30.1) 7(4.5)	61(50.4) 46(38.0) 14(11.6)	66(60.0) 35(31.8) 9(8.2)	89(55.6) 65(40.6) 6(3.8)	**0.030**

Bold = Statistically significant; ER: oestrogen receptor; PR: progesterone receptor; Triple negative: ER-/PR-/HER2- . Adjusted p values were calculated using Benjamini-Hochberg false discovery rate method to adjust for multiple testing.

5.3.13 c-MYC and RECQL4 co-expression has prognostic significance in breast cancer

In tumours with high RECQL4 nuclear expression, the patient could be stratified on the basis of c-MYC expression. Strong c-MYC expression was associated with poor survival compared to absent/low/moderate c-MYC staining (Figure 5.10 A) . On the other hand, tumours with low RECQL4 cytoplasmic expression, c-MYC status stratified patients however the significance was borderline (p=0.05) (Figure 5.10 D). This data provides the clinical evidence that c-MYC and RECQL4 co-expression could have prognostic significance in breast cancer.

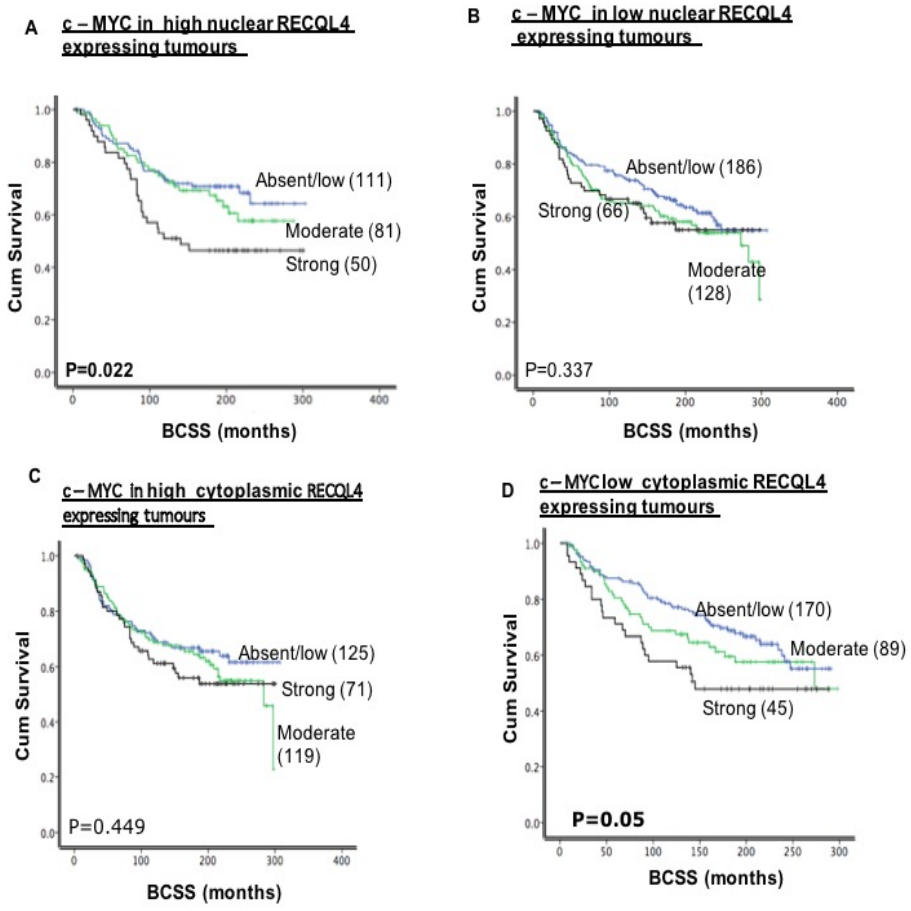


Figure 5.10 RECQL4 and cMYC expression and breast cancer survival

A. cMYC in high nuclear RECQL4 expressing tumours **B.** cMYC in low nuclear RECQL4 expressing tumours **C.** cMYC in high cytoplasmic RECQL4 expressing tumours **D.** cMYC in low cytoplasmic RECQL4 expressing tumours

5.3.14 3.3.2 RECQL4 gene amplification or gain in copy number is associated with aggressive breast cancer

0/1970 (0%) of the tumours had RECQL4 homozygous deletion, 19/1970 (1%) of tumours had RECQL4 heterozygous deletion, 1348/1970 (68.4%) tumours had RECQL4 neutral gene copy number, 543/1970 (27.6%) tumours had gain in RECQL4 copy number and 60/1970 (3%) of tumours had amplification of the RECQL4 gene. As shown in Table 5.9, high stage, grade 3 tumours, lymph node positivity, PAM50.Her2, PAM50 Lum B, integrative cluster 1 (intClust.1) ($p<0.00001$), intClust.5 ($p<0.00001$), intClust.9 ($p<0.00001$) and intClust.10 ($p<0.00001$) were more common in tumours with gain/amplification of RECQL4.

Table 5.9 RECQL4 gene copy number alterations in METABRIC cohort (n=1980)

	RECQL4 Gene Copy Number changes				
	HETD N(%)	**NEUT N(%)**	**GAIN N(%)**	**AMP N(%)**	**P value**
Stage					
1	5 (35.7)	369 (36.9)	121(30.9)	6(14.0)	**0.036**
2	8 (57.1)	556 (55.6)	225(57.5)	31(72.1)	
3	1 (7.2)	69 (6.9)	41(10.5)	6(13.9)	
4	0 (0.0)	6 (0.6)	4(1.1)	0(0.0)	
Grade					
G1	0 (0.0)	143 (11.2)	25 (4.7)	1(1.8)	**0.0001**
G2	8 (44.4)	576 (45.0)	160(30.4)	21(37.5)	
G3	10(55.6)	562 (43.8)	342(64.9)	34(60.7)	
Lymph node Status					

Negative	11(57.9)	739 (54.8)	268(49.4)	22(36.7)	**0.01**
Positive	8(42.1)	609(45.2)	275(50.6)	38(63.3)	
PAM50 subtype					
PAM50.Her2	2(10.5)	151(12.8)	75(14.5)	11(19.6)	**0.00001**
PAM50.Basal	6(31.6)	194(16.5)	117(22.7)	10(17.9)	
PAM50.LumA	3(15.8)	561(47.7)	143(27.7)	8(14.3)	
PAM50.LumB	8(42.1)	270(23.0)	181(35.1)	27(48.2)	
IntClust subgroup					
intClust.1	6(31.6)	65(5.0)	67(12.3)	1(1.7)	**$3.2x10^{-149}$**
intClust.2	1(5.3)	45(3.3)	25(4.6)	1(1.7)	
intClust.3	2(10.5)	269(20.0)	17(3.1)	0(0.0)	
intClust.4	1(5.3)	316(23.3)	23(4.2)	1(1.7)	
intClust.5	0(0.0)	126(9.3)	59(10.9)	4(6.6)	
intClust.6	0(0.0)	38(2.8)	42(7.7)	5(8.3)	
intClust.7	1(5.3)	113(8.4)	70(12.9)	4(6.7)	
intClust.8	3(15.8)	244(18.1)	51(9.5)	0(0.0)	
intClust.9	0(0.0)	8(0.6)	95(17.5)	41(68.3)	
intClust.10	5(26.3)	124(9.2)	94(17.3)	3(5.0)	

5.3.15 RECQL4 gain/amplification is associated with poor breast cancer survival

As expected, breast cancer-specific survival (BCSS) was significantly worse ($p=3.6x10^{-6}$) in tumours with gain/amplification of RECQL4 compared to tumours with neutral or loss of RECQL4 (Figure 5.11).

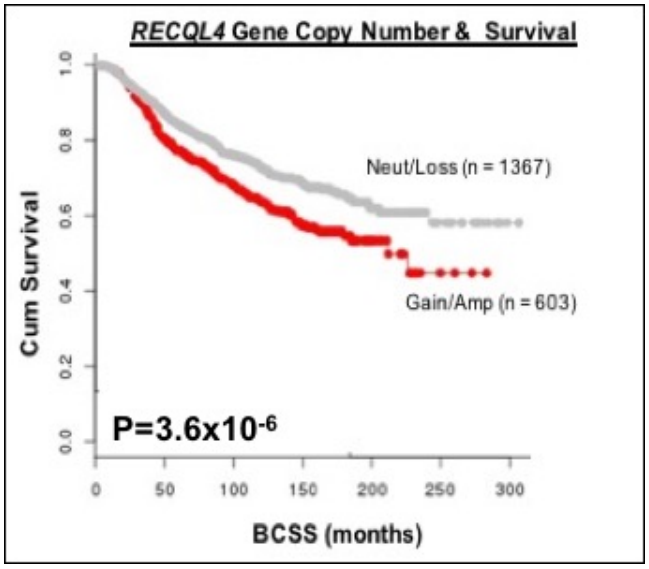


Figure 5.11 RECQL4 gene copy number and breast cancer survival

5.3.16 Breast Cancer cell lines show robust RECQL4 mRNA expression

We initially profiled a panel of breast cancer cell lines. MDA-MB-231, MDA-MB-436, and MDA-MB-468 showed higher expression of RECQL4 mRNA as compared to RECQL4 mRNA expression in control MCF7 cell line. (Figure 5.12)

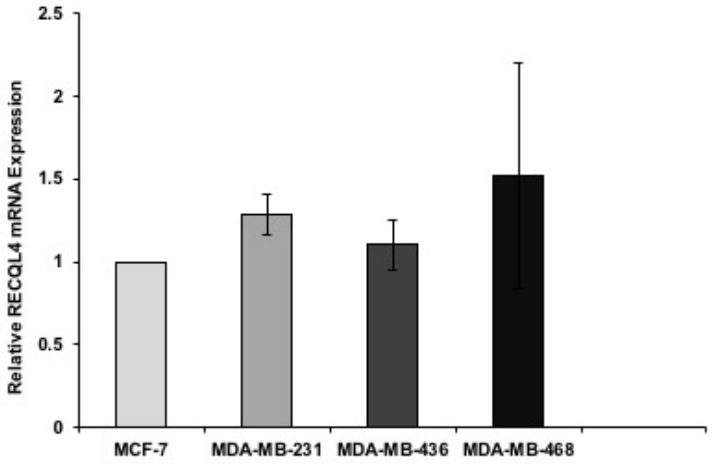


Figure 5.12 Relative mRNA expression of RECQL4 in Breast cancer cells lines

A graph of mRNA expression of RECQL4 in MDA-MB-231, MDA-MB-436, and MDA-MB-468 breast cancer cell lines relative to MCF7 control breast cancer cell line. Data presented as mean ± SEM of two independent experiments, each performed in triplicate.

5.3.17 High RECQL4 mRNA expression is associated with an aggressive breast cancer phenotype

High RECQL4 mRNA levels correlate to aggressive breast cancer: 966/1977 (49%) of tumours had low RECQL4 mRNA expression and 1011/1977 (51%) tumours had high RECQL4 mRNA expression. High RECQL4 mRNA expression was highly significantly associated with aggressive clinicopathological features (Table 5.10), high histological grade, Lymph node stage, larger tumour size, Nottingham prognostic index (NPI) >3.4, Her-2 overexpression, ER-negative, PR negative and triple negative phenotypes (ps<0.001). High RECQL4 mRNA expression was also found to be significantly

associated with previously described molecular phenotypes in breast cancer: PAM50.Her2 ($p<0.00001$), PAM50.Basal ($p<0.00001$) and PAM50.LumB ($p<0.00001$), Genufu subtype (ER-/Her2-) ($p<0.00001$), Genufu subtype (ER+/Her2-/High proliferation) ($p<0.00001$) and Genufu subtype (Her2 positive) ($p = 0.001$) breast tumours. However, PAM50.LumA tumours and Genufu subtype (ER+/Her2-/low proliferation) were more likely to express low levels of RECQL4 mRNA ($ps<0.00001$). Similarly, RECQL4 mRNA level was significantly associated with the various integrative clusters described in the METABRIC study which was based on gene copy number changes and gene expression data (Curtis et al, 2012b). High RECQL4 mRNA expression was significantly associated with intClust.1 ($p<0.00001$), intClust.2 ($p<0.00001$), intClust.5 ($p<0.00001$), intClust.9 ($p<0.00001$) and intClust.10 ($p<0.00001$), which had the worst clinical outcome in the METABRIC study (Curtis et al, 2012b). Low RECQL4 mRNA expression was associated with intClust.3 ($p<0.0001$), intClust.4 ($p<0.0001$) and intClust.8 ($p<0.0001$), which had intermediate to a good prognosis in the METABRIC study (Curtis et al, 2012b).

Table 5.10 Association between RECQL4 mRNA expression and clinicopathologic variables in the METABRIC cohort

VARIABLE	RECQL4 mRNA Expression		P Values
	Low	High	
	N(%)	N (%)	
A) Pathological Parameters			
Lymph node stage			
Negative	537(55.9%)	498(49.3%)	**0.003**
Positive (1-3)	129(13.4%)	185(18.3%)	
Positive (>3)	295(30.7%)	327(32.4%)	
Grade			
G1	137(15.0%)	32(3.3%)	**$2.4x10^{-47}$**
G2	466(51.2%)	304(31.1%)	
G3	308(33.8%)	642(65.6%)	
Tumour Size (cm)			
T 1a+b(1.0)	58(6.0%)	34(3.4%)	**$1.3x10^{-5}$**
T 1c(>1.0-2.0)	413(43.1%)	353(35.4%)	
T2 (>2.0-5)	450(46.9%)	551(55.2%)	
T3 (>5)	38(4.0%)	60(6.0%)	
NPI			
≤ 3.4	298(35.3%)	120(12.4%)	**$7.2x10^{-27}$**
>3.4	603(66.9%)	849(87.6%)	
Her2 overexpression (No)	883(91.4%)	849(84.0%)	**$5.3x10^{-7}$**
(Yes)	83(8.6%)	162(16.0%)	
Triple negative (No)	866(89.6)	794(78.5)	**$1.6x10^{-11}$**
(Yes)	100(10.4)	217(21.5)	
ER (Negative)	156(16.1%)	314(31.1%)	**$7.0x10^{-15}$**
(Positive)	810(83.9%)	697(68.9%)	
PR (Negative)	359(37.2%)	577(57.1%)	**$7.8x10^{-19}$**

(Positive)	607(62.8%)	434(42.9%)	
Genefu subtype			
ER-/Her-2 negative	47(9.8%)	103(20.0%)	**$6.0x10^{-6}$**
ER+/Her-2 negative/high proliferation	97(20.2%)	269(52.3%)	**$9.2x10^{-26}$**
ER+/Her-2 negative/low proliferation	299(62.3%)	69(13.4%)	**$3.1x10^{-57}$**
Her-2 positive	37(7.7%)	73(14.2%)	**0.001**
PAM50 subtype			
PAM50.Her2	66(8.1%)	172(17.9%)	**$1.9x10^{-9}$**
PAM50.Basal	90(11.1%)	240(25.0%)	**$7.6x10^{-14}$**
PAM50.LumA	515(63.5%)	200(20.8%)	**$2.1x10^{-74}$**
PAM50.LumB	140(17.3%)	349(36.3%)	**$3.9x10^{-19}$**
IntClust subgroups			
intClust.1	32(3.3%)	105(10.4%)	**$6.0x10^{-10}$**
intClust.2	19(2.0%)	53(5.2%)	**$1.0x10^{-4}$**
intClust.3	238(24.6%)	52(5.1%)	**$1.7x10^{-34}$**
intClust.4	253(26.2%)	90(8.9%)	**$3.4x10^{-24}$**
intClust.5	68(7.0%)	121(12.0%)	**$1.9x10^{-4}$**
intClust.6	50(3.8%)	36(5.4%)	**$7.0x10^{-5}$**
intClust.7	97(10.0%)	92(9.1%)	0.477
intClust.8	187(19.4)	113(11.2%)	**$4.1x10^{-7}$**
intClust.9	7(0.7%)	139(13.7%)	**$1.7x10^{-28}$**
intClust.10	41(4.2%)	184(18.2%)	**$1.5x10^{-22}$**

Bold = Statistically significant;HER2: human epidermal growth factor receptor 2; ER: oestrogen receptor; PR: progesterone receptor; Triple negative: ER-/PR-/HER2- .

5.3.18 High RECQL4 mRNA expression is associated with poor breast cancer survival

High RECQL4 mRNA expression in tumours was associated with poor breast cancer specific survival (BCSS) in the whole cohort ($p<0.00001$) (Figure 5.13 A). In the ER+ sub-group, high RECQL4 mRNA expression was associated with poor BCSS ($p<0.00001$) (Figure 5.13 B). In the ER+ sub-group that received

adjuvant endocrine therapy, high RECQL4 mRNA expression remains associated with adverse BCSS (p<0.00001) (Figure 5.13 C). The prognostic significance of high RECQL4 mRNA expression was even more noticeable in the ER+ sub-group that received no endocrine therapy (p<0.00001) (Figure 5.13 D) In the ER- sub-group, high RECQL4 mRNA expression was associated with poor BCSS with borderline significance (p=0.05) (Figure 5.14 A). In the ER- sub-group that received adjuvant chemotherapy or no chemotherapy, RECQL4 mRNA expression did not significantly impact outcome (p=0.158 and p=0.245 respectively) (Figure 5.14 B,C).

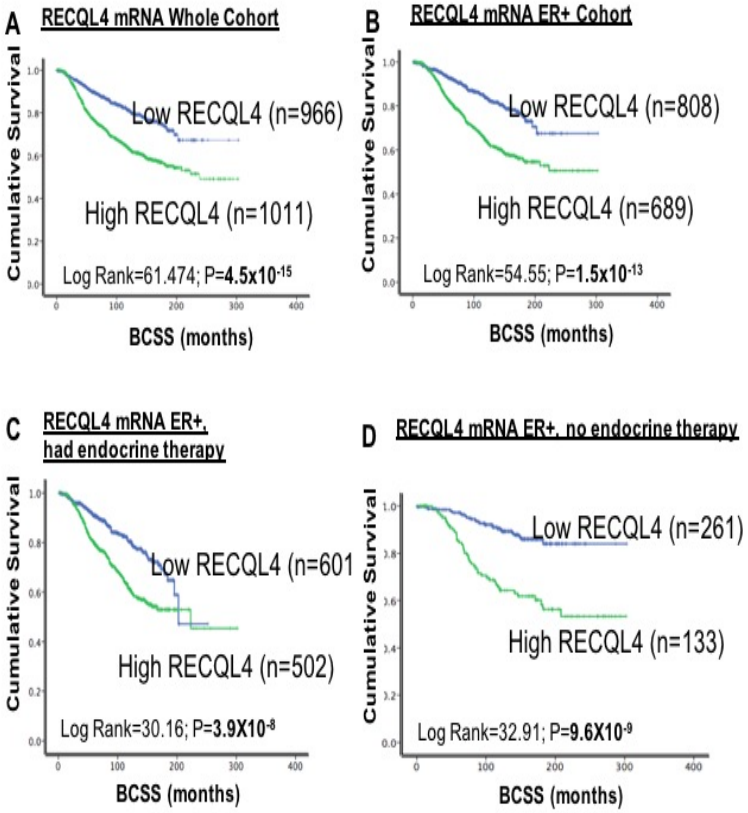


Figure 5.13 RECQL4 mRNA expression and breast cancer survival

A. Whole cohort **B.** ER+ cohort **C.** ER+ cohort, had endocrine therapy **D.** ER+ cohort, who had no endocrine therapy

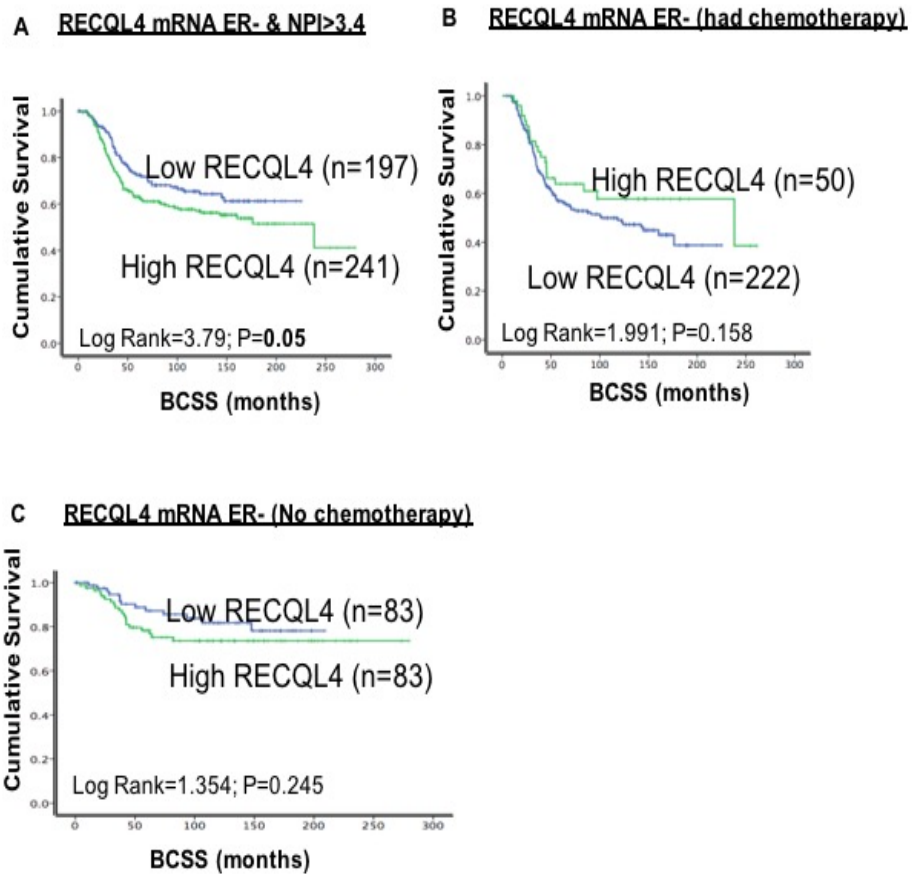


Figure 5.14 RECQL4 mRNA expression and breast cancer survival in the ER- cohort

A. ER- whole cohort **B.** ER- cohort who had chemotherapy **C.** ER- cohort who had no chemotherapy

5.3.19 RECQL4 mRNA remains an independent predictor of breast cancer survival

In multivariate Cox regression analysis that included other validated prognostic factors, such as lymph node stage, NPI and tumour size, RECQL4 mRNA expression was a powerful independent predictor for breast cancer-specific survival ($p<0.00001$) (Table 5.11).

Table 5.11 Multivariate analysis in the METABRIC cohort confirms that RECQL4 mRNA overexpression is a powerful independent prognostic factor

	P-Value	**HR**	**95% CI for HR**	
			Lower	**Upper**
Breast Cancer Specific Survival				
RECQL4 Expression	**0.000015**	1.366	1.185	1.573
NPI	**0.00016**	1.319	1.142	1.524
Tumour Size				
G1	**0.039**	0.893	0.538	1.481
G2	0.246	0.992	0.629	1.563
G3	0.178	1.357	0.734	2.509
LN Status				
LN (1-3)	**0.00046**	1.980	1.328	2.950
LN(>3)	0.312	1.277	0.973	1.678

5.3.20 High RECQL4 mRNA is a result of RECQL4 gain/amplification

To get an insight into the mechanism of high RECQL4 mRNA, we correlated mRNA and gene copy numbers in the METABRIC cohort, to see whether high RECQL4 mRNA is a result of RECQL4 gain or amplification. As shown in Figure 5.15, there was a strong correlation between gene copy number changes and m RNA levels ($p<0.00001$). The data therefore, advocate that in a proportion of aggressive tumours, high mRNA level is due to increased gene copy numbers in tumours.

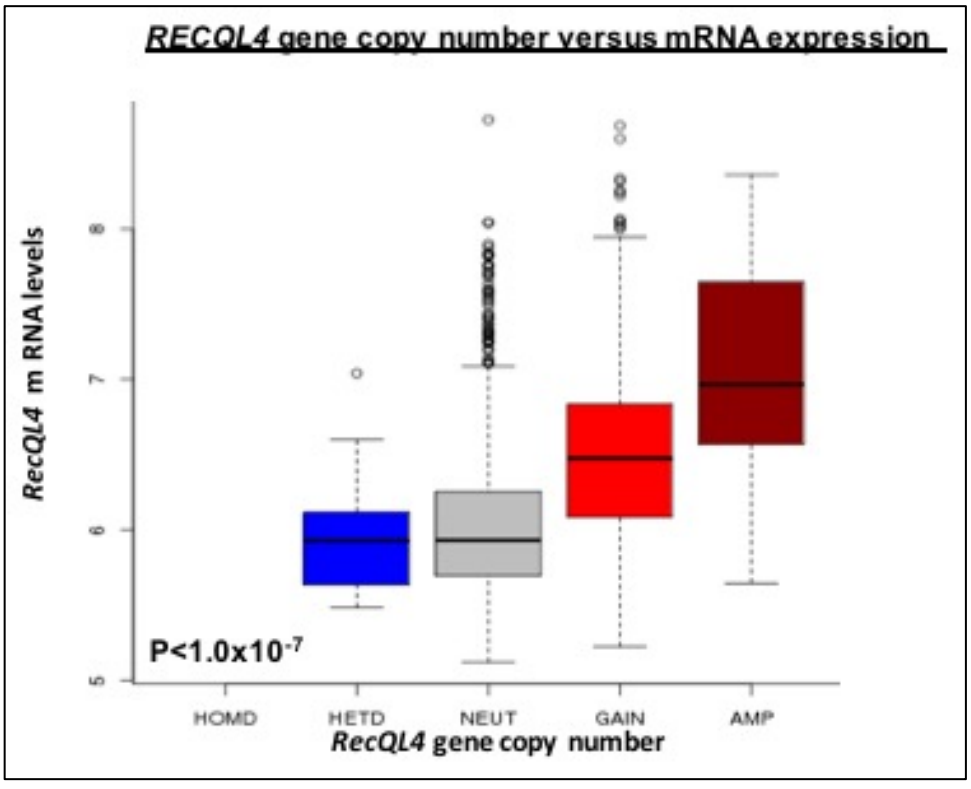


Figure 5.15 Correlation between RECQL4 gene copy number and RECQL4 mRNA expression in the whole cohort

5.3.21 Rothmund-Thomson syndrome (RTS) patients are not at increased risk of breast cancer

The above data suggest that RECQL4 promotes sporadic breast cancer. However, a germline mutation in RECQL4 is the cause of two-thirds of patients with Type II RTS, and it has previously been shown that RECQL4 mutation status correlates with risk of developing osteosarcoma (Siitonen et al, 2009; Wang et al, 2003). We therefore, proceeded to investigate if Type II RTS patients are at increased risk of breast cancer. We collaborated with Ta-Tara Rideau, Research Coordinator at Texas Children's Cancer Centre, Houston, TX for data collection on RTS patients. Of the 58 patients (24 females and 34 males), there were no cases of breast cancer reported. The median follow-up time for the entire group of patients was 12 years (range 10 months to 42 years).

5.4 Discussion

RECQL4 is a key member of the RECQ family of DNA helicases which unwind DNA, a process that is essential during DNA repair and replication. Although RECQL4 is a recognised tumour suppressor, in the current study we have provided the first comprehensive clinical evidence that RECQL4 promotes sporadic breast cancers. Amplification or gain in gene copy numbers, high mRNA expression, and high protein level were significantly associated with aggressive phenotypes and poor survival.

Fang et al. has shown gene amplification and elevated levels of RECQL4 expression in several breast cancer cell lines (Fang et al, 2013). Moreover, suppression of RECQL4 expression resulted in reduced breast cancer cell proliferation *in vitro* and impaired tumorigenicity *in vivo* in that study (Fang et al, 2013). In prostate cancer cell lines, RECQL4 is frequently overexpressed, and its knockdown resulted in reduced proliferation *in vitro* and suppressed tumour growth in tumour bearing mice (Su et al, 2010b). In human prostate cancer, RECQL4 was also overexpressed and correlated to aggressive cancer in that study (Su et al, 2010b). RECQL4 overexpression has also been described in sporadic osteosarcomas (Maire et al, 2009). Similarly, in a small cohort of 43 breast tumours, RECQL4 mRNA was frequently overexpressed. However, no clinicopathological associations or survival outcomes were described in that study (Fang et al, 2013). Recent studies have shown that RECQL4 is overexpressed in gastric cancer, hepatocellular carcinoma and breast cancer and high RECQL4 mRNA expression is associated with poor outcome (Chen et al, 2018; Li et al, 2018; Zhu et al, 2018).

Our study has comprehensively investigated RECQL4 in breast cancers. RECQL4 gene is localized to chromosome 8q24. Gains of 8q, as well as amplifications of the long arm of chromosome 8, is frequent in sporadic breast cancer (Ioannidis et al, 2003; Thomassen et al, 2009; Yokota et al, 1999). Therefore we initially investigated RECQL4 gene copy number changes in breast cancer and have provided the first clinical evidence that gain/amplification of RECQL4 gene is not only associated with aggressive

pathological features but is also linked to adverse breast cancer-specific survival. We then proceeded to evaluate RECQL4 at the mRNA level in the METABRIC cohort. Similar to gain/amplified *RECQL4* gene copy number changes, high RECQL4 mRNA overexpression was significantly associated with high grade, lymph node positivity, PAM50 Her-2, PAM50. LumB, intClus 5, intClus 6 and intClus 9. Interestingly, low *RECQL4* mRNA expression was associated with PAM50. LumA implying a role for RECQL4 in proliferation. In fact, ER+/Her-2 negative/low proliferation was more frequently seen in tumours with a low *RECQL4* mRNA level. In addition, high *RECQL4* mRNA level was frequent in intClust 10 subgroup which is the most highly genomically unstable sub group with basal-like features. Low *RECQL4* mRNA level was also seen in intClust 3 subgroup that is characterised by low genomic instability. Together the data suggest that *RECQL4* mRNA level may also inform proliferation and genomic stability status in breast cancers. In addition, high *RECQL4* mRNA level was also frequently seen in intClust 5 (HER-2 enriched with worst survival), intClust 9 (8q cis-acting/20qamplified mixed subgroup), and intClust 1 (17q23/20q cis-acting luminal B subgroup) subgroups that were described as intermediate prognostic groups (Curtis et al, 2012b). On the other hand, low *RECQL4* mRNA level is linked to intClust 4 (includes both ER-positive and ER-negative cases with a flat copy number landscape and termed the 'CNA-devoid' subgroup with extensive lymphocytic infiltration), and intClust 8 subgroups (classical 1q gain/16q loss luminal A subgroup). An important observation here is that the *RECQL4* mRNA expression data is strikingly similar to the data we recently

reported for FEN1 (flap endonuclease 1) and BLM (Bloom syndrome helicase) in the METABRIC cohort (Abdel-Fatah et al, 2014a; Arora et al, 2015c). The clinical data would concur with pre-clinical studies that suggest functional interactions between RECQL4, BLM, and FEN1 (Croteau et al, 2012b). As there was a strong correlation between mRNA level and gene copy number changes, our data suggest that high mRNA level is likely due to the direct consequence of gain or amplification of *RECQL4* gene copy number changes in the METABRIC cohort.

RECQL4 is a 1208 amino acid containing multifunctional protein. The N-terminal region of RECQL4 contains nuclear as well as mitochondrial targeting sequences and is important for sub-cellular localisation of RECQL4. In addition, acetylation of the N- terminal region by p300 may regulate nuclear to cytoplasmic localisation of RECQL4 (Croteau et al, 2012b). Not surprisingly, we observed complex RECQL4 staining patterns in breast cancer with tumours exhibiting no staining, nuclear only staining, cytoplasmic only staining or nuclear and cytoplasmic staining. In contrast, in normal breast tissue, we observed only nuclear staining implying that there is altered sub-cellular localisation in breast cancer tissue. Low nuclear RECQL4 level was associated with an aggressive phenotype. In addition, low nuclear RECQL4 level was linked to low nuclear levels of FEN1, Polβ, RAD51 and BLM. The data is consistent with previous preclinical studies that demonstrate an interaction between RECQL4 and FEN1, Polβ, RAD51 as well as BLM. In contrast, high cytoplasmic RECQL4 level was inversely associated with FEN1, Polβ, RAD51

and BLM. We speculate that cytoplasmic accumulation of RECQL4 may reflect altered sub-cellular localisation due to additional defects such as post-translational acetylation mediated mechanisms. An additional possibility is that cytoplasmic accumulation may reflect a mitochondrial role of RECQL4 (Chi et al, 2012; Croteau et al, 2012a; Gupta et al, 2014). However, further preclinical studies will be required to clarify the biological significance of the mitochondrial function of RECQL4 in breast cancer. When nuclear and cytoplasmic expression was investigated together, we observed that tumours with low nuclear/high cytoplasmic expression have aggressive features and associated with poor breast cancer-specific survival.

In the current study, we have observed that RECQL4 amplification/gain in copy numbers, mRNA overexpression, and altered protein levels have prognostic and predictive significance in breast cancer. Interestingly, both RECQL4 and c-MYC are localized to chromosome 8q24, a site frequently amplified in sporadic breast cancers. We therefore, hypothesised a clinicopathological association between RECQL4 and c-MYC. Herein we have provided the first evidence that in tumours with high RECQL4 nuclear expression, c-MYC status can stratify tumours. We speculate that either c-MYC can be targeted for therapy in RECQL4 overexpressing tumours or RECQL4 can be targeted in c-MYC driven breast cancers. These new observations suggest a functional link between c-MYC and RECQL4 although pre-clinical mechanistic studies are required to confirm this hypothesis. Interestingly, WRN helicase deficiency can suppress c-MYC driven tumorigenesis (Robinson et al, 2009) and BLM helicase was shown

to promote c-MYC degradation and delay tumour initiation in xenograft models suggesting a tumour suppressing function of BLM (Chandra et al, 2013).

Germline mutation in RECQL4 is the cause of type II RTS where patients are predisposed to develop cancer particularly osteosarcomas and lymphomas. In the largest available cohort of type II RTS patients, we did not observe any incidence of breast cancer. This suggests that either RECQL4 deficiency does not influence breast cancer pathogenesis or that RTS patients have not lived long enough to develop breast cancer.

In conclusion, this chapter provides evidence that RECQL4 is a key biomarker and potential anti-cancer drug target in breast cancer.

Chapter 6

WERNER helicase expression in sporadic breast cancers

6 WERNER helicase expression in sporadic breast cancers

6.1 Introduction

Werner (WRN) enzyme, also known as Recombinase Q like helicase 2 (RECQL2), has a DNA-helicase and exonuclease activity towards double-stranded DNA (Bohr, 2008; Croteau et al, 2014b). 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 (Bohr, 2008; Goto et al, 1992; Schellenberg et al, 1992). WRN protein has been shown to play an important role in DNA repair, replication, transcription, telomere maintenance and consequently genomic stability (Bohr, 2008; Harrigan et al, 2006; Thompson & Schild, 2002a). WRN is phosphorylated by multiple kinases and these post-translational modifications are essential for WRN to perform its vital functions (Mukherjee et al, 2018). WRN colocalizes and shows direct interaction with TOPO I. WRN enhances the ability of TOPO I to relax negatively supercoiled DNA (Laine et al, 2003). RECQL5 co-operates with WRN at stalled replication forks and stimulates its helicase activity on DNA fork duplexes (Popuri et al, 2017b).

Mutations in the human WRN gene leading to the loss of WRN gene product are associated with Werner syndrome (WS) (Goto et al, 1992). WS is a rare autosomal recessive disease which is characterized by chromosomal instability,

premature aging, and propensity to malignancies (Bohr, 2008; Lauper et al, 2013). The most common neoplasms in patients with WS are soft tissue sarcoma, osteosarcoma, thyroid cancer, malignant melanoma, breast cancer, benign meningioma and myeloid disorders (Lauper et al, 2013). 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 (Shen & Loeb, 2000b; van Brabant et al, 2000; Yu et al, 1997).

RECQ helicases have a highly conserved RECQ C-terminal group which interacts with DNA damage response proteins (Bohr, 2008; Opresko et al, 2007; Sharma et al, 2006b). 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 (Szekely et al, 2005). 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 (Opresko et al, 2007). 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 (Otterlei et al, 2006a).

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

6.2 Methods

6.2.1 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 carcinoma patients who were diagnosed between 1986 and 1999 and entered into the Nottingham tenovus series. The second cohort was an independent series of 252 ER-negative invasive breast cancers diagnosed and treated at Nottingham University Hospitals between 1999 and 2007. Patient demographics of the Nottingham tenovus series (NTS) and ER-negative cohort are summarised in Table 2.4 and 2.5 respectively.

6.2.2 Tissue Microarray (TMA)

Breast tumours were arrayed in tissue microarrays (TMAs) constructed with two replicate 0.6 mm cores from the centre and periphery of the tumours. Details of TMA construction is detailed in section 2.1.1.

6.2.3 Antibody optimisation

Optimal concentration and conditions for staining were ascertained for WRN antibody using standard IHC technique, as described in section 2.1.2 . to keep the experimental conditions standardized, we tested each dilution on the same type of sample and also on the same version of TMA. We proceeded to optimisation on Breast TMA. The WRN antibody (Rabbit Antibody, polyclonal) was purchased from Novus Biological, catalogue number NBP1-87143. The

recommended optimal dilution for IHC by the manufacturer was 1:50-1:200. Antigen retrieval method used was Citrate buffer pH 6.0 as per manufacturer recommendation. We initially tried 1:40, 1:50 and 1:75 dilution, using citrate as antigen retrieval and incubation time 30 min and 60 min at room temperature for each dilution. We did not see any staining. We then tried optimisation in 1:50, 1:75, 1:100, and 1:150 dilutions and incubation time used was overnight at room temperature.

All optimisation slides were then evaluated by an expert pathologist (MA and EAR) to decide if further optimisation was required or to identify the optimal concentration for staining the breast tumours. After careful review by an expert pathologist, we selected 1:100 as optimal dilution because it gave the best differential pattern of staining with minimum background / non-specific binding as shown in Figure 6.1.

Both positive and negative controls were included to ensure the quality of staining. A negative control was included by omitting primary antibody and exposing to antibody diluent only. This was to determine if the secondary antibody was binding non-specifically resulting in false positives. It will be ideal to use absorption control to test if the primary antibody binds specifically to the antigen of interest. Beta 2 microglobulin (B2M) is highly expressed in breast cancer (Li et al., 2012), hence this was used as positive control. The same positive control section was included in each run to ensure consistency to be evaluated.

Table 6.1 Primary antibody details

Antibody	Clonality	Source	Catalogue No.	Optimal Dilution/ Incubation time
WRN	Rabbit Ab (Polyclonal)	Novus Biological	NBP1-87143	1:100 Overnight

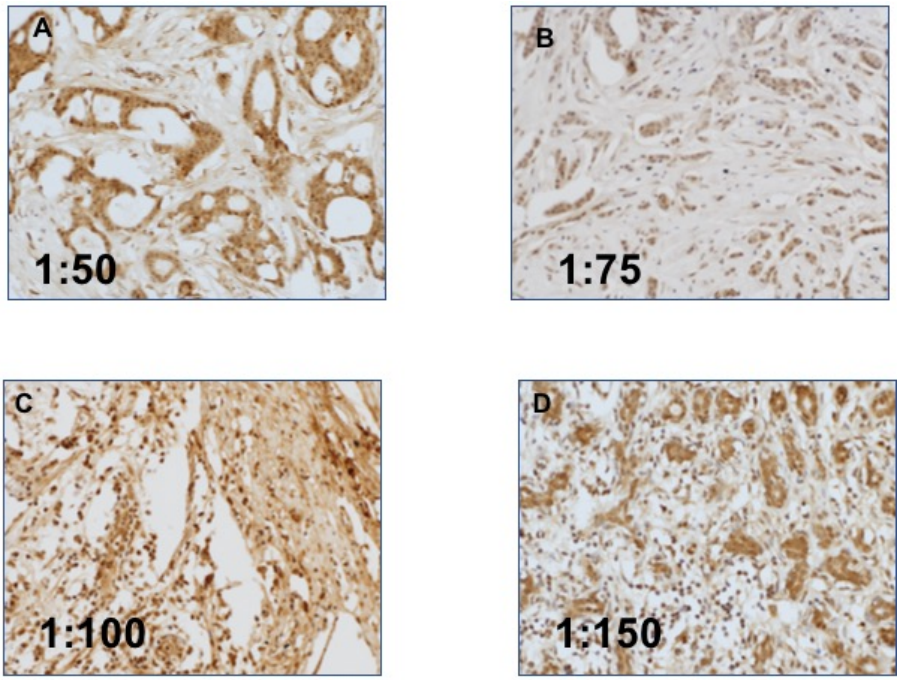


Figure 6.1 Photomicrographs of WRN protein expression in breast TMA with different optimisation dilutions

A. 1:50 dilution , overnight Incubation **B.** 1:75 dilution , overnight Incubation **C.** 1:100 dilution , overnight Incubation **D.** 1:150 dilution ,overnight Incubation (1:100 was selected for staining as it showed differential pattern of staining and little or no background staining).

6.2.4 3.2.4 Western Blot analysis

Western blot analysis as described in section 2.3.2.2 was used to evaluate the specificity of WRN antibody, before using them for IHC. WRN protein expression was assessed in four breast cancer cell lines MCF7, MDA-MB-231 , MDA-MB-436, and MDA-MB-468. The characteristics of these cell lines are explained in detail in section. The details of cell lysate preparation and sample preparation are also detailed in section 2.3.2.2.1.

Table 6.2 The Primary antibody and its optimal concentration used for western blot

Antibody	**Clonality**	**Source**	**Catalogue No.**	**Optimal Dilution/ Incubation time**
WRN	Rabbit Ab (Polyclonal)	Novus Biological	NBP1-87143	1:1500 (Overnight at 4^0C)

6.2.5 IHC technique

Details of the IHC technique used is described in detail in section 2.1.2.

6.2.6 Histopathological scoring of stained Breast TMAs

Scoring of all Breast TMAs was performed by me and an expert pathologist blinded to the clinicopathological characteristics of patients. H scoring was used to score breast tumours as described in detail in section 2.1.6. 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. A median H-Score of <116 was categorised as low WRN nuclear expression and a median H-score of <20 was regarded as high WRN cytoplasmic expression.

6.2.7 Quantitative real-time PCR

Total RNA was extracted from MCF-7, MDA-MB-231, MDA-MB-468 and MDA-MB-436 cells using RNeasy Mini kit (QIAGEN, UK). The RNA quantification was done using a NanoDrop 2000c Spectrophotometer (Thermo Scientific). The cDNA was synthesised from 0.5 μg of total RNA using RT^2 first strand kit (QIAGEN). The real-time qPCR was performed using SYBR Green PCR Master mix (Applied Biosystems) with a primer set (WRN QuantiTect Primer Assay, catalogue no. QT00074809, QIAGEN) targeting the WRN gene. The glyceraldehyde-3-phosphate dehydrogenase housekeeper gene was used as an internal control (GAPDH QuantiTect Primer Assay, catalog no. QT00079247, QIAGEN). The real-time PCR for each RNA sample was performed in triplicate. NTC (No Template Control) was included to rule out cross-contamination of reagents and surfaces. To make NTC, we included all the RT-PCR reagents except the RNA template. Minus reverse transcriptase (- RT) control was used to do an assessment of genomic DNA contamination. A melting curve analysis was included to get information about the purity of the

amplified product. A single peak was seen which indicated the presence of only one double stranded product.

6.2.8 Methodology for WRN mRNA expression in Breast Cancer

WRN mRNA expression was investigated in the METABRIC (Molecular Taxonomy of Breast Cancer International Consortium) cohort. The METABRIC study protocol and the molecular profiling methodology in a cohort of 1977 breast cancer samples are detailed by Curtis et al (Curtis et al, 2012b). Patient demographics, details of data acquisition and analysis are summarised in Table 2.7.

WRN probe (id: ILMN_1679881) was used because it was a perfect match and quality for its target, having a GC content of 58%, 0 SNPs and it does not possess a poly G tail at the end. For TOPO1, the probe used was ILMN_2192316. The cut off for the high vs low WRN and TOPO1 gene expression was done using x-tile software (Version 3.6.1) so that the resultant subgroups had a significant difference in survival outcomes.

6.2.9 Statistical analysis

Data analysis was performed using SPSS (SPSS, version 22 Chicago, IL) as described in detail in section 2.1.9.

6.2.10 Ethical approval

Ethical approval was obtained from the Nottingham Research Ethics Committee (Reference number C202313, Approval date : 21/10/2010). Tumour Marker Prognostics Studies (REMARK) criteria, recommended by McShane et al (McShane et al, 2005), were followed throughout this project.

6.3 Results

6.3.1 WRN Protein Expression in Breast Cancer

We initially assessed WRN protein expression in a panel of breast cancer cell lines. As shown in Figures 6.2 and 6.3, 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.

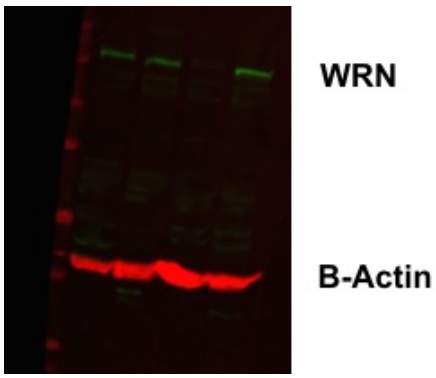


Figure 6.2 Western Blot of WRN Expression in MCF7, MDA-MB-231 & MDA-MB-436 and MDA-MB-468 cell lines

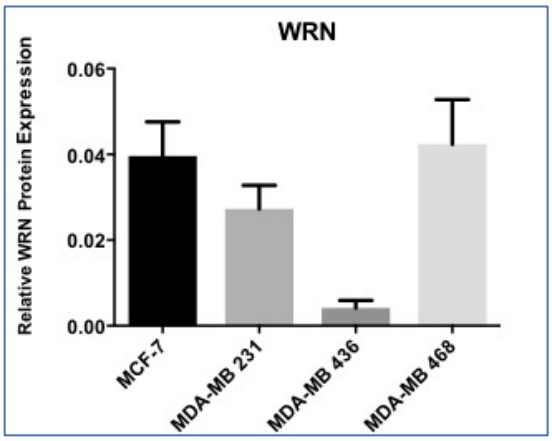


Figure 6.3 WRN protein expression in breast cancer cell lines

Graph shows WRN protein expression in MCF-7, MDA-MB-231, MDA-MB-436 and MDA-MB-468 breast cancer cell lines relative to β-Actin. Data presented as mean ± SEM of two independent experiments.

6.3.2 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 noticed both nuclear and cytoplasmic localisation of WRN in normal and tumour breast tissue. (Figure 6.4). 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 6.5)

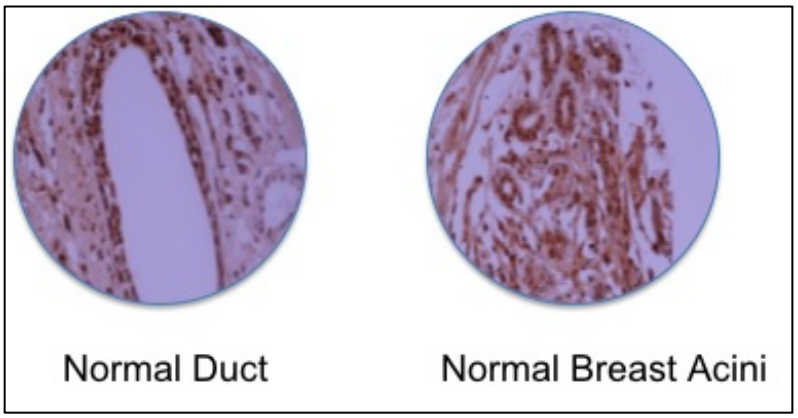


Figure 6.4 Photomicrographs of WRN protein (nuclear & cytoplasmic) expression in normal breast tissue

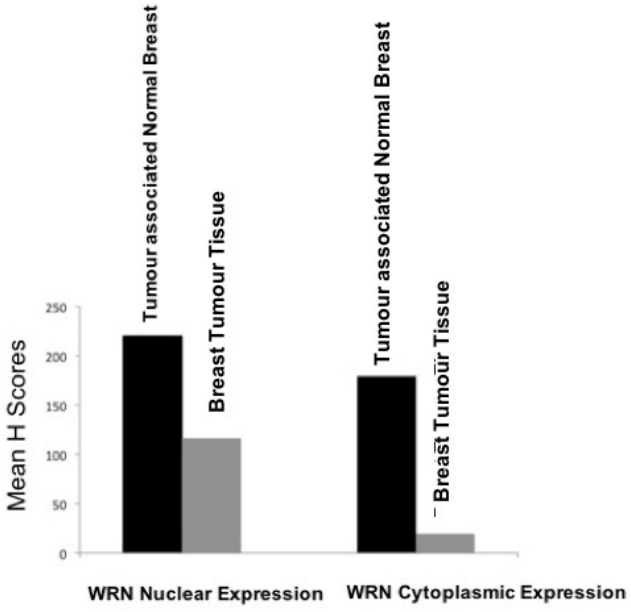


Figure 6.5 WRN nuclear and cytoplasmic in normal (tumour associated) breast tissue and breast tumour tissue

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

We did not see any significant association between WRN nuclear expression and clinical and pathological features in breast cancer (Table 6.3). 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).

Table 6.3 Werner Nuclear Expression in Breast Cancer

	Werner Nuclear Protein Expression		**P- values**	
	Low N (%)	High N (%)	Un-adjusted	Adjusted
A) Pathological Parameters				
Tumour Size <1cm >1-2cm >2-5cm >5cm	 7 (5.9) 50 (42.4) 60 (50.8) 1 (0.8)	 13 (8.5) 62 (40.5) 75 (49) 3 (2.0)	0.740	0.848
Tumour Stage 1 2 3	 53 (44.9) 48 (40.7) 17 (14.4)	 64 (41.8) 74 (48.4) 15 (9.8)	0.330	0.585

Tumour Grade G1 G2 G3	 6 (5.1) 49 (41.5) 63 (53.4)	 5 (3.3) 70 (45.8) 78 (51)	0.642	0.807
Mitotic Index M1 (low; mitoses < 10) M2 (medium; mitoses 10-18) M3 (high; mitosis >18)	 33 (28.4) 30 (25.9) 53 (45.7)	 47 (30.9) 42 (27.6) 63 (41.4)	0.785	0.850
Tubule Formation 1 (>75% of definite tubule) 2 (10%-75% definite tubule) 3 (<10% definite tubule)	 1 (0.9) 40 (34.5) 75 (64.7)	 2 (1.3) 41 (27.0) 109 (71.7)	0.401	0.680
Pleomorphism 1 (small-regular uniform) 2 (Moderate variation) 3 (Marked variation)	 1 (0.9) 42 (36.2) 73 (62.9)	 1 (0.7) 66 (43.7) 84 (55.6)	0.462	0.667
Tumour Type IDC-NST Tubular Carcinoma Medullary Carcinoma ILC Others Mixed NST/lobular/Special Type	 70 (59.8) 19 (16.2) 19 (16.2) 9 (7.7)	 86 (56.6) 22 (14.1) 33 (21.7) 11 (7.2)	0.730	0.862
Lymph Node Status Negative Positive (1-3) Positive (>3)	 51 (44.7) 50 (43.9) 13 (11.4)	 64 (42.7) 74 (49.3) 12 (8.0)	0.530	0.712
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 Phenotype 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
PR 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	100 (50.3)	107 (43.5)		

High	99 (49.7)	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	 146 (55.1) 119 (44.9)	 134 (42.3) 183 (57.7)	 **0.002**	 **0.013**

High				
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; PR: progesterone receptor; Triple negative: ER-/PR-/HER2-. Adjusted p values were calculated using Benjamini-Hochberg false discovery rate method to adjust for multiple testing.

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

We did not observe any significant association between WRN cytoplasmic expression and clinical and pathological features in breast cancer (Table 6.4). 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).

Table 6.4 WRN cytoplasmic protein expression and breast cancer

	Werner Cytoplasmic Protein Expression		**P Value**	
	Low N (%)	High N (%)	Un-adjusted	Adjusted
A) Pathological Parameters				
Tumour Size				
<1cm	52 (11.2)	61 (12.4)	0.432	0.673
>1-2cm	234 (50.4)	233 (47.6)		
>2-5cm	175 (37.7)	188 (38.4)		
>5cm	3 (0.6)	8 (1.6)		
Tumour Stage				
1	300 (64.7)	304 (61.9)	0.335	0.593
2	120 (25.9)	147 (29.9)		
3	44 (9.5)	40 (8.1)		
Tumour Grade				
G1	83 (17.9)	84 (17.1)	0.330	0.612
G2	149 (32.1)	180 (36.7)		
G3	232 (50.0)	227 (46.2)		
Mitotic Index				
M1 (low; mitoses < 10)	158 (35.7)	191 (39.7)	0.213	0.639
M2 (medium; mitoses 10-18)	72 (16.3)	87 (18.1)		
M3 (high; mitosis >18)	212 (48.0)	203 (42.2)		
Tubule Formation				
1 (>75% of definite tubule)	24 (5.4)	28 (5.8)	0.926	0.976
2 (10%-75% definite tubule)	140 (31.7)	156 (32.4)		
3 (<10% definite tubule)	278 (62.9)	297 (61.7)		
Pleomorphism				
1 (small-regular uniform)	15 (3.4)	8 (1.7)	0.123	0.533
2 (Moderate variation)	166 (37.7)	202 (42.2)		
3 (Marked variation)	259 (58.9)	269 (56.2)		

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 Phenotype 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
PR 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)	173 (51.3)	164 (51.7)	0.919	0.995

Low 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
<u>E) Cell cycle and Apoptosis Regulators</u>				
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

Bold = Statistically significant; BRCA1: Breast cancer 1, early onset; HER2: human epidermal growth factor receptor 2; ER: oestrogen receptor; PR: progesterone receptor; CK: cytokeratin; Basal-like: ER-, HER2 and positive expression of either CK5/6, CK14 or EGFR; Triple negative: ER-/PR-/HER2- . Adjusted p values were calculated using Benjamini-Hochberg false discovery rate method to adjust for multiple testing.

6.3.5 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 not associated with an aggressive molecular phenotype. 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 6.5)

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

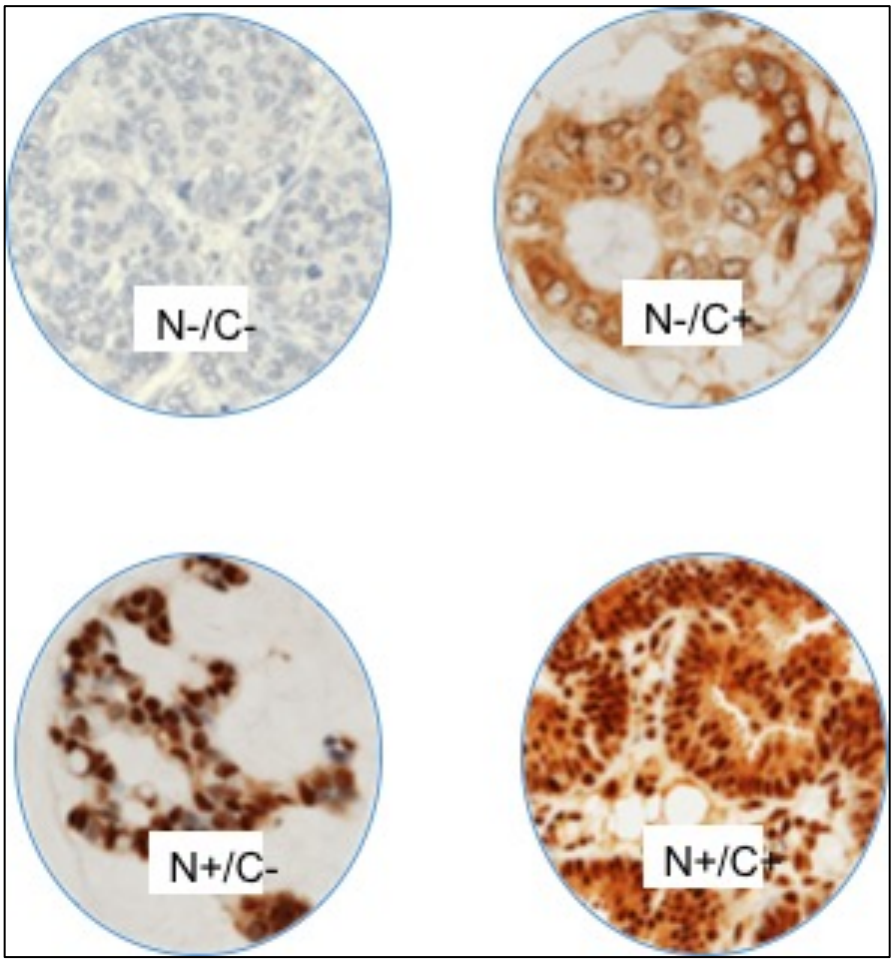


Figure 6.6 Micro photomicrographs of WRN protein expression in breast cancers

N-/C- :Nuclear negative & cytoplasmic negative; **N+/C+** :Nuclear negative & cytoplasmic positive; **N+/C-** :Nuclear positive & cytoplasmic negative; **N-/C+** :Nuclear negative & cytoplasmic positive

Table 6.5 WRN nuclear and cytoplasmic co-expression and breast cancer

VARIABLE	WRN (Nuclear & Cytoplasmic) Protein Co-expression				P- value (unadjusted)	P-Value Adjusted
	Wn-/Wc- N (%)	Wn+/Wc- N (%)	Wn-/Wc+ N (%)	Wn+/Wc+		
A) Pathological Parameters						
Tumour Size						
≤1cm	26 (9.0)	26 (14.9)	16 (11.3)	45 (12.9)	0.382	0.677
>1-2cm	144 (49.7)	90 (51.7)	68 (48.2)	165 (47.3)		
>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)	0.767	0.830
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)		
Tumour Grade						
G1	53 (18.3)	30 (17.2)	27 (19.1)	57 (16.3)	0.664	0.809
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 M1 (low; mitoses < 10) M2 (medium; mitoses 10-18) M3 (high; mitosis >18)	 92 (33.9) 45 (16.6) 134 (49.4)	 66 (38.6) 27 (15.8) 78 (45.6)	 61 (43.6) 22 (15.7) 57 (40.7)	 130 (38.1) 65 (19.1) 146 (42.8)	0.470	0.789
Tubule Formation 1 (>75% definite tubule) 2 (10%-75% definite tubule) 3 (<10% definite tubule)	 13(4.8) 89 (32.8) 169 (62.4)	 11 (6.4) 51 (29.8) 109 (63.7)	 9 (6.4) 45 (32.1) 86 (61.4)	 19 (5.6) 111 (32.6) 211 (61.9)	0.981	38.25
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
B) Aggressive Phenotype						

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 Phenotype 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
C) Hormone Receptors						
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
PR 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
A) 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 (Cyto) 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 (Nu) 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)	**$2.3x10^{-4}$**	**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)	**$2.3x10^{-4}$**	**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 (Nu)						

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 (Cyto) 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
B) <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 (Cyto) 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 (Nu) 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 High	 39 (25.5) 114 (74.5)	 24 (23.8) 77 (76.2)	 24 (28.6) 60 (71.4)	 32(16.4) 163 (83.6)	0.078	0.253
RECQL5(Nu) Low High	 118 (55.9) 93 (44.1)	 41(33.6) 81 (66.4)	 57 (58.2) 41 (41.8)	 101(41.9) 140 (58.1)	**$4.2X10^{-5}$**	**0.001**
RECQL4 (Nu) Low High	 120 (65.9) 62 (34.1)	 54 (53.5) 47 (46.5)	 57 (67.9) 27 (32.1)	 125(57.3) 93 (42.7)	0.067	0.237

RECQL4 (Cyto) Low High	107 (59.1) 74 (40.9)	43 (43.0) 57 (57.0)	39 (46.4) 45 (53.6)	91(41.9) 126 (58.1)	**0.004**	**0.031**
RECQL1 (Nu) Low High	99 (56.6) 76 (43.4)	52 (53.1) 46 (46.9)	38 (50.7) 37 (49.3)	76(41.8) 106 (58.2)	**0.039**	0.169
BLM (Nu) Low High	78 (34.7) 147 (65.3)	33 (28.0) 85 (72.0)	22 (19.8) 89 (80.2)	57(22.9) 192 (77.1)	**0.008**	**0.044**
BLM (Cyto) Low High	174 (77.7) 50 (22.3)	75 (64.7) 41 (35.3)	81 (72.3) 31 (27.7)	171(69.2) 76 (30.8)	**0.05**	0.195
C-Myc Low High	105 (51.5) 99 (48.5)	48 (47.5) 53 (52.5)	35 (40.2) 52 (59.8)	83 (47.2) 93 (52.8)	0.372	0.690

Bold = Statistically significant; HER2: human epidermal growth factor receptor 2; ER: oestrogen receptor; PR: progesterone receptor; Triple negative: ER-/PR-/HER2- . Adjusted p values were calculated using Benjamini-Hochberg false discovery rate method to adjust for multiple testing.

6.3.6 WRN nuclear and TOPO1 co-expression is not associated with clinical and pathological features in breast cancer

As discussed previously WRN interacts with TOPO1 and enhances the ability of TOPO1 to relax the supercoiled DNA. Hence, we did WRN nuclear and TOPO1 co-expression analysis in the sporadic breast but did not see any significant associations with clinical and pathological variables.

6.3.7 Low WRN nuclear expression is associated with worse survival

In univariate analysis, patients whose tumour had low WRN nuclear expression had significant (p=0.02) worse survival (Figure 6.7 A). Furthermore, a statistically significant worst outcome was more pronounced in the ER-negative cohort (p=0.012) (Figure 6.7 C,E)

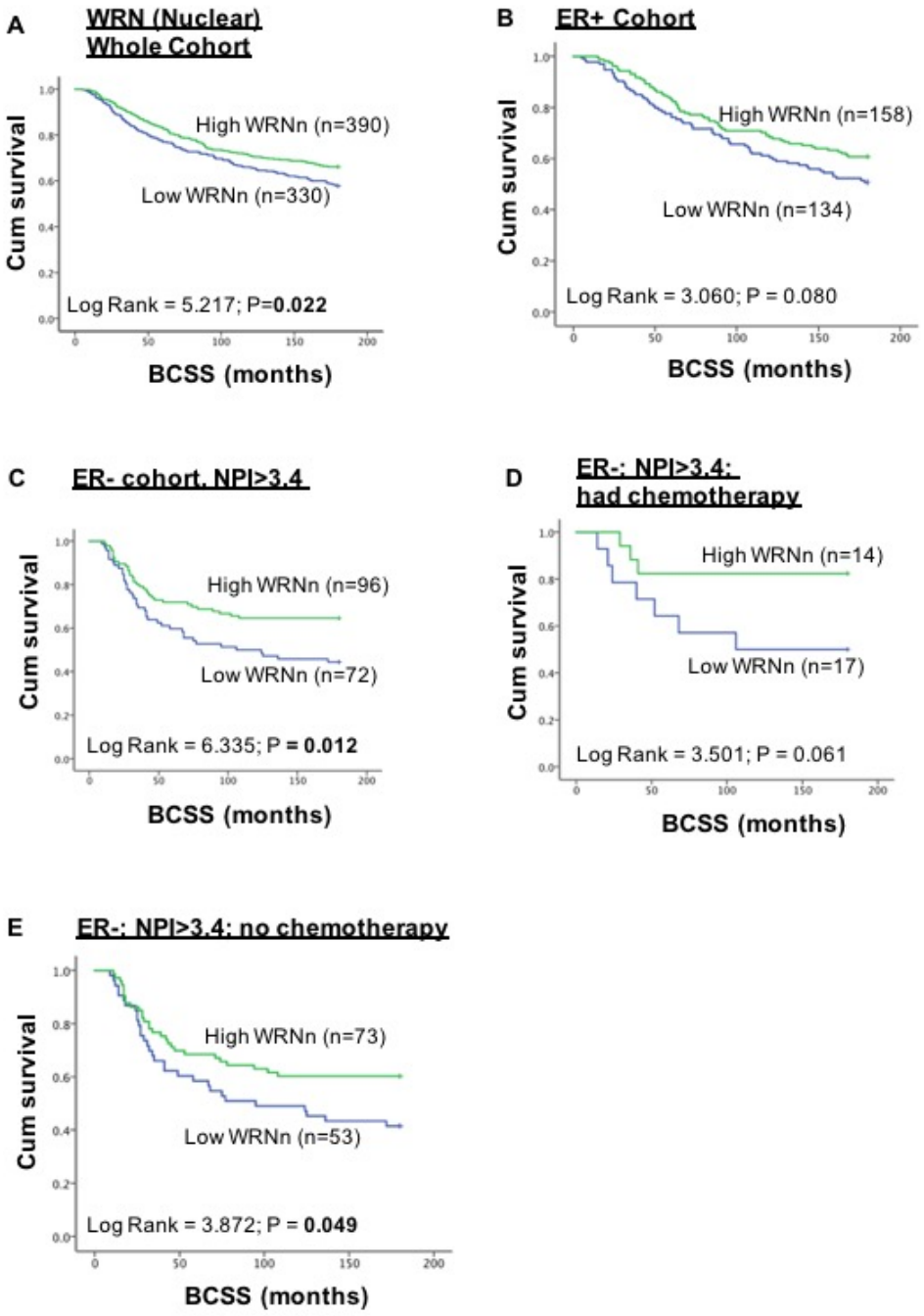


Figure 6.7 WRN Nuclear Expression and Breast cancer survival

A. Whole cohort **B.** ER+, NPI>3.4 Cohort **C.** ER-, NPI>3.4 cohort **D.** ER-, NPI>3.4 cohort who had chemotherapy **E.** ER-, NPI>3.4 cohort who had no chemotherapy.

BCSS: Breast cancer-specific survival in months.

6.3.8 Low cytoplasmic WRN expression is associated with worse survival

Tumours which were low in WRN cytoplasmic expression had a poor outcome and it was statistically significant (p=0.017) Figure. We did not see any significant impact on survival in ER+ and ER- cohort (Figure 6.8A)

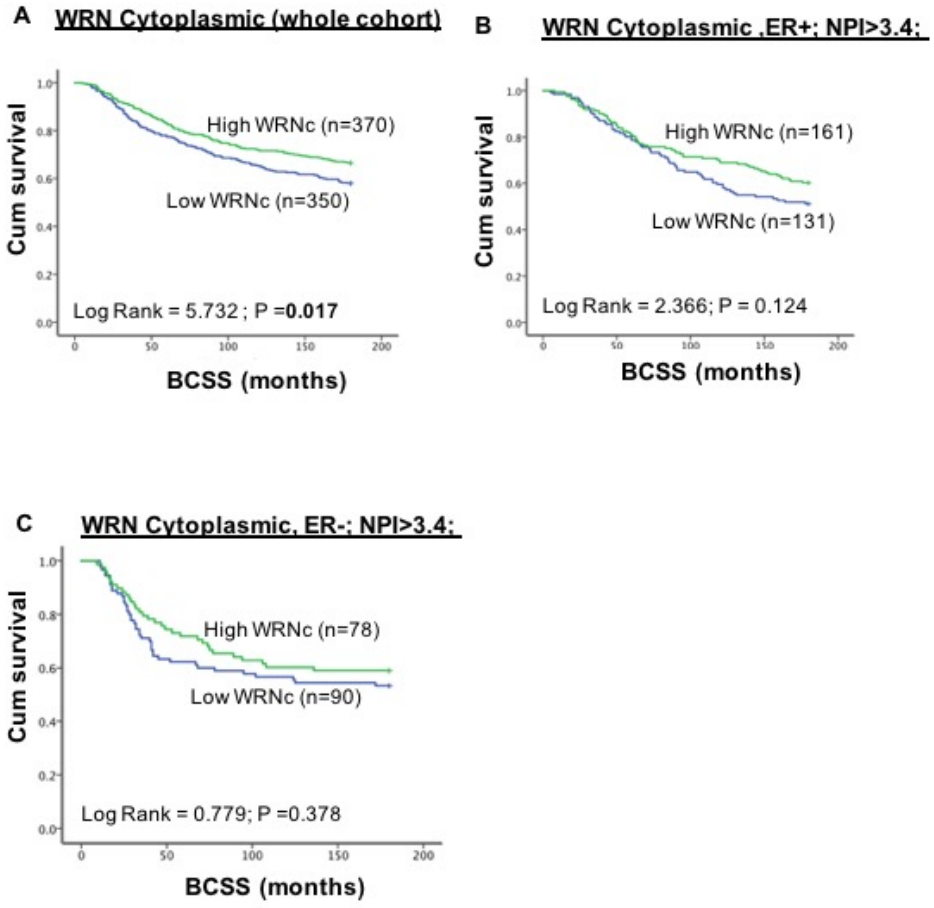


Figure 6.8 WRN cytoplasmic expression and Breast cancer survival

A. Whole cohort **B.** ER+, NPI>3.4 Cohort **C.** ER-, NPI>3.4 cohort; BCSS: Breast cancer-specific survival in months.

6.3.9 Tumours with low nuclear/low cytoplasmic WRN expression had poor breast cancer specific survival

We then evaluated the impact of WRN nuclear and cytoplasmic co-expression on breast cancer survival. In the whole cohort, patients with low nuclear/low cytoplasmic WRN expression had poor breast cancer-specific survival (p=0.04). This suggests that low expression has prognostic significance. In the ER+ and ER- cohort, WRN nuclear and cytoplasmic co-expression did not have any statistically significant impact on survival.

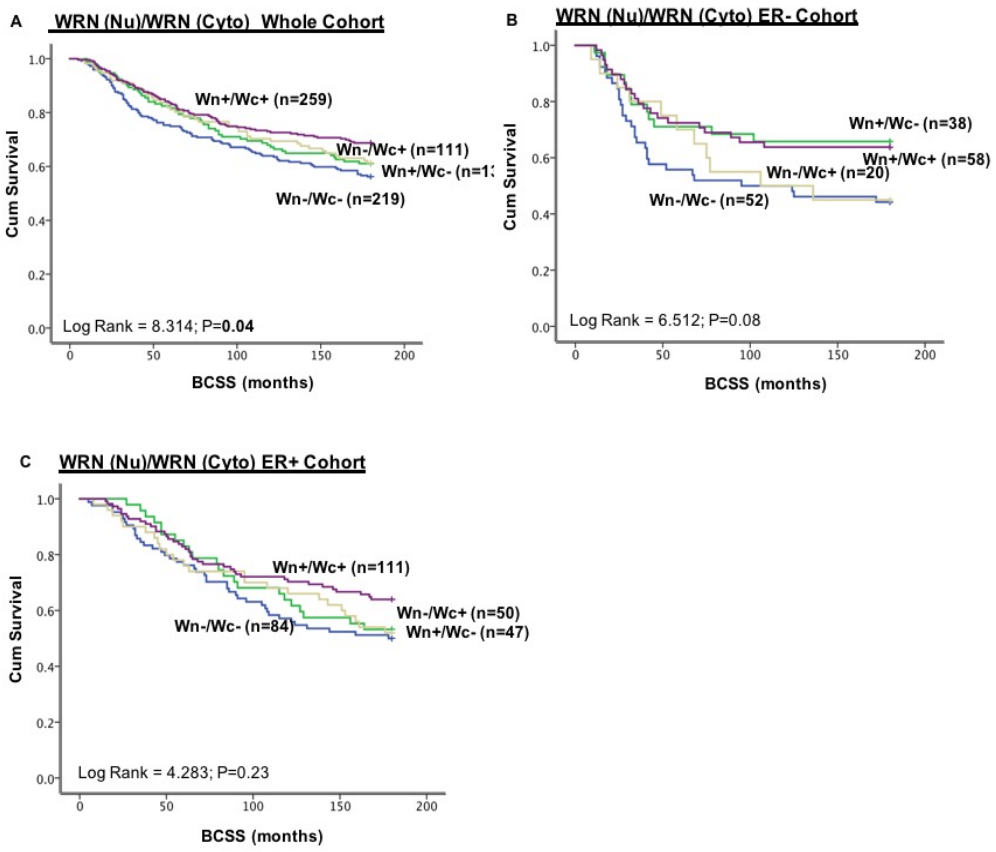


Figure 6.9 WRN nuclear and cytoplasmic co-expression in Breast cancer

A. Whole cohort **B.** ER- Cohort **C.** ER+ Cohort; BCSS: Breast cancer-specific survival

6.3.10 Tumours with low WRN nuclear expression and high TOPO1 expression show poor survival

In WRN (nuclear) and TOPO1 (nuclear) co-expression analysis, tumours with low WRN nuclear expression and high TOPO1 expression had poor survival in the whole cohort (Figure 6.10). We did not see any significant impact on survival in the subgroup analysis.

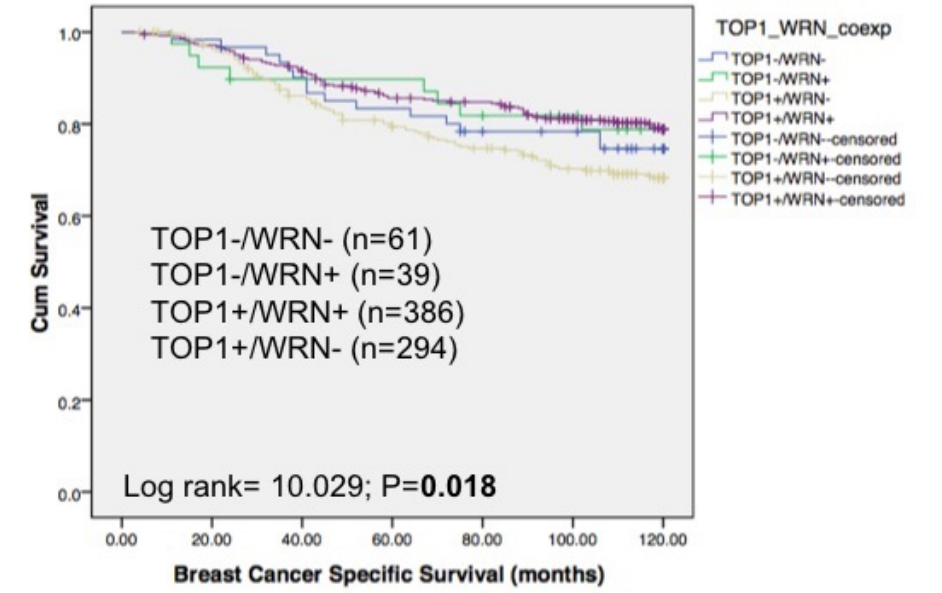


Figure 6.10 WRN nuclear and TOPO1 nuclear co-expression and Breast cancer-specific survival

6.3.11 WRN nuclear and WRN cytoplasmic expression are independent predictors of survival

We then proceeded to multivariate analysis. In multivariate analysis (Table 6.6), WRN nuclear and WRN cytoplasmic expression independently influenced

survival (p=0.39 and 0.032 respectively). Tumour stage and grade were other factors independently associated with breast cancer-specific survival.

Table 6.6 Multivariate analysis in the Nottingham cohort

	P value	**Exp (B)**	**95% CI of Exp (B)**	
			Lower	**Upper**
Breast Cancer Specific Survival				
Stage	**0.000017**	1.954	1.442	2.649
Grade	**0.000016**	2.473	1.636	3.738
HER2 expression	0.242	1.313	.832	2.073
NPI	0.531	.793	.383	1.639
WRN (Cytoplasmic)	**0.039**	.677	.467	.980
WRN (Nuclear)	**0.032**	.672	.466	.967
TOPO1 (Nuclear)	0.270	1.234	.849	1.794

6.3.12 WRN mRNA Expression in Breast Cancer

We profiled a panel of breast cancer cell lines. MDA-MB-231 and MDA-MB-436 showed lower, and MDA-MB-468 showed higher expression of WRN mRNA relative to control MCF-7 cell line. (Figure 6.11)

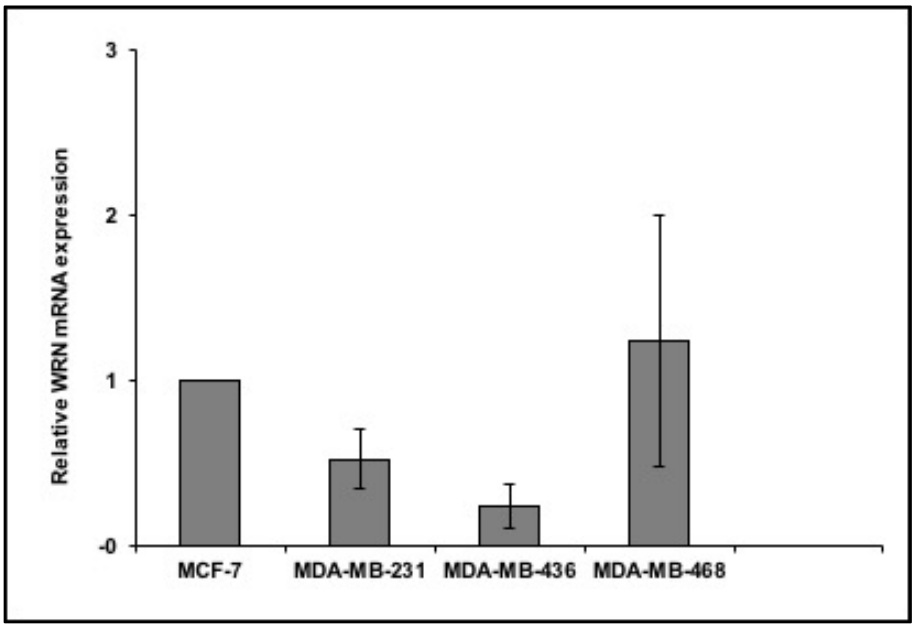


Figure 6.11 Relative mRNA expression of WRN in Breast cancer cell lines

A graph of mRNA expression of WRN in MDA-MB-231, MDA-MB-436, and MDA-MB-468 breast cancer cell lines relative to MCF7 control breast cancer cell line. Data presented as mean ± SEM of two independent experiments, each performed in triplicate.

6.3.13 Low WRN mRNA expression is significantly associated with aggressive clinicopathological features

Nearly 16.5% (326/1977) of tumours had low WRN mRNA expression and ~85.5% (1651/1977) tumours had high WRN mRNA expression. Low WRN mRNA expression was significantly associated with aggressive clinicopathological features including high histological grade, lymph node stage, high-risk Nottingham prognostic index (NPI) > 3.4 and Her-2 overexpression (ps≤0.01) (Table 6.7). Interestingly, ER-negative and triple negative phenotypes were more common in tumours with high WRN mRNA expression (ps≤0.01).

Low WRN mRNA expression was significantly associated with molecular phenotypes: PAM50.Her2, PAM50.LumB, Genufu subtype (ER+/Her2-/High proliferation) and Genufu subtype (Her2 positive) breast tumours (ps≤0.01). On the other hand, PAM50.LumA tumours, PAM50.basal and Genufu subtype (ER+/Her2-/low proliferation) express high levels of WRN mRNA (ps≤0.01). Similarly, the WRN mRNA level was significantly linked to the various integrative clusters (Table 6.7) as described in the METABRIC study. This study was based on gene copy number changes and gene expression data (Curtis et al). Low WRN mRNA expression was significantly associated with clusters, which had a poor clinical outcome in the METABRIC study (Curtis et al).

Table 6.7 Association between WRN mRNA expression and clinicopathologic variables in METABRIC cohort

VARIABLE	*WRN* mRNA Expression		P Values
	Low	High	
	N(%)	N (%)	
A) Pathological Parameters			
Lymph node stage			
Negative	139(42.8%)	896(54.4%)	**0.001**
Positive (1-3)	65(20.0%)	249(15.1%)	
Positive (>3)	121(37.2%)	501(30.4%)	
Grade			
G1	17(5.4%)	152(9.6%)	**0.002**
G2	113(36.1%)	657(41.7%)	
G3	183(58.5%)	767(48.7%)	
Tumour Size (cm)			
T 1a+b(1.0)	14(4.3%)	78(4.8%)	0.073
T 1c(>1.0-2.0)	106(32.9%)	660(40.8%)	
T2 (>2.0-5)	183(56.8%)	818(50.0%)	
T3 (>5)	19(5.9%)	79(4.8%)	
NPI			
≤ 3.4	46(14.8%)	342(21.9%)	**0.005**
>3.4	265(85.2%)	1217(78.1%)	
Her2 overexpression (No)	264(81.0%)	1468(88.9%)	**$7.1x10^{-5}$**
(Yes)	62(19.0%)	183(11.1%)	
Triple negative (No)	303(92.9)	1357(82.2)	**$1.0x10^{-6}$**
(Yes)	23(7.1)	294(17.8)	
ER (Negative)	60(18.4%)	410(24.8%)	**0.013**
(Positive)	266(81.6%)	1241(75.2%)	
PR (Negative)	155(47.5%)	781(47.3%)	**0.936**
(Positive)	171(52.5%)	870(52.7%)	

Genefu subtype			
ER-/Her-2 negative	9(5.5%)	141(17.0%)	**$1.7x10^{-4}$**
ER+/Her-2 negative/high proliferation	81(49.4%)	285(34.3%)	**$2.6x10^{-4}$**
ER+/Her-2 negative/low proliferation	45(27.4%)	323(38.9%)	**0.005**
Her-2 positive	29(17.7%)	81(9.8%)	**0.003**
PAM50 subtype			
PAM50.Her2	52(17.9%)	186(12.6%)	**0.014**
PAM50.Basal	28(9.7%)	302(20.4%)	**$1.8x10^{-5}$**
PAM50.LumA	98(33.8%)	617(41.6%)	**0.013**
PAM50.LumB	112(38.6%)	377(25.4%)	**$4.0x10^{-6}$**
IntClust subgroups			
intClust.1	35(10.7%)	102(6.2%)	**0.003**
intClust.2	17(5.2%)	55(3.3%)	0.097
intClust.3	28(8.6%)	262(15.9%)	**0.001**
intClust.4	32(9.8%)	311(18.8%)	**$8.5x10^{-5}$**
intClust.5	53(16.3%)	136(8.2%)	**$7.0x10^{-6}$**
intClust.6	29(8.9%)	57(3.5%)	**$1.1x10^{-5}$**
intClust.7	42(12.9%)	147(8.9%)	**0.026**
intClust.8	30(9.2)	270(16.4%)	**0.001**
intClust.9	48(14.7%)	98(5.9%)	**$2.9x10^{-8}$**
intClust.10	12(3.7%)	213(12.9%)	**$2.0x10^{-6}$**

Bold = Statistically significant;HER2: human epidermal growth factor receptor 2; ER: oestrogen receptor; PR: progesterone receptor; Triple negative: ER-/PR-/HER2-

6.3.14 Low WRN mRNA expression in tumours is associated with adverse Breast Cancer Specific Survival

Low WRN mRNA expression in tumours was associated with adverse Breast Cancer Specific Survival (BCSS) in the whole cohort ($p = 0.002$) (Figure 6.8 A). The data provide evidence that low WRN mRNA expression is associated with aggressive phenotypes and poor survival in patients. The worst outcome was even more significant in ER+ cohort ($p<0.001$) (Figure 6.8 B) , including ER+

patients who had received adjuvant endocrine therapy (p value=0.001) (Figure 6.8 C).

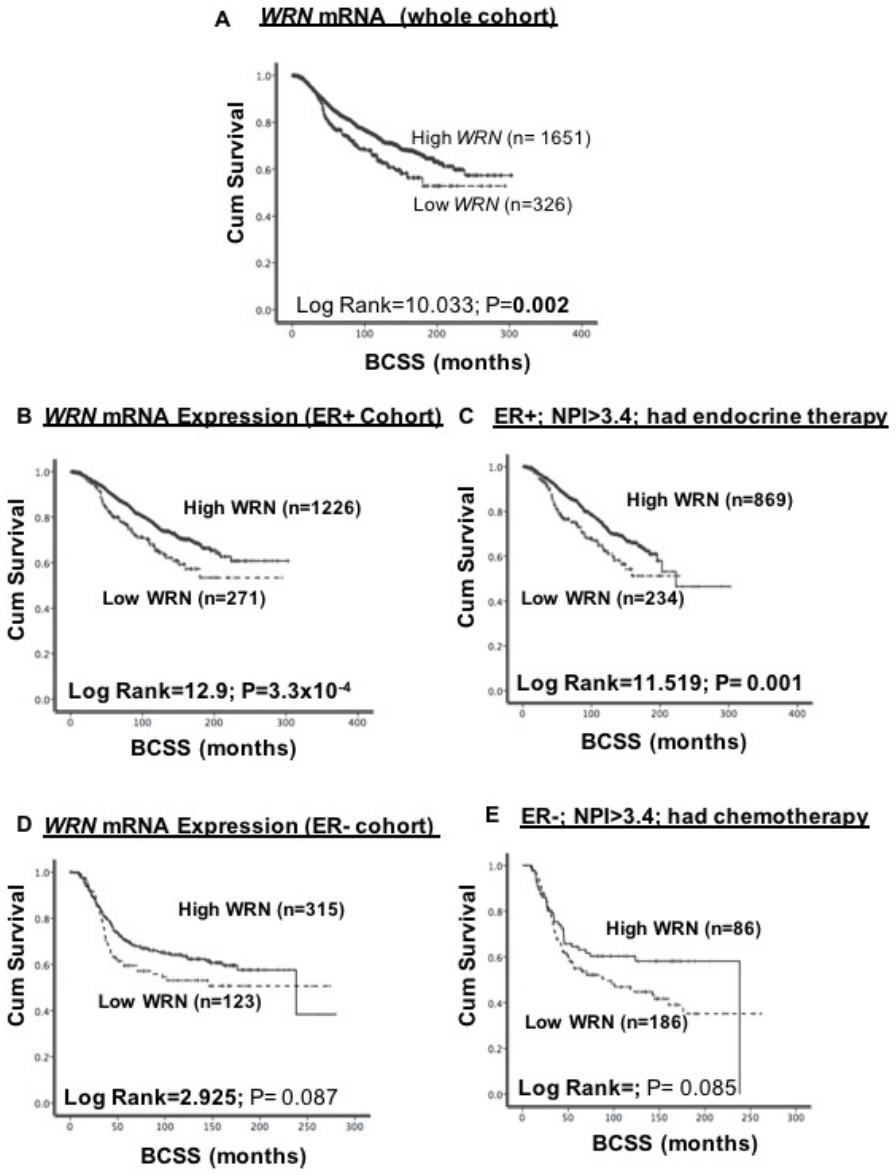


Table 6.8 Kaplan Meier curves showing BCSS (Breast cancer-specific survival) based on WRN mRNA expression

A. whole cohort; **B.** ER+ cohort; **C.** ER+ patients, NPI >3.4, who had endocrine therapy; **D.** ER- cohort and **E.** ER- patients with NPI >3.4, who received chemotherapy.

6.3.15 Tumours with high TOPO1 mRNA and low WRN mRNA have poor survival in the whole cohort

Similar to WRN-TOPO1 protein expression data, tumours with low WRN mRNA and high TOPO1 mRNA were significantly ($p<0.0001$) associated with poor breast cancer-specific survival in the whole cohort (Figure 6.12 A). Interestingly, in ER+ tumours including those who had endocrine therapy high WRN and high TOPO1 was associated with poor survival ($p<0.0001$) (Figure 6.12 B,C). In the ER- cohort, tumours with low WRN mRNA and low TOPO1 mRNA expression had poor survival outcome (p values=<0.05) (Figure 6.12 D,E).

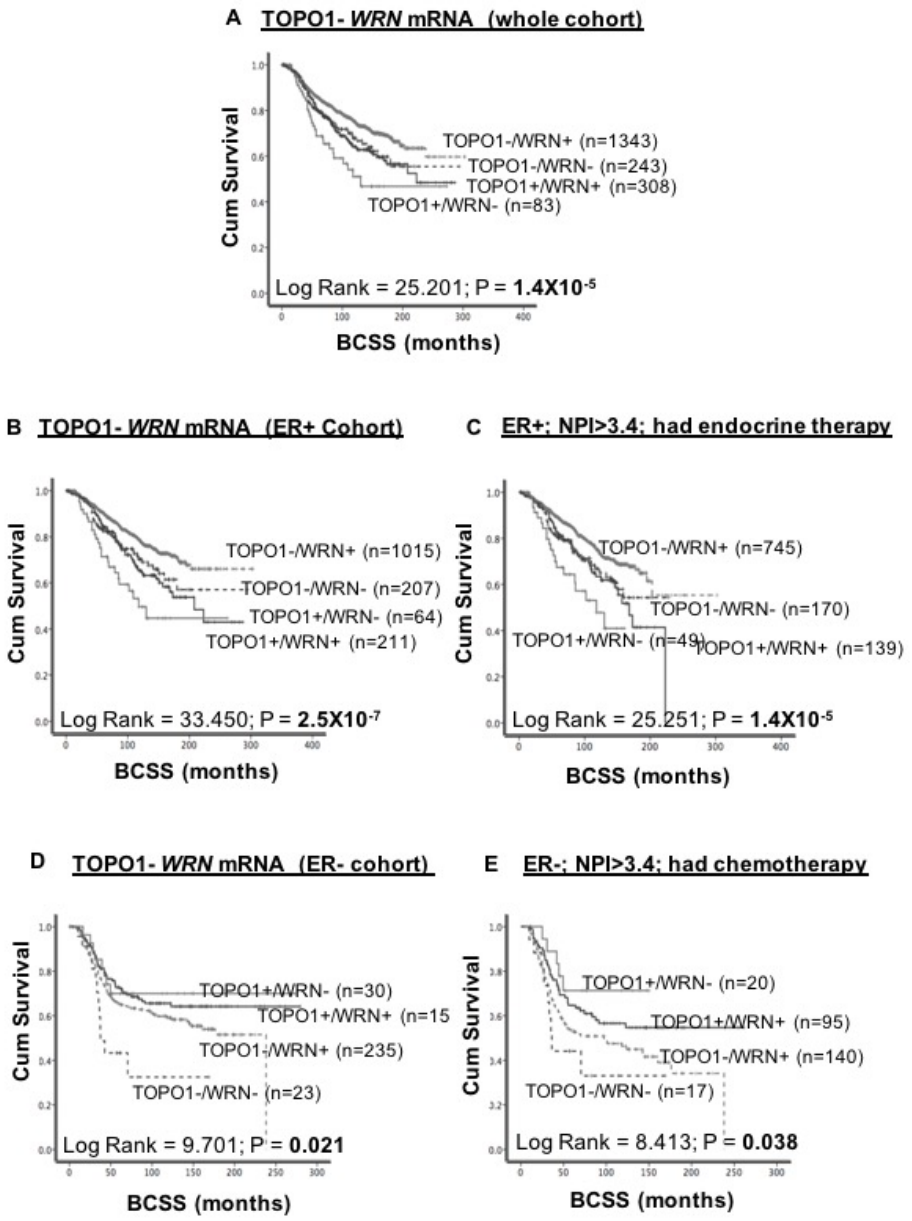


Figure 6.12 Kaplan Meier curves showing BCSS (Breast cancer-specific survival) based on TOPO1-WRN mRNA co-expression

A. whole cohort; **B.** ER+ cohort; **C.** ER+ patients with NPI >3.4, who received endocrine therapy; **D.** ER- cohort and **E.** ER- patients with NPI >3.4, who received chemotherapy.

6.3.16 WRN mRNA expression is not an independent predictor for breast cancer specific survival on multivariate analysis

In multivariate Cox regression analysis that included other validated prognostic factors, such as lymph node stage, histological grade, and tumour size, WRN mRNA expression did not come out as an independent predictor for breast cancer-specific survival (p=0.631) (Table 2).

Table 6.9 Multivariate analysis in the METABRIC cohort

	P-Value	**HR**	**95% CI for HR**	
			Lower	**Upper**
Breast Cancer Specific Survival				
WRN mRNA expression	0.631	1.054	-0.163902	0.270303
Size (>5Cm)	0.0141	2.055	0.144908	1.295992
Grade				
G1		1.0		
G2	**0.013849**	1.844	0.124659	1.099638
G3	**0.000157**	2.522	0.445475	1.405029
LN Status				
LN (1-3)	**$1.0x10^{-6}$**	1.7112	0.000001	0.000001
LN(>3)	**$1.0x10^{-6}$**	3.4951	1.016750	1.485988

6.4 Discussion

WRN is the largest family member of the human RECQ helicase protein. WRN is the only DNA RECQ helicase which 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 (Chun et al, 2011; Chun & Yee, 2010; Ding et al, 2007). Genetic epidemiological studies identified certain polymorphisms of the WRN gene which are associated with increased risk of breast cancer (Wang et al, 2014; Wang et al, 2009; Zins et al, 2015). Specifically, the CC genotype of WRN rs1346044 has been associated with the 2-fold risk of developing breast cancer (Zins et al, 2015). 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 (Wang et al, 2014). 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 (Wang et al, 2009).

In this chapter, we have shown that, at the transcriptomic level, low WRN mRNA expression was associated with aggressive clinicopathological features (high grade, lymph node stage and Her-2 overexpression) and distinct aggressive molecular phenotypes (as described by (Curtis et al) including PAM50.Her2,

PAM50.LumB, Genufu subtype (ER+/Her2-/High proliferation) and Genufu subtype (Her2 positive) breast tumours. Low WRN mRNA level was also associated with poor breast cancer-specific survival. Our results are consistent with recently published study by Zhu et al. In this study high expression of WRN mRNA was associated with improved overall and relapse free survival in breast cancer patients (Zhu et al, 2018). At the protein level, we noticed complex staining patterns with tumours showing negative, nuclear and/or cytoplasmic WRN staining. Similar to the WRN mRNA expression data, low cytoplasmic and low nuclear WRN protein levels were correlated to poor survival in patients. However, low WRN protein expression was not significantly linked to clinical and pathological variables. 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 (Agrelo et al, 2006). However, authors did not describe any clinicopathological associations in this study. In our study, 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 observations indicate that low WRN expression in human tumours may lead to a 'mutator phenotype' expressing 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 (Han et al, 2002; Lu et al, 2014; Rodier et al, 2009). In colorectal tumours, hypermethylation of WRN promoter CpG island was correlated with good response and better overall survival after treatment with irinotecan (Agrelo et al, 2006). Specifically, WRN knockdown and Camptothecin treatment both induce DNA damage and cause increased p21 expression and SA-β-gal activity in colon cancer (Han et al, 2002). 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 (Shamanna et al, 2016).

In view of the interaction between WRN and TOPO1, we carried out combined WRN and TOPO1 analysis and observed that tumours with high TOPO1/high WRN expression have poor survival in the ER-positive cohort. 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 (Aggarwal et al, 2011b). Our data suggest that this subgroup of ER+ patient with high TOPO1 and WRN expression could be targeted with combination TOPO1 inhibitor therapy and small molecules targeting WRN protein.

Taken together, this study has shown that WRN expression has prognostic significance as tumours with low expression have poor survival. We have also shown the predictive significance of high WRN/TOPO1 co-expression in ER+ tumours as these patients might not get benefit from endocrine therapy so an alternative approach such as TOPO1 inhibitors might be better. This should be further explored in prospective clinical trial of TOPO1 inhibitor therapy in ER-positive breast cancers.

Chapter 7

BLM helicase expression in sporadic breast tumours

7 BLM helicase expression in sporadic breast cancers

7.1 Introduction

Bloom's syndrome helicase (BLM) is an important member of the RECQ family of DNA helicases which plays a crucial part in the maintenance of genomic stability. BLM is an ATP-dependent 3'-5' DNA helicase involved in various key biological functions. .To perform its vital biological functions, BLM interacts with many other DNA repair proteins including topoisomerase III, hRMI1, hRMI1, and Rad51. BLM is also a part of the BRCA1-associated genome surveillance complex (BASC). BASC contains BRCA1, MSH2, MSH6, MLH1, ATM, PMS2, the RAD50-MRE11-NBS1 protein complex and BLM (Brosh Jr, 2013; Chu & Hickson, 2009; Croteau et al, 2014a; Dautin & Bernstein, 2007; Payne & Hickson, 2009).

BLM helps in unwinding a variety of DNA substrates that can occur during the process of DNA replication and repair. BLM also play an important part in the initiation and regulation of homologous recombination (HR) repair of DSB (double-strand breaks). Furthermore, during the terminal stages of HR, BLM is required for dissolution of Holliday junction. Besides, its DNA repair function, BLM is involved in telomere maintenance in cells and processing of stalled replication forks during replication (Brosh Jr, 2013; Chu & Hickson, 2009; Croteau et al, 2014a; Dautin & Bernstein, 2007; Payne & Hickson, 2009). During HR, BLM performs both pro- and anti- recombogenic roles. Anti-recombogenic role of BLM is important for normal retention of RAD51 at

double strand DNA break sites and regulation of homologous recombination (Patel et al, 2017).

Germ-line mutation in the BLM gene leads to a rare disorder called Bloom's syndrome (BS) which is characterised by cancer predisposition, growth retardation, immunodeficiency, photo hypersensitivity and impaired fertility (Cheok et al, 2005). BLM germ-line mutation results in substantial reduction in BLM mRNA levels and BLM protein expression leading to huge chromosomal instability expressed classically as increased frequency of sister chromatid exchanges (SCEs) in BS cells (Brosh Jr, 2013; Chu & Hickson, 2009; Croteau et al, 2014a; Dautin & Bernstein, 2007; Payne & Hickson, 2009). BS patients are also prone to develop cancers such as leukaemia, lymphomas and a variety of epithelial cancers including breast cancers (Cheok et al, 2005). Interestingly, BLM gene polymorphism has been linked to a greater risk of developing sporadic breast tumours (Sassi et al, 2013). In preclinical studies, depletion of BLM expression by shRNA lead to decreased proliferation in cells (Mao et al, 2010b) and also increased sensitivity to chemotherapeutic drugs such as camptothecins, cisplatin, 5-fluorouracil and hydroxyurea (Brosh Jr, 2013; Chu & Hickson, 2009; Croteau et al, 2014a; Dautin & Bernstein, 2007; Payne & Hickson, 2009). BLM seems to be an interesting drug target, hence small molecular inhibitors of BLM are being developed (Banerjee et al, 2013; Rosenthal et al, 2010).

The predictive and prognostic significance of BLM helicase in sporadic breast cancer is unknown, so we hypothesised that BLM may be de-regulated in

sporadic breast cancers and have clinicopathological significance in breast cancer patients. In this chapter, we comprehensively studied the BLM protein and mRNA expression in a large cohort of sporadic breast tumours.

7.2 Methods

7.2.1 Patient selection for Protein Data

Comprehensive evaluation of the protein expression of BLM in breast cancer was performed in two breast cancer patient cohorts. The first cohort consisted of 1650 primary invasive breast carcinoma patients who were diagnosed between 1986 and 1999 and entered into the Nottingham tenovus series. The second cohort was an independent series of 252 ER-negative invasive breast cancers diagnosed and treated at Nottingham University Hospitals between 1999 and 2007. Patient demographics of the Nottingham tenovus series (NTS) and ER-negative cohort are summarised in Table 2.4 and 2.5 respectively.

7.2.2 Tissue Microarray (TMA)

Breast tumours were arrayed in tissue microarrays (TMAs) constructed with two replicate 0.6 mm cores from the centre and periphery of the tumours. Details of TMA construction is detailed in section 2.1.1.

7.2.3 Identification of optimal BLM antibody concentration for IHC staining

Optimal concentration and conditions for staining were ascertained for BLM antibody using standard IHC technique, as described in section . We tested each dilution on the same type of sample and also the same version of TMA, to keep the experimental conditions standardized. We consulted online resource 'Human Protein Atlas' **(**www.proteinatlas.org**)** and identified that BLM protein expression is seen in various tissues including Kidney, ovary and breast tissue. We first tried optimisation in the Kidney (a full section) using citrate pH 6.0 as an antigen retrieval method and incubation time 60 min room temperature (RT). We tried 1:50;1:100;1:150 and 1:200 dilutions, but we did not see any staining (Figure 7.1 A). We then tried optimisation using 1:50 dilution in kidney and ovary, using citrate pH6.0 as antigen retrieval method and incubation time overnight at room temperature. We saw nice staining as seen in Figure 7.1 B & C. We then proceeded to optimisation on Breast TMA. The BLM antibody (Rabbit Antibody, polyclonal) was purchased from Novus Biological, catalogue number NBP1-89929. The recommended optimal dilution for IHC by the manufacturer was 1:10-1:500. Antigen retrieval method used was Citrate buffer pH 6.0 as per manufacturer recommendation. Incubation time used was overnight at room temperature. We used 1:50,1:75 and 1:100 dilutions for optimisation.

All optimisation slides were then evaluated by an expert pathologist (MA and EAR) to decide if further optimisation was required or to identify the optimal

concentration for staining the breast tumours. After careful review by an expert pathologist, we selected 1:100 as optimal dilution because it gave the best differential pattern of staining with minimum background / non-specific binding as shown in Figure 7.2.

Both positive and negative controls were included to ensure the quality of IHC staining. A negative control was included by omitting primary antibody and exposing to antibody diluent only. This was to determine if the secondary antibody was binding non-specifically resulting in false positives. It will be ideal to use absorption control to test if the primary antibody binds specifically to the antigen of interest. Beta 2 microglobulin (B2M) is known to be highly expressed in breast cancer (Li et al., 2012), hence this was used as positive control. The same positive control section was included in each run to ensure consistency to be evaluated.

Table 7.1 Primary antibody details

Antibody	**Clonality**	**Source**	**Catalogue No.**	**Optimal Dilution/ Incubation time**
BLM	Rabbit Ab (Polyclonal)	Novus Biological	NBP1-89929	1:100 Overnight

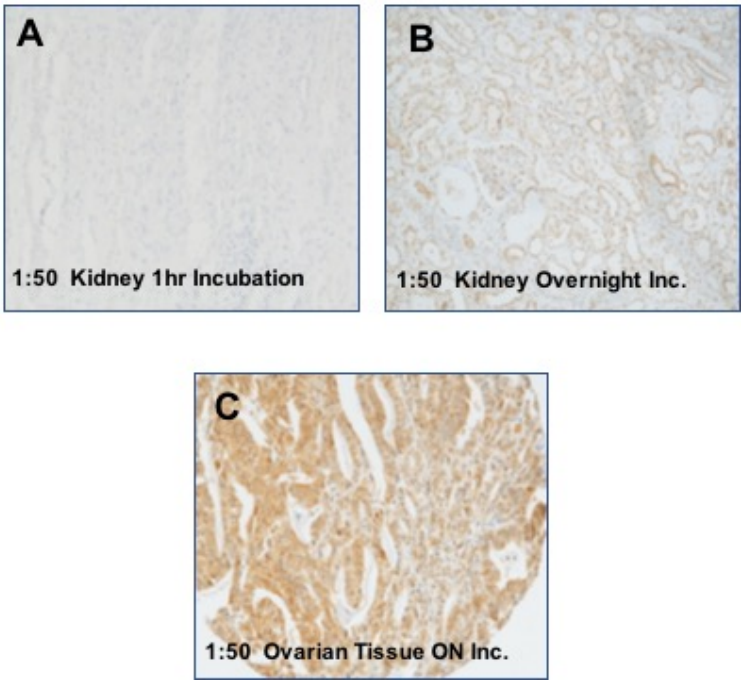


Figure 7.1 Photomicrographs of BLM protein expression in Kidney and ovarian tissue with different optimisation dilutions

A. 1:50 dilution, Kidney full face, 1 Hr Incubation time **B. 1**:50 dilution, Kidney full face, overnight Incubation **C.** 1:50 dilution, ovarian tissue , overnight Incubation

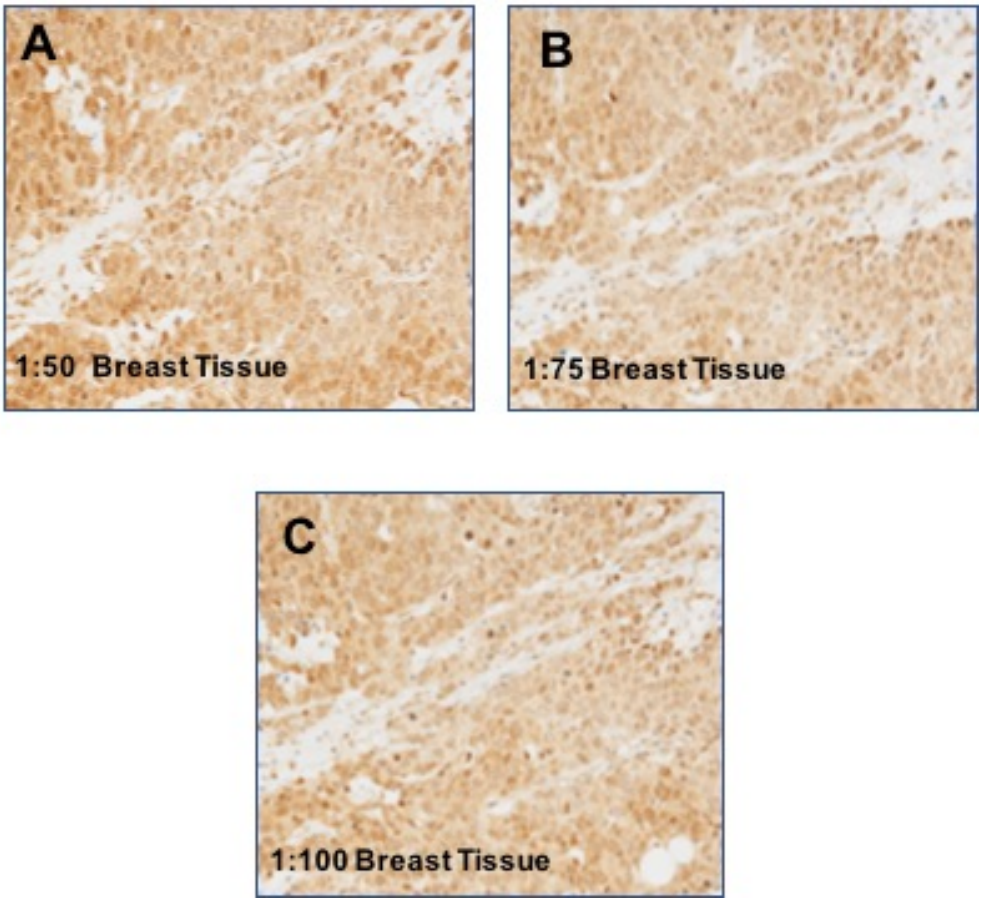


Figure 7.2 Photomicrographs of BLM protein expression in breast TMA with different optimisation dilutions

A. 1:50 dilution, overnight Incubation **B.** 1:75 dilution, overnight Incubation **C.** 1:100 dilution, overnight Incubation

7.2.4 Western Blot analysis

Western blot analysis, as described in section 2.3.2.2, was used to evaluate the specificity of BLM antibody, before using them for IHC. BLM protein expression was assessed in four breast cancer cell lines MCF7, MDA-MB-231, MDA-MB-436, and MDA-MB-468. The characteristics of these cell lines are explained in detail in Table. The details of cell lysate preparation and sample preparation are also detailed in section 2.3.2.2.1.

Table 7.2 The Primary antibody and its optimal concentration used for western blot

Antibody	**Clonality**	**Source**	**Catalogue No.**	**Optimal Dilution/ Incubation time**
BLM	Rabbit Ab (Polyclonal)	Novus Biological	NBP1-89929	1:1500 (Overnight at 4^0C)

7.2.5 IHC technique

Details of the IHC technique used is described in detail in section.

7.2.6 Histopathological scoring of stained Breast TMAs

Scoring of all Breast TMAs was performed by me and an expert pathologist blinded to the clinicopathological characteristics of patients. H scoring was used to score breast tumours as described in detail in section 2.1.9. 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 BLM into low and high expression. A median H score of ≥ 50 was taken as the cut-off for high BLM nuclear and cytoplasm expression. A median H-Score of <50 was categorised as low BLM nuclear and cytoplasmic expression.

7.2.7 Quantitative real-time PCR

Total RNA was extracted from MCF-7, MDA-MB-231, MDA-MB-468 and MDA-MB-436 cells using RNeasy Mini kit (QIAGEN, UK). The RNA quantification was done using a NanoDrop 2000c Spectrophotometer (Thermo Scientific). The cDNA was synthesised from 0.5 µg of total RNA using RT^2 first strand kit (QIAGEN). The real-time qPCR was performed using SYBR Green PCR Master mix (Applied Biosystems) with a primer set (BLM QuantiTect Primer Assay, catalogue no. QT00027671, QIAGEN) targeting the BLM gene. The GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as a housekeeper for internal control (GAPDH QuantiTect Primer Assay, catalogue no. QT00079247, QIAGEN). The real-time PCR for each RNA sample was done in triplicate. NTC (No Template Control) was used to assess for cross-contamination of reagents and surfaces. To make NTC, we included all the RT-PCR reagents except the RNA template. Minus reverse transcriptase (- RT) control was used to do an assessment for genomic DNA contamination. A melting curve analysis was included to get information about the purity of the amplified product. A single peak was seen which indicated the presence of only one double stranded product.

7.2.8 Methodology and patient selection for BLM mRNA expression in Breast Cancer

BLM mRNA expression was investigated in the METABRIC (Molecular Taxonomy of Breast Cancer International Consortium) cohort. The METABRIC study protocol and the molecular profiling methodology in a cohort of 1977 breast cancer samples are detailed by Curtis et al (Curtis et al, 2012b). Patient demographics, details of data acquisition and analysis are summarised in Table 2.7.

BLM probe (id: ILM_1709484) was used because it was a perfect match and quality for its target, having a GC content of 58%, 0 SNPs and it does not possess a poly G tail at the end. The cut off for the high vs low BLM gene expression was done using x-tile software (Version 3.6.1) so that the resultant subgroups had significant difference in survival outcomes.

7.2.9 Methodology for cell line work

The methodology for BLM knock down cell line study is discussed in section 2.3. The method used for cell survival assays and functional cytotoxicity study are detailed in section 2.3.2.3 and 2.3.2.4.

7.2.10 Statistical analysis

Data analysis was performed using SPSS (SPSS, version 22 Chicago, IL) as described in detail in section 2.1.9.

7.2.11 Ethical approval

Ethical approval was obtained from the Nottingham Research Ethics Committee (Reference number C202313, Approval date : 21/10/2010). Tumour Marker Prognostics Studies (REMARK) criteria, recommended by McShane et al (McShane et al, 2005), were followed throughout this project.

7.3 Results

7.3.1 Western blot analysis confirms the specificity of BLM antibody

A panel of breast cancer cell lines (MCF-7, MDA-MB231, MDA-MB-436, and MDA-MB-468) were profiled and they showed robust expression of BLM protein relative to β-Actin. MDA-MBA-231 has a maximum expression of BLM protein. In contrast, MCF-7 has low BLM expression (Figure 7.3B).

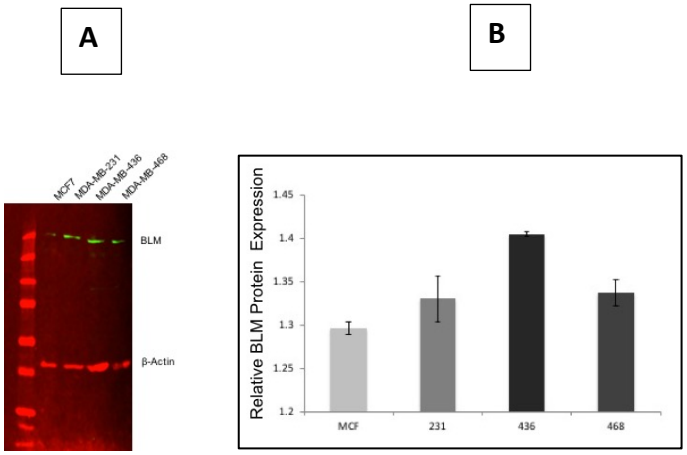


Figure 7.3 BLM protein expression in breast cancer cell lines

A. Western blot showing a single band at 159kDa **B.** Graph shows WRN protein expression in MCF-7, MDA-MB-231, MDA-MB-436 and MDA-MB-468 breast cancer cell lines relative to β-Actin. Data presented as mean ± SEM of two independent experiments.

7.3.2 Low nuclear BLM protein expression is associated with aggressive breast cancer

Out of 1253 tumours analysed, low nuclear BLM protein level was seen in 682 tumours (54%) and high nuclear BLM protein level was observed in 571 (46%) tumours . As shown in the Table 7.3, low nuclear BLM protein level was significantly associated with larger tumours, high tumour grade, higher mitotic index, pleomorphism and tumour type ($p<0.05$). ER-, PR-, AR-, triple negative and basal-like phenotypes were also more likely in tumours with low nuclear BLM protein level ($p<0.01$).

7.3.3 Low nuclear BLM protein expression is significantly associated with other DNA repair proteins.

BRCA1 negative, low XRCC1, low FEN1, low APE1, low Polβ, low CDK18 and low DNA-PKcs were significantly associated with tumours that have low nuclear BLM protein level. In addition, high p16, low p21, high MIB1, p53 mutants, low Bcl-2, low Top2A, low nuclear pCHK1, low RECQL5 and low nuclear Chk2 were more common in tumours with low nuclear BLM protein level ($p<0.05$) (Table 7.3).

Table 7.3 BLM nuclear protein expression and breast cancer

VARIABLE	BLM (Nuclear) Protein Expression		Unadjusted P- values	*Adjusted P -Values
	Low N (%)	High N (%)		
A) Pathological Parameters				
Tumour Size				
<1cm	50 (7.3)	67 (11.7)	**0.012**	**0.018**
>1-2cm	345 (50.6)	283 (49.6)		
>2-5cm	265 (38.9)	213 (37.3)		
>5cm	22 (3.2)	8 (1.4)		
Tumour Stage				
1	422 (61.8)	356 (62.2)	0.874	36.70
2	198 (29.0)	168 (29.4)		
3	63 (9.2)	48 (8.4)		
Tumour Grade				
G1	98 (14.4)	111 (19.4)	**$7.0X10^{-6}$**	**$1.0X10^{-5}$**
G2	189 (27.7)	208 (36.4)		
G3	395 (57.9)	252 (44.1)		
Mitotic Index				
M1 (low; mitoses < 10)	184 (27.2)	246 (43.2)	**$1.0X10^{-6}$**	**$1.0X10^{-5}$**
M2 (medium; mitoses 10-18)	129 (19.1)	94 (16.5)		
M3 (high; mitosis >18)	364 (53.8)	229 (40.2)		
Tubule Formation				
1 (>75% of definite tubule)	34 (5.0)	34 (6.0)	0.348	0.365
2 (10%-75% definite tubule)	219 (32.3)	201 (35.3)		
3 (<10% definite tubule)	424 (62.6)	334 (58.7)		
Pleomorphism				
1 (small-regular uniform)	14 (2.1)	12 (1.5)	**0.020**	**0.029**
2 (Moderate variation)	231 (34.2)	236 (41.5)		
3 (Marked variation)	431 (63.8)	318 (56.0)		
Tumour Type				
IDC-NST	374 (62.3)	275 (56.2)	**$1.2X10^{-4}$**	**$4.0X10^{-4}$**
Tubular Carcinoma	114 (19.0)	105 (21.5)		
Medullary Carcinoma	24 (4.0)	3 (0.6)		
ILC	45 (7.5)	48 (9.8)		
Others	43 (7.2)	58 (11.9)		

Lymphovascular Invasion No Yes	454 (67.6) 218 (32.4)	362 (64.0) 204 (36.0)	0.183	0.207
B) Aggressive phenotype				
Her2 overexpression No Yes	603 (89.9) 68 (10.1)	487 (87.7) 68 (12.3)	0.240	0.265
Triple Negative Phenotype No Yes	492 (74.0) 173 (26.0)	495 (88.9) 62 (11.1)	**$1.0X10^{-6}$**	**$1.0X10^{-5}$**
Basal Like Phenotype No Yes	527 (82.7) 110 (17.3)	503 (92.0) 44 (8.0)	**$3.0X10^{-6}$**	**$1.0X10^{-5}$**
Cytokeratin 6 (CK6) Negative Positive	475 (80.4) 116 (19.6)	416 (88.1) 56 (11.9)	**0.001**	**0.002**
Cytokeratin 14 (CK14) Negative Positive	499 (85.3) 86 (14.7)	419 (89.7) 48 (10.3)	**0.033**	**0.042**
Cytokeratin 18 (CK18) Negative Positive	84 (15.4) 460 (84.6)	19 (4.4) 411 (95.6)	**$1.0X10^{-6}$**	**$1.0X10^{-5}$**
Cytokeratin 19 (CK19) Negative Positive	46 (7.8) 545 (92.2)	20 (4.3) 446 (95.7)	**0.020**	**0.028**
C) Hormone receptors				
ER Negative Positive	222 (33.1) 449 (66.9)	108 (19.0) 459 (81.0)	**$1.0X10^{-6}$**	**$1.0X10^{-5}$**
PR Negative Positive	307 (47.8) 335 (52.2)	202 (38.5) 323 (61.5)	**0.001**	**0.002**

AR Negative Positive	248 (44.5) 309 (55.5)	123 (28.0) 316 (72.0)	**$1.0X10^{-6}$**	**$1.0X10^{-5}$**
D) DNA Repair				
BRCA1 Absent Normal	111 (22.4) 385 (77.6)	61 (15.7) 327 (84.3)	**0.013**	**0.019**
XRCC1 Low High	103 (20.5) 400 (79.5)	50 (12.2) 361 (87.8)	**0.001**	**0.002**
FEN1 Low High	361 (79.0) 96 (21.0)	269 (67.4) 130 (32.6)	**$1.0X10^{-5}$**	**$3.0X10^{-4}$**
SMUG1 Low High	177 (40.9) 256 (59.1)	128 (34.6) 242 (65.4)	0.067	0.082
APE1 Low High	340 (58.7) 239 (41.3)	192 (39.1) 299 (60.9)	**$1.0X10^{-6}$**	**$1.0X10^{-5}$**
PolB Low High	277 (46.3) 321 (53.7)	147 (28.7) 366 (71.3)	**$1.0X10^{-6}$**	**$1.0X10^{-5}$**
ATR Low High	35 (6.1) 538 (93.9)	18 (3.9) 449 (96.1)	**$1.8X10^{-4}$**	**$5.0X10^{-4}$**
ATM Low High	223 (52.0) 206 (48.0)	179 (53.8) 154 (46.2)	0.627	0.642
DNA-PK Low High	250 (43.6) 324 (56.4)	126 (25.8) 363 (74.2)	**$1.0X10^{-6}$**	**$1.0X10^{-5}$**
E) Cell cycle/apoptosis regulators				

P16 Low High	396 (80.8) 94 (19.2)	347 (93.8) 23 (6.2)	**$1.0X10^{-6}$**	**$1.0X10^{-5}$**
P21 Low High	316 (60.5) 206 (39.5)	202 (53.2) 178 (46.8)	**0.027**	**0.036**
MIB1 Low High	227 (41.0) 326 (59.0)	244 (50.2) 242 (49.8)	**0.003**	**0.006**
P53 Low expression High expression	420 (75.0) 140 (25.0)	381 (82.6) 80 (17.4)	**0.003**	**0.005**
Bcl-2 Negative Positive	246 (40.2) 366 (59.8)	155 (30.6) 351 (69.4)	**0.001**	**0.002**
TOP2A Low Overexpression	239 (49.5) 244 (50.5)	162 (40.7) 236 (59.3)	**0.009**	**0.015**
pCHK1 (Nuclear) Low High	616 (90.2) 67 (9.8)	415 (72.3) 159 (27.7)	**$1.0X10^{-6}$**	**$1.0X10^{-5}$**
pCHK1 (Cytoplasmic) Low High	191 (28.0) 492 (72.0)	124 (21.6) 450 (78.4)	**0.010**	**0.016**
Non-phospho CHK1 (Cyto.) Low High	284 (52.0) 262 (48.0)	205 (45.2) 249 (54.8)	**0.031**	**0.041**
CHK2 Low High	258 (50.5) 253 (49.5)	164 (41.0) 236 (59.0)	**0.004**	**0.007**
Bax Low High	272 (68.9) 123 (31.1)	235 (72.1) 91 (27.9)	0.345	0.371
CDK1 Low High	303 (67.2) 148 (32.8)	247 (72.0) 96 (28.0)	0.144	0.172

CDK18 (Cytoplasmic) Low High	 426 (78.5) 117 (21.5)	 318 (70.7) 132 (29.3)	**0.005**	**0.008**
RECQL5 Low High	 295 (56.0) 232 (44.0)	 167 (36.9) 285 (63.1)	**$1.0X10^{-6}$**	**$1.0X10^{-5}$**
MDM2 Low Overexpression	 386 (77.4) 113 (22.6)	 272 (73.3) 99 (26.7)	0.170	0.198

Bold = Statistically significant; BRCA1: Breast cancer 1, early onset; HER2: human epidermal growth factor receptor 2; ER: oestrogen receptor; PR: progesterone receptor; CK: cytokeratin; Basal-like: ER-, HER2 and positive expression of either CK5/6, CK14 or EGFR; Triple negative: ER-/PR-/HER2- . Adjusted p values were calculated using Benjamini-Hochberg false discovery rate method to adjust for multiple testing.

7.3.4 High cytoplasmic BLM protein expression is associated with high levels of DNA repair proteins.

1212 tumour cores were suitable for analysis. High cytoplasmic BLM level was seen in 642 tumours (53%) and low cytoplasmic BLM level was seen in 570 tumours (47%) (Figure 2). As shown in Table, high cytoplasmic BLM protein expression was significantly linked to pleomorphism (p=0.012), tumour type (p=0.034), high XRCC1 (p=0.039), high FEN1(p=0.031), high APE1 (p<0.001), high ATR (p=0.003), high MIB1 (p=0.021), high Chk1 and Chk2 (p values<0.01), high Bax levels (p=0.018), high CDK18 (P<0.001) and high RECQL5 (p<0.001) [Table7.4].

Table 7.4 BLM cytoplasmic protein expression and breast cancer

VARIABLE	BLM (Cytoplasmic) Protein Expression		Unadjusted P-Values	*Adjusted P-Values
	Low N (%)	High N (%)		
A) Pathological Parameters				
Tumour Size				
<1cm	59 (6.9)	69 (8.5)	0.204	0.343
>1-2cm	274 (48.1)	325 (51.8)		
>2-5cm	219 (38.4)	235 (37.9)		
>5cm	18 (3.2)	13 (1.8)		
Tumour Stage				
1	360 (63.0)	418 (61.1)	0.545	0.789
2	158 (27.7)	181 (30.4)		
3	53 (9.3)	62 (8.5)		
Tumour Grade				
G1	105 (18.4)	104 (15.2)	0.137	0.274
G2	187 (32.8)	210 (30.7)		
G3	278 (48.8)	369 (54.0)		
Mitotic Index				
M1 (low; mitoses < 10)	210 (31.3)	220 (36.5)	0.108	0.238
M2 (medium; mitoses 10-18)	104 (17.6)	119 (19.4)		
M3 (high; mitosis >18)	251 (51.5)	342 (44.0)		
Tubule Formation				
1 (>75% of definite tubule)	31 (5.5)	37 (5.4)	0.998	41.91
2 (10%-75% definite tubule)	190 (33.6)	230 (33.8)		
3 (<10% definite tubule)	344 (60.9)	414 (60.8)		
Pleomorphism				
1 (small-regular uniform)	18 (3.2)	10 (1.5)	**0.002**	**0.012**
2 (Moderate variation)	234 (41.4)	233 (34.3)		
3 (Marked variation)	313 (55.4)	436 (64.2)		
Tumour Type				
IDC-NST	275 (56.8)	374 (61.8)	**0.009**	**0.034**
Tubular Carcinoma	94 (19.4)	125 (20.7)		
Medullary Carcinoma	12 (2.5)	15 (2.5)		
ILC	58 (12.0)	35 (5.8)		
Others	45 (9.3)	56 (9.3)		

Lymphovascular Invasion No Yes	 363 (65.1) 195 (34.9)	 453 (66.6) 227 (33.4)	0.564	0.789
B) Aggressive phenotype				
Her2 overexpression No Yes	 495 (88.9) 62 (11.1)	 595 (88.9) 74 (11.1)	0.969	1.02
Triple Negative Phenotype No Yes	 454 (80.9) 107 (19.1)	 533 (80.6) 128 (19.4)	0.897	0.99
Basal Like Phenotype No Yes	 476 (87.8) 66 (12.2)	 554 (86.3) 88 (13.7)	0.436	0.704
Cytokeratin 6 (CK6) Negative Positive	 391 (83.4) 78 (16.6)	 500 (84.2) 94 (15.8)	0.723	0.893
Cytokeratin 14 (CK14) Negative Positive	 396 (85.7) 66 (14.3)	 522 (88.5) 68 (11.5)	0.183	0.334
Cytokeratin 18 (CK18) Negative Positive	 55 (12.5) 385 (87.5)	 48 (9.0) 486 (91.0)	0.076	0.187
Cytokeratin 19 (CK19) Negative Positive	 35 (7.4) 436 (92.6)	 31 (5.3) 555 (94.7)	0.153	0.292
C) Hormone receptors				
ER Negative Positive	 150 (26.5) 415 (73.5)	 180 (26.7) 493 (73.3)	0.938	1.01
PR Negative Positive	 238 (44.2) 300 (55.8)	 271 (43.1) 358 (56.9)	0.692	0.880

AR Negative Positive	170 (38.3) 274 (61.7)	201 (36.4) 351 (63.6)	0.543	0.884
D) DNA Repair				
BRCA1 Absent Normal	79 (20.2) 312 (79.8)	93 (18.9) 400 (81.1)	0.617	0.835
XRCC1 Low High	84 (20.1) 333 (79.9)	69 (13.9) 428 (86.1)	**0.012**	**0.039**
FEN1 Low High	309 (77.8) 88 (22.2)	321 (69.9) 138 (30.1)	**0.009**	**0.031**
SMUG1 Low High	155 (41.4) 219 (58.6)	150 (35.0) 279 (65.0)	0.059	0.154
APE1 Low High	278 (57.3) 207 (42.7)	254 (43.4) 331 (56.6)	**$6.0X10^{-6}$**	**$1.0X10^{-5}$**
PolB Low High	203 (40.2) 302 (59.8)	221 (36.5) 385 (63.5)	0.203	0.355
ATR Low High	382 (73.3) 139 (26.7)	315 (61.6) 204 (38.4)	**$2.4X10^{-5}$**	**0.0003**
ATM Low High	182 (53.4) 159 (46.6)	220 (52.3) 201 (47.7)	0.759	0.884
DNA-PK Low High	184 (39.0) 288 (61.0)	192 (32.5) 399 (67.5)	**0.028**	0.084
E) Cell cycle/apoptosis regulators				

P16 Low High	338 (86.4) 53 (13.6)	405 (86.4) 64 (13.6)	0.969	0.992
P21 Low High	235 (57.5) 174 (42.5)	283 (57.4) 210 (42.6)	0.76	0.86
MIB1 Low High	238 (50.1) 237 (49.9)	233 (41.3) 331 (58.7)	**0.005**	**0.021**
P53 Low expression High expression	370 (81.0) 87 (19.0)	431 (76.4) 133 (23.6)	0.079	0.184
Bcl-2 Negative Positive	175 (35.1) 324 (64.9)	226 (36.5) 393 (63.5)	0.618	0.811
TOP2A Low Overexpression	193 (49.6) 196 (50.4)	208 (42.3) 284 (57.7)	**0.030**	0.084
pCHK1 (Nuclear) Low High	458 (80.2) 113 (19.8)	573 (83.5) 113 (16.5)	0.127	0.266
pCHK1 (Cytoplasmic) Low High	187 (32.7) 384 (67.3)	128 (18.7) 558 (81.3)	**$1.0X10^{-6}$**	**$1.0X10^{-5}$**
Non-phospho CHK1 (Cyto.) Low High	243 (54.1) 206 (45.9)	246 (44.6) 305 (55.4)	**0.003**	**0.015**
CHK2 Low High	217 (54.1) 184 (45.9)	205 (40.2) 305 (59.8)	**$2.9X10^{-5}$**	**$2.0X10^{-4}$**
Bax Low High	237 (76.0) 75 (24.0)	270 (66.0) 139 (34.0)	**0.004**	**0.018**
CDK1 Low High	240 (70.6) 100 (29.4)	310 (68.3) 144 (31.7)	0.486	0.756

CDK18 (Cytoplasmic) Low High	 367 (81.7) 82 (18.3)	 377 (69.3) 167 (30.7)	**$7.0X10^{-6}$**	**$1.0X10^{-4}$**
RECQL5 Low High	 235 (53.5) 204 (46.5)	 227 (42.0) 313 (58.0)	**$3.4X10^{-4}$**	**$2.4X10^{-3}$**
MDM2 Low Overexpression	 296 (75.1) 98 (24.9)	 362 (76.1) 114 (23.9)	0.752	0.902

Bold = Statistically significant; BRCA1: Breast cancer 1, early onset; HER2: human epidermal growth factor receptor 2; ER: oestrogen receptor; PR: progesterone receptor; CK: cytokeratin; Basal-like: ER-, HER2 and positive expression of either CK5/6, CK14 or EGFR; Triple negative: ER-/PR-/HER2- . Adjusted p values were calculated using Benjamini-Hochberg false discovery rate method to adjust for multiple testing.

7.3.5 High cytoplasmic and low nuclear BLM levels are associated with aggressive breast cancer

Nuclear and cytoplasmic co-expression of BLM in breast cancer: 28% (333/1253) were low nuclear/high cytoplasmic, 26.5% (332/1253) were low nuclear/low cytoplasmic, 26.5% (333/1253) were high nuclear/high cytoplasmic and 19% (238/1253) were high nuclear/low cytoplasmic. Tumours with high cytoplasmic/low nuclear BLM levels were more likely to be high grade, high mitotic index, pleomorphism, IDC-NST tumour type, PR-, triple negative and basal-like phenotype tumours ($p<0.0001$). High p16, low p21, high MIB1, p53 mutants and high Bax levels more common in tumours with high cytoplasmic/low nuclear BLM levels. Interestingly, low cytoplasmic/low nuclear BLM tumours were more likely to manifest low DNA repair levels

including BRCA1 negative, low XRCC1, low FEN1, low SMUG1, low APE1, low polβ, low ATR, and low DNA-PKcs expression [Table 7.5].

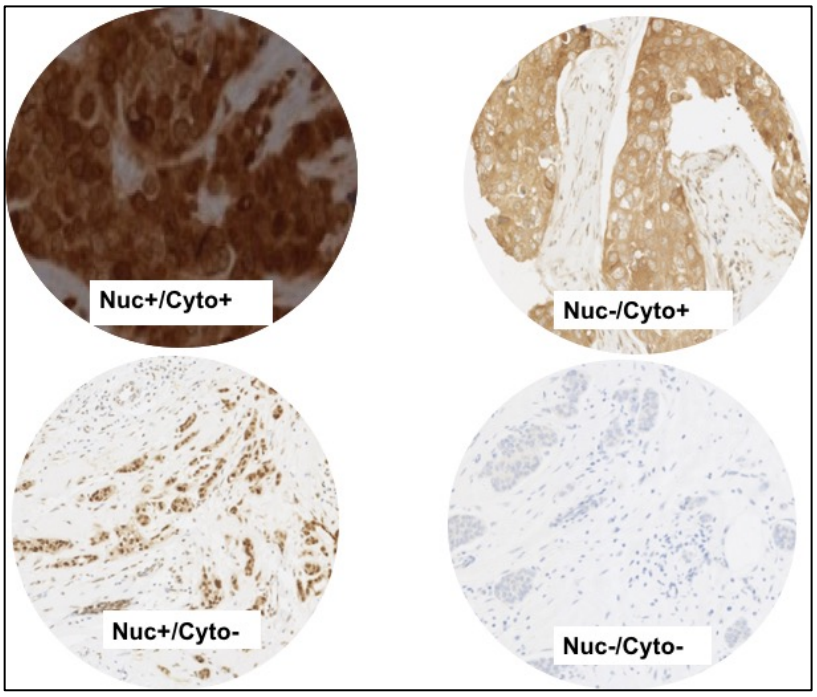


Figure 7.4 Micro photomicrographs of BLM protein expression in breast cancers

Nu-/Cyto- (Nuclear negative & cytoplasmic negative); Nu+/Cyto+ (Nuclear negative & cytoplasmic positive); Nu+/Cyto- (Nuclear positive & cytoplasmic negative); Nu-/Cyto+ (Nuclear negative & cytoplasmic positive)

Table 7.5 . BLM (nuclear and cytoplasmic protein co-expression) in breast cancer (n=1253)

VARIABLE	BLM (Nuclear) Protein Expression				*P -Value	
	Nuc-/Cyto- N (%)	Nuc+ /Cyto- N (%)	Nuc-/Cyto+ N (%)	Nuc+/ Cyto+ N (%)	Unadjusted	Adjusted
A) Pathological Parameters						
Tumour Size						
<1cm	29 (8.7)	30 (12.6)	21 (6.0)	37 (11.1)	0.065	0.083
>1-2cm	163 (49.1)	111 (46.6)	182 (52.0)	172 (51.7)		
>2-5cm	127 (38.3)	92 (38.7)	138 (39.4)	121 (36.3)		
>5cm	13 (3.9)	5 (2.1)	9 (2.6)	3 (0.9)		
Tumour Stage						
1					0.946	39.73
2	207 (62.3)	153 (64.0)	215 (61.3)	203 (61.0)		
3	92 (27.7)	66 (27.6)	106 (30.2)	102 (30.6)		
	33 (9.9)	20 (8.4)	30 (8.5)	28 (8.4)		

Tumour Grade						
G1					**$3.0X10^{-6}$**	**$1.0X10^{-5}$**
G2	53 (16.0)	52 (21.8)	45 (12.9)	59 (17.7)		
G3	87 (26.2)	208 (42.0)	102 (29.1)	108 (32.4)		
	192 (57.8)	86 (36.1)	203 (58.0)	166 (49.8)		
Mitotic Index						
M1 (low; mitoses < 10)	93 (28.4)	117 (49.4)	91 (26.1)	129 (38.9)	**$1.0X10^{-6}$**	**$1.0X10^{-5}$**
M2 (medium; mitoses 10-18)	65 (19.8)	39 (16.5)	64 (18.3)	55 (16.6)		
M3 (high; mitosis >18)	170 (51.8)	81 (34.2)	194 (55.6)	148 (44.6)		
Tubule Formation						
1 (>75% definite tubule)	17 (5.2)	14 (5.9)	17 (4.9)	20 (6.0)	0.90	0.92
2 (10%-75% definite tubule)	107 (32.6)	83 (35.0)	112 (32.1)	118 (35.5)		
3 (<10% definite tubule)	204 (62.2)	140 (59.1)	220 (63.0)	194 (58.4)		
Pleomorphism						
1 (small-regular uniform)	12 (3.7)	6 (2.5)	2 (0.6)	8 (2.4)	**$1.2X10^{-5}$**	**$1.0X10^{-5}$**
2 (Moderate variation)	112 (34.1)	122 (51.5)	119 (34.2)	114 (34.4)		
3 (Marked variation)	204 (62.2)	109 (46.0)	227 (65.2)	209 (63.1)		

Tumour Type IDC-NST Tubular Carcinoma Medullary Carcinoma ILC Others	170 (59.2) 55 (19.2) 12 (4.2) 28 (9.8) 22 (7.7)	105 (53.3) 39 (19.8) 0 (0.0) 30 (15.2) 23 (11.7)	204 (65.2) 59 (18.8) 12 (3.8) 17 (5.4) 21 (6.7)	170 (58.2) 66 (22.6) 3 (1.0) 18 (6.2) 35 (12.0)	**$6.6X10^{-5}$**	**$1.0X10^{-4}$**
Lympho-vascular Invasion No Yes	219 (67.2) 107 (32.8)	144 (62.1) 88 (37.9)	235 (67.9) 111 (32.1)	218 (65.3) 116 (34.7)	0.486	0.551
<u>B) Aggressive phenotype</u>						
Her2 overexpression No Yes	290 (89.2) 35 (10.8)	205 (88.4) 27 (11.6)	313 (90.5) 33 (9.5)	282 (87.3) 41 (12.7)	0.617	0.664
Triple Negative Phenotype No Yes	244 (74.8) 82 (25.2)	210 (89.4) 25 (10.6)	248 (73.2) 91 (26.8)	285 (88.5) 37 (11.5)	**$1.0X10^{-6}$**	**$1.0X10^{-5}$**
Basal Like Phenotype No Yes	260 (83.3) 52 (16.7)	216 (93.9) 14 (6.1)	267 (82.2) 58 (17.8)	287 (90.5) 30 (9.5)	**$2.9X10^{-5}$**	**$1.0X10^{-4}$**

Cytokeratin 6 (CK6) Negative Positive	223 (79.6) 57 (20.4)	168 (88.9) 21 (11.1)	252 (81.0) 59 (19.0)	248 (87.6) 35 (12.4)	**0.007**	**0.011**
Cytokeratin 14 (CK14) Negative Positive	233 (84.1) 44 (15.9)	163 (88.1) 22 (11.9)	266 (86.4) 42 (13.6)	256 (90.8) 26 (9.2)	0.114	0.141
Cytokeratin 18 (CK18) Negative Positive	49 (18.6) 215 (81.4)	6 (3.4) 170 (96.6)	35 (12.5) 245 (87.5)	13 (5.1) 241 (94.9)	**$1.0X10^{-6}$**	**$1.0X10^{-5}$**
Cytokeratin 19 (CK19) Negative Positive	29 (10.2) 254 (89.8)	6 (3.2) 182 (96.8)	17 (5.5) 291 (94.5)	14 (5.0) 264 (95.0)	**0.008**	**0.012**
C) Hormone receptors						
ER Negative Positive	110 (33.5) 218 (66.5)	40 (16.9) 197 (83.1)	112 (32.7) 231 (67.3)	68 (20.6) 262 (79.4)	**$1.0X10^{-6}$**	**$1.0X10^{-5}$**
PR Negative Positive	151 (47.6) 166 (52.4)	87 (39.4) 134 (60.6)	156 (48.0) 169 (52.0)	115 (37.8) 189 (62.2)	**0.016**	**0.022**

AR Negative Positive	126 (47.0) 142 (53.0)	44 (25.0) 316 (75.0)	122 (42.2) 167 (57.6)	79 (30.0) 184 (70.0)	**$1.0X10^{-6}$**	**$1.0X10^{-5}$**
D) DNA Repai						
BRCA1 Absent Normal	59 (24.6) 181 (75.4)	20 (13.2) 131 (86.8)	52 (20.3) 204 (79.7)	41 (17.3) 196 (82.7)	**0.036**	**0.047**
XRCC1 Low High	61 (25.6) 177 (74.4)	23 (12.8) 156 (87.2)	27 (11.6) 205 (88.4)	153 (16.7) 761 (83.3)	**$1.7X10^{-4}$**	**$3.0X10^{-4}$**
FEN1 Low High	192 (83.8) 37 (16.2)	117 (69.6) 51 (30.4)	169 (74.1) 59 (25.9)	152 (65.8) 79 (34.2)	**$1.0X10^{-4}$**	**$2.0X10^{-4}$**
SMUG1 Low High	104 (47.1) 117 (52.9)	51 (33.3) 102 (66.7)	73 (34.4) 139 (65.6)	77 (35.5) 140 (64.5)	**0.013**	**0.018**
APE1 Low High	185 (66.8) 92 (33.2)	93 (44.7) 115 (55.3)	99 (35.0) 184 (65.0)	532 (49.7) 538 (50.3)	**$1.0X10^{-6}$**	**$1.0X10^{-5}$**

PolB Low High	147 (50.9) 142 (49.1)	56 (25.9) 160 (74.1)	130 (42.1) 179 (57.9)	91 (30.6) 206 (69.4)	**$1.0X10^{-6}$**	**$1.0X10^{-5}$**
ATR Low High	236 (75.9) 75 (24.1)	146 (69.5) 64 (30.5)	221 (67.4) 107 (32.6)	175 (55.6) 140 (44.4)	**$1.0X10^{-6}$**	**$1.0X10^{-5}$**
ATM Low High	109 (54.0) 93 (46.0)	73 (52.5) 66 (47.5)	114 (50.2) 113 (49.8)	106 (54.6) 88 (45.4)	0.806	0.846
DNA-PK Low High	126 (45.8) 149 (54.2)	58 (29.4) 139 (70.6)	124 (41.5) 175 (58.5)	68 (23.3) 224 (76.7)	**$1.0X10^{-6}$**	**$1.0X10^{-5}$**
<u>E) Cell cycle/apoptosis regulators</u>						
P16 Low High	199 (81.9) 44 (18.1)	139 (93.9) 9 (6.1)	197 (79.8) 50 (20.2)	208 (93.7) 14 (6.3)	**$1.0X10^{-6}$**	**$1.0X10^{-5}$**
P21 Low High	151 (59.4) 103 (40.6)	84 (54.2) 71 (45.8)	165 (61.6) 103 (38.4)	118 (52.4) 107 (47.6)	0.154	0.184

MIB1 Low High	121 (44.5) 151 (55.5)	117 (57.6) 86 (42.4)	106 (37.7) 175 (62.3)	127 (44.9) 156 (55.1)	**$4.2X10^{-5}$**	**$1.0X10^{-4}$**
P53 Low High	214 (78.1) 60 (21.9)	156 (85.2) 27 (14.8)	206 (72.0) 80 (28.0)	225 (80.9) 53 (19.1)	**0.005**	**0.008**
Bcl-2 Negative Positive	119 (40.3) 176 (59.7)	56 (27.5) 148 (72.5)	127 (27.5) 148 (72.5)	99 (32.8) 203 (67.2)	**0.006**	**0.009**
TOP2A Low High	129 (56.6) 99 (43.4)	64 (39.8) 97 (60.2)	110 (43.1) 145 (56.9)	98 (41.4) 139 (58.6)	**0.001**	**0.002**
pCHK1 (Nuclear) Low High	298 (90.0) 33 (10.0)	160 (66.7) 80 (33.3)	318 (90.3) 34 (9.7)	255 (76.3) 79 (23.7)	**$1.0X10^{-6}$**	**$1.0X10^{-5}$**
pCHK1 (Cyto) Low High	123 (37.2) 208 (62.8)	64 (26.7) 176 (73.3)	68 (19.3) 284 (80.7)	60 (18.0) 274 (82.0)	**$1.0X10^{-6}$**	**$1.0X10^{-5}$**
Non-phospho CHK1 Low High	151 (57.0) 114 (43.0)	92 (50.0) 92 (50.0)	133 (47.3) 148 (52.7)	113 (41.9) 157 (58.1)	**0.005**	**0.008**
CHK2 Low	145 (59.7)	72 (45.6)	113 (42.2)	92 (38.0)	**$9.0X10^{-6}$**	**$1.0X10^{-5}$**

High	98 (40.3)	86 (54.4)	155 (57.8)	150 (62.0)		
Bax Low High	138 (75.4) 123 (24.6)	99 (76.7) 30 (23.3)	134 (63.2) 78 (36.8)	136 (69.0) 61 (31.0)	**0.018**	**0.024**
CDK1 Low High	142 (68.3) 66 (31.7)	98 (74.2) 34 (25.8)	161 (66.3) 82 (33.7)	149 (70.6) 62 (29.4)	0.416	0.485
CDK18 (Cytoplasmic) Low High	223 (84.8) 40 (15.2)	144 (77.4) 42 (22.6)	203 (72.5) 77 (27.5)	174 (65.9) 90 (34.1)	**$7.0X10^{-6}$**	**$1.0X10^{-5}$**
RECQL5 Low High	161 (63.6) 92 (36.4)	74 (39.8) 112 (60.2)	134 (48.9) 140 (51.1)	93 (35.0) 173 (65.0)	**$1.0X10^{-6}$**	**$1.0X10^{-5}$**
MDM2 Low High	184 (76.3) 57 (23.7)	112 (73.2) 41 (26.8)	202 (78.3) 56 (21.7)	160 (73.4) 58 (26.6)	0.544	0.601

Bold = Statistically significant; BRCA1: Breast cancer 1, early onset; HER2: human epidermal growth factor receptor 2; ER: oestrogen receptor; PR: progesterone receptor; CK: cytokeratin; Basal-like: ER-, HER2 and positive expression of either CK5/6, CK14 or EGFR; Triple negative: ER-/PR-/HER2- . Adjusted p values were calculated using Benjamini-Hochberg false discovery rate method to adjust for multiple testing. *Fischer test was used to obtain p values where one or more of cells has an expected frequency of five or less.

7.3.6 BLM-/RAD51- tumours show association with aggressive breast cancers

As discussed previously, BLM interacts with Rad51 to play an essential role in HR repair. We therefore, conducted nuclear co-expression analysis in breast cancer. As shown in Table 7.6, significant associations were observed between BLM-/RAD51- tumours and tumour size, NPI>3.4, high grade, high mitotic index, pleomorphism, tumour type, ER- and PR- tumours ($p<0.05$).

Table 7.6 BLM-Rad51(Nuclear) Protein Co-Expression

VARIABLE	BLM-Rad51(Nuclear) Protein Co-Expression				P- value Unadjusted	P -Value Adjusted
	BLM-/Rad51- N (%)	BLM+ /Rad51- N (%)	BLM- /Rad51+ N (%)	BLM+ /Rad51+ N (%)		
A) Pathological Parameters						
Tumour Size						
≤1cm	6 (5.6)	18 (8.0)	4 (5.6)	26 (9.8)	**0.004**	**0.008**
>1-2cm	49 (45.8)	97 (43.1)	49 (69.0)	138 (52.3)		
>2-5cm	49 (45.8)	106 (49.1)	16 (22.5)	99 (37.5)		
>5cm	3 (2.8)	4 (1.8)	2 (2.8)	1 (0.4)		
Tumour Stage						
1	65 (60.7)	116 (51.3)	46 (63.9)	153 (58.0)	0.251	0.326
2	30 (28.0)	88 (38.9)	23 (31.9)	85 (32.2)		
3	12 (11.2)	22 (9.7)	3 (4.2)	26 (9.8)		
Tumour Grade						
G1	9 (16.0)	27 (11.9)	9 (12.7)	48 (18.2)	**$7.0X10^{-5}$**	**$3.0X10^{-4}$**
G2	21 (26.2)	70 (31.0)	22 (31.0)	103 (39.0)		
G3	77 (57.8)	129 (57.1)	40 (56.3)	113 (42.8)		

Mitotic Index M1 (low; mitoses < 10) M2 (medium; mitoses 10-18) M3 (high; mitosis >18)	 16 (15.2) 19 (18.1) 70 (66.7)	 65 (29.7) 42 (19.2) 112 (51.1)	 19 (27.1) 10 (14.3) 41 (58.6)	 103 (39.8) 51 (19.7) 105 (40.5)	**$1.0X10^{-4}$**	**$3.0X10^{-4}$**
Tubule Formation 1 (>75% definite tubule) 2 (10%-75% definite tubule) 3 (<10% definite tubule)	 2 (1.9) 30 (28.6) 73 (69.5)	 9 (4.1) 65 (35.0) 145 (66.2)	 3 (4.3) 24 (34.3) 43 (61.4)	 12 (4.6) 97 (37.5) 150 (57.9)	 0.366	 4.75
Pleomorphism 1 (small-regular uniform) 2 (Moderate variation) 3 (Marked variation)	 0 (0.0) 26 (25.0) 78 (75.0)	 0 (0.0) 78 (35.6) 141 (64.4)	 1 (1.4) 24 (34.3) 45 (64.3)	 4 (1.6) 111 (43.0) 143 (55.4)	 **0.011**	 **0.02**
Tumour Type IDC-NST Tubular Carcinoma Medullary Carcinoma ILC Others	 81 (75.7) 8 (7.5) 7 (6.5) 4 (3.8) 7 (6.5)	 142 (62.8) 37 (16.4) 5 (2.2) 23 (10.1) 23 (9.5)	 47 (65.3) 13 (18.1) 1 (1.4) 6 (8.4) 21 (6.8)	 139 (52.7) 69 (26.2) 3 (1.2) 27 (10.3) 35 (9.6)	**0.040**	**0.06**
Lymph Node Status Negative Positive (1-3) Positive (>3)	 52 (61.9) 26 (31.0) 6 (7.1)	 198 (50.3) 80 (41.0) 17 (8.7)	 38 (61.3) 23 (37.1) 1 (1.6)	 135 (56.3) 88 (36.7) 17 (7.1)	0.320	0.34
B) Aggressive Phenotype						
Her2 overexpression No Yes	 97 (90.7)	 184 (82.9)	 62 (87.3)	 217 (84.4)	0.278	0.319

	10 (9.3)	38 (17.1)	9 (12.7)	40 (15.6)		
Triple Negative Phenotype No Yes	 88 (82.2) 19 (17.8)	 191 (84.5) 35 (15.5)	 54 (75.0) 18 (25.0)	 228 (86.4) 36 (13.6)	 0.129	 0.18
NPI ≤3.4 >3.4	 14 (14.1) 85 (85.9)	 49 (22.6) 168 (77.4)	 19 (27.5) 50 (72.5)	 86 (34.0) 167 (66.0)	 **0.001**	 **0.0026**
C) Hormone Receptors						
ER Negative Positive	 49 (47.1) 55 (52.9)	 67 (30.5) 153 (69.5)	 21 (30.9) 47 (69.1)	 46 (17.9) 211 (82.1)	 **$3.8X10^{-7}$**	 **$1.0X10^{-5}$**
PR Negative Positive	 65 (64.4) 36 (35.6)	 102 (47.9) 111 (52.1)	 30 (42.9) 40 (57.1)	 89 (35.3) 163 (64.7)	 **$1.0X10^{-5}$**	 **$1.0X10^{-4}$**

Bold = Statistically significant; HER2: human epidermal growth factor receptor 2; ER: oestrogen receptor; PR: progesterone receptor; Triple negative: ER-/PR-/HER2- . Adjusted p values were calculated using Benjamini-Hochberg false discovery rate method to adjust for multiple testing.

7.3.7 BLM nuclear expression is not associated with breast cancer specific survival

In univariate analysis, BLM nuclear expression did not influence survival as shown in Figure 7.5 & 7.6.

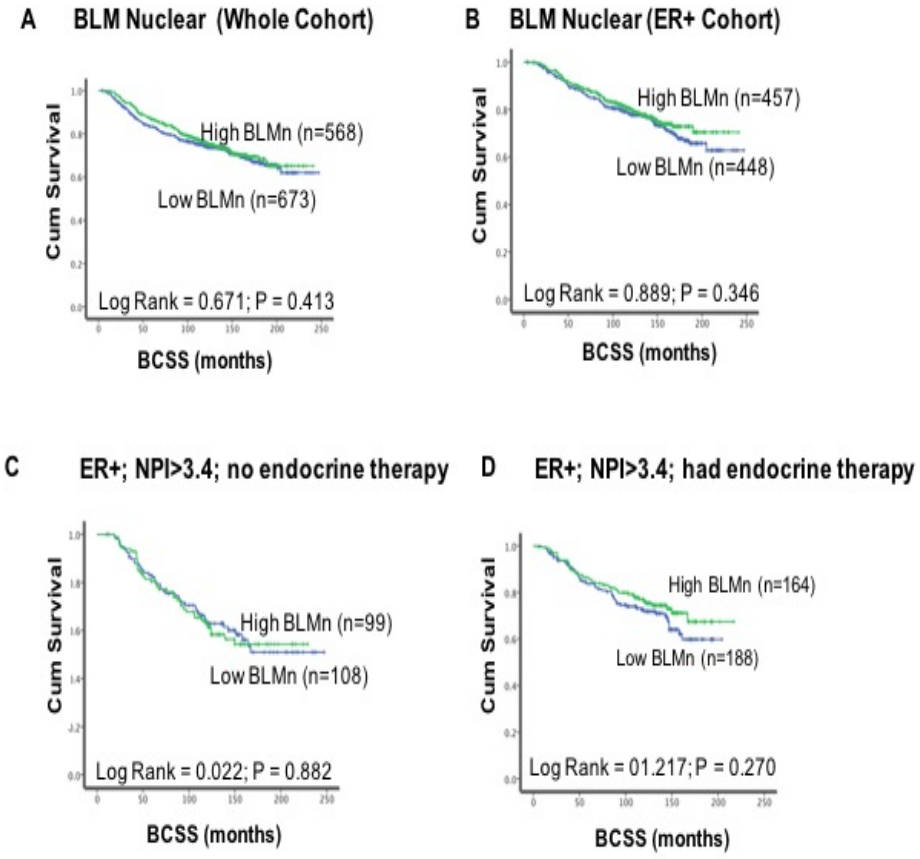


Figure 7.5 BLM Nuclear Expression and Breast cancer survival in Whole and ER+ Cohort

A. BLM nuclear expression and survival in the whole cohort **B.** BLM nuclear expression and survival in ER+ Cohort **C.** BLM expression and survival in ER+, NPI>3.4 cohort who had no endocrine therapy **D.** BLM nuclear expression and survival in ER+, NPI>3.4 cohort who had endocrine therapy. BCSS: Breast cancer-specific survival in months.

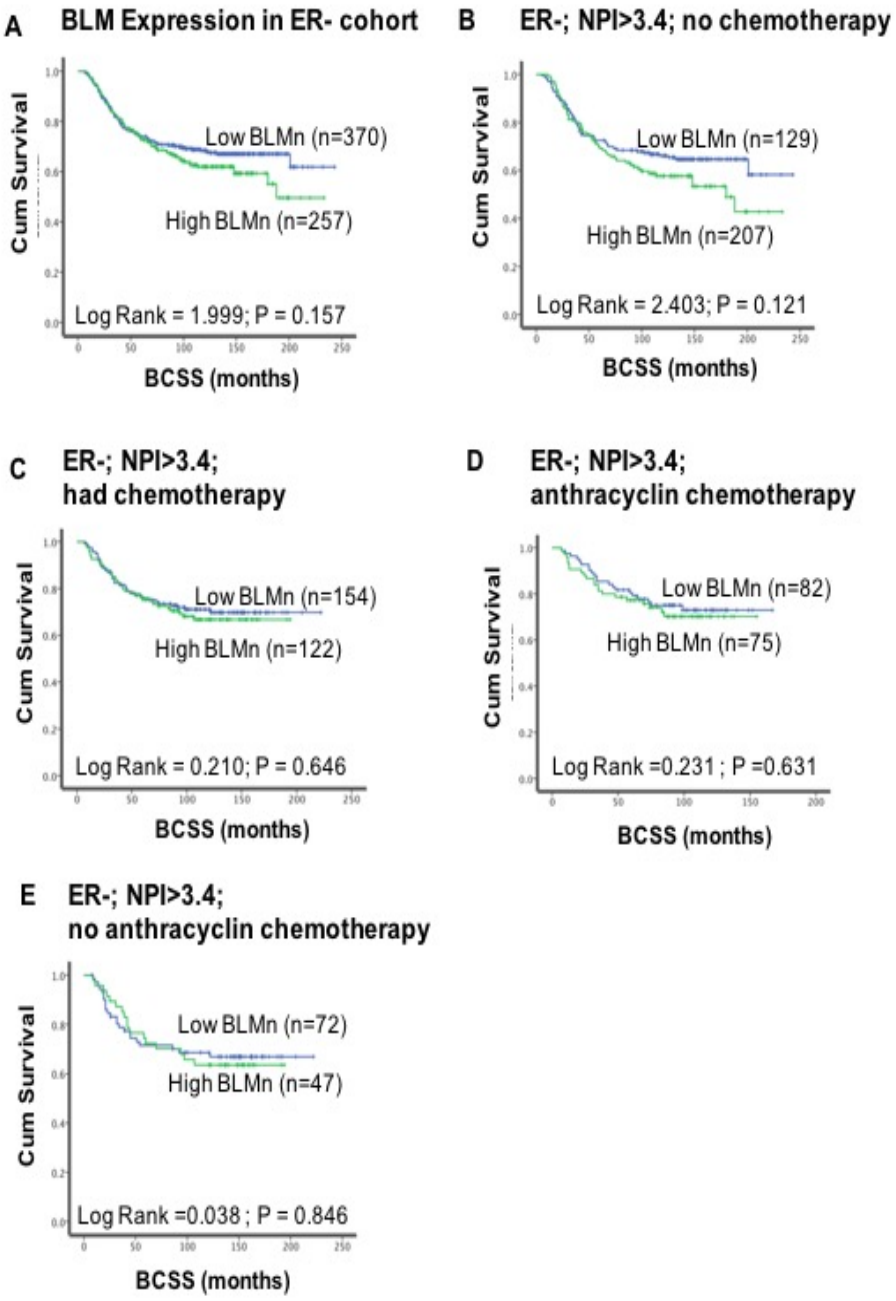


Figure 7.6 BLM Nuclear Expression and Breast cancer survival in ER- Cohort

A. ER- whole cohort **B.** ER- ,NPI>3.4 Cohort who had no CMF chemotherapy **C.** ER-, NPI>3.4 cohort who had CMF chemotherapy **D.** ER-, NPI>3.4 cohort who had anthracycline chemotherapy **E.** ER-, NPI>3.4 cohort who had no anthracycline chemotherapy.

BCSS: Breast cancer-specific survival in months.

7.3.8 BLM cytoplasmic protein expression is not associated with breast cancer survival

BLM cytoplasmic expression did not significantly impact breast cancer Specific survival (Figure 7.7).

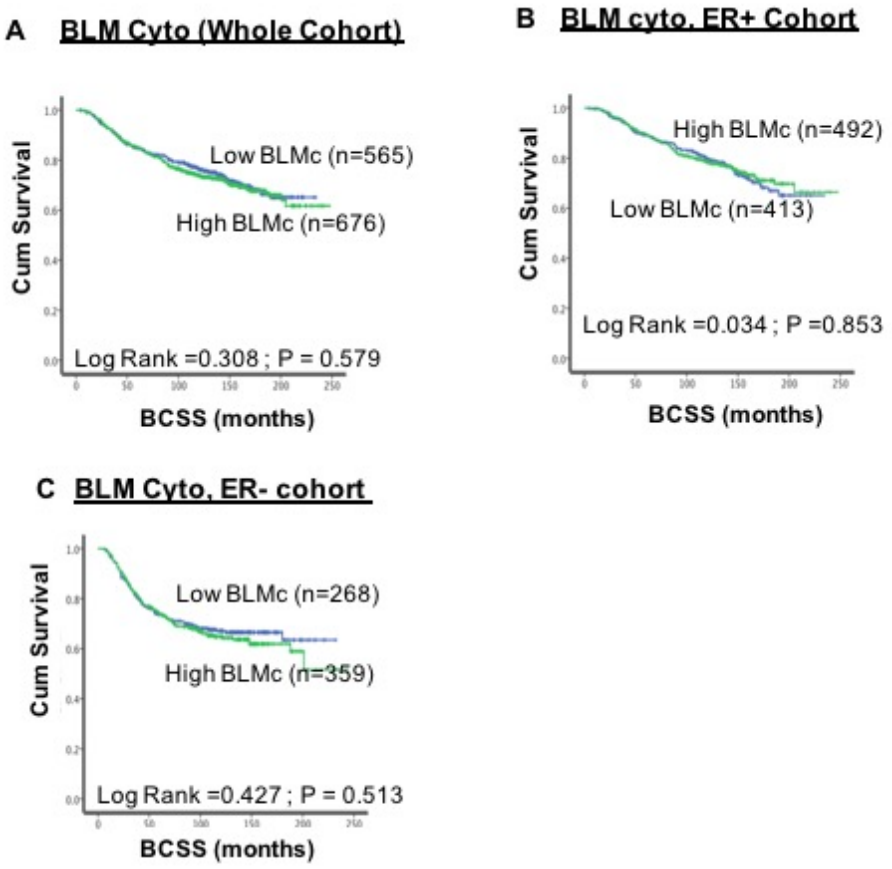


Figure 7.7 BLM cytoplasmic expression and Breast cancer survival

A. BLM cytoplasmic expression and survival in whole cohort **B.** BLM cytoplasmic expression and survival in ER+ Cohort **C.** BLM expression and survival in ER+, NPI>3.4 cohort who had no endocrine therapy; BCSS: Breast cancer-specific survival in months.

7.3.9 High nuclear/low cytoplasmic BLM protein expression is associated with poor breast cancer specific survival

We then evaluated BLM nuclear and cytoplasmic co-expression and its impact on breast cancer survival. In high-risk ER-positive tumours who did not receive any endocrine therapy, patients with high nuclear/low cytoplasmic BLM expression in tumour had poor breast cancer specific survival (p=0.036) . This suggests that altered expression has prognostic significance (Figure 7.8 C).On the other hand, in patients who received endocrine therapy, although low nuclear/high cytoplasmic BLM tumours have the worst survival it was not statistically significant. Similarly, in the whole cohort and ER- cohort, BLM nuclear and cytoplasmic co-expression did not show significant influence on survival outcome.

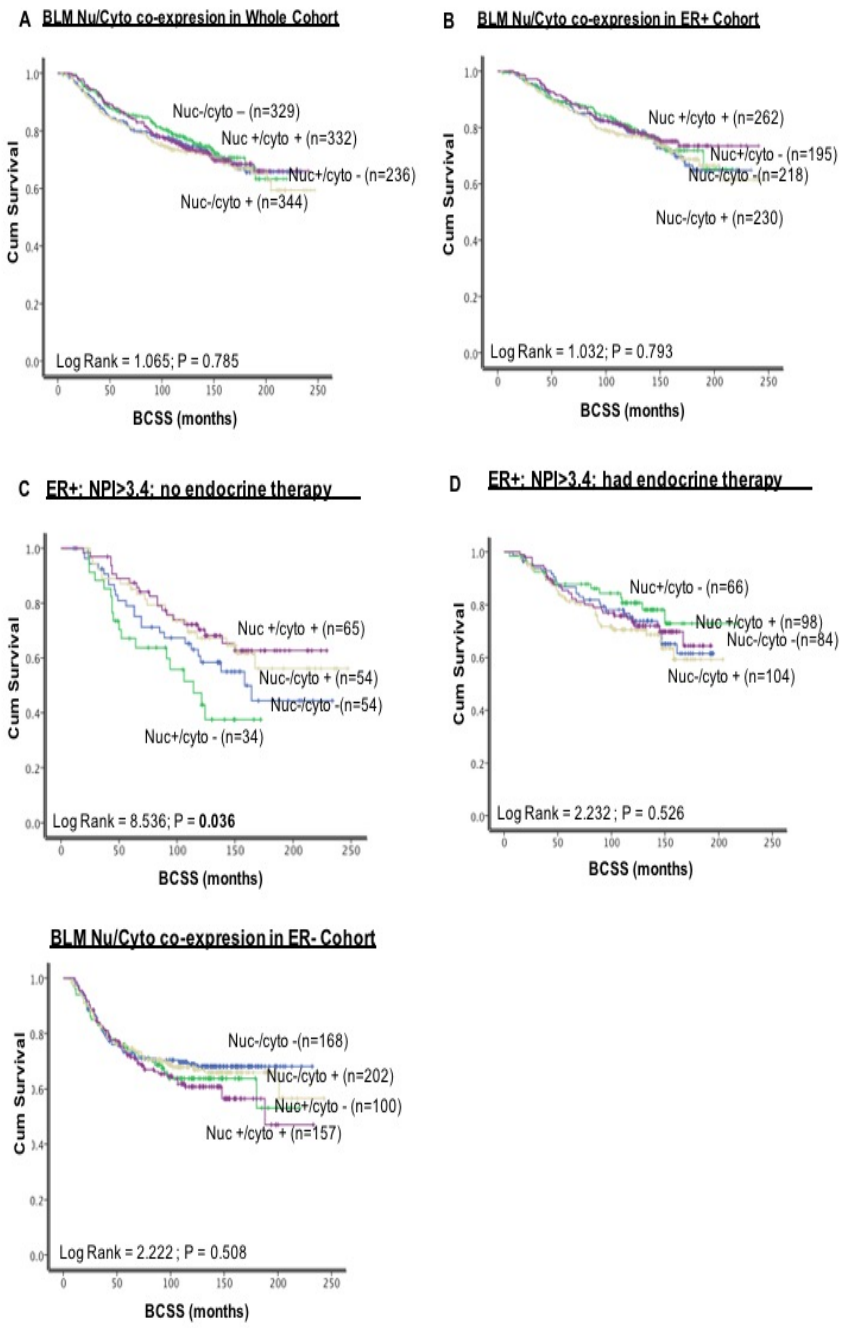


Figure 7.8 Kaplan Meier curves showing BCSS based on BLM nuclear /BLM cytoplasmic protein co-expression

A. whole cohort; **B.** ER+ cohort **C.** ER+ patients with NPI >3.4, who received no endocrine therapy **D.** ER+ patients with NPI >3.4, who received endocrine therapy **E.** ER- cohort

7.3.10 BLM-/Rad51- tumours are associated with poor breast cancer specific survival

In BLM (nuclear) and Rad51 (nuclear) co-expression analysis, BLM-/Rad51- tumours have poor survival in the whole cohort (Figure 7.9 A) and the in the ER- subgroup that received adjuvant chemotherapy (Figure 7.9 D). BLM/Rad51 expression did not influence survival in ER + tumours (Figure 7.10).

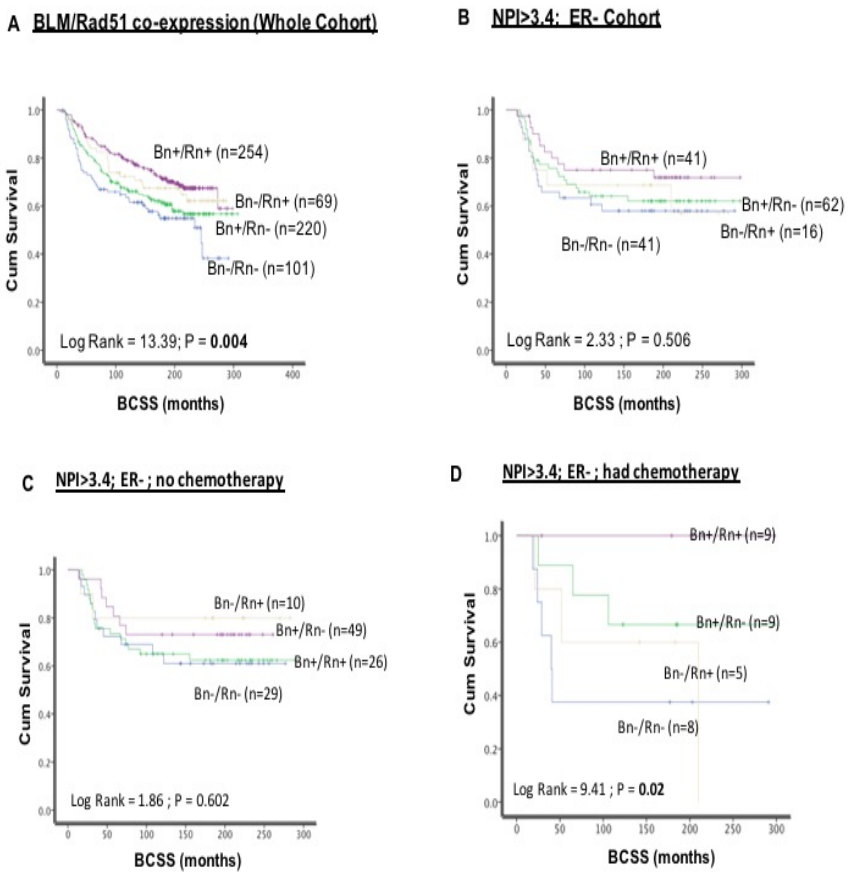


Figure 7.9 Kaplan Meier curves showing BCSS based on BLM/Rad51 protein co-expression in whole and ER- cohort

A. whole cohort; **B.** ER- cohort with NPI >3.4; **C.** ER- patients with NPI >3.4, who received no chemotherapy; **D.** ER- patients with NPI >3.4, who received chemotherapy.

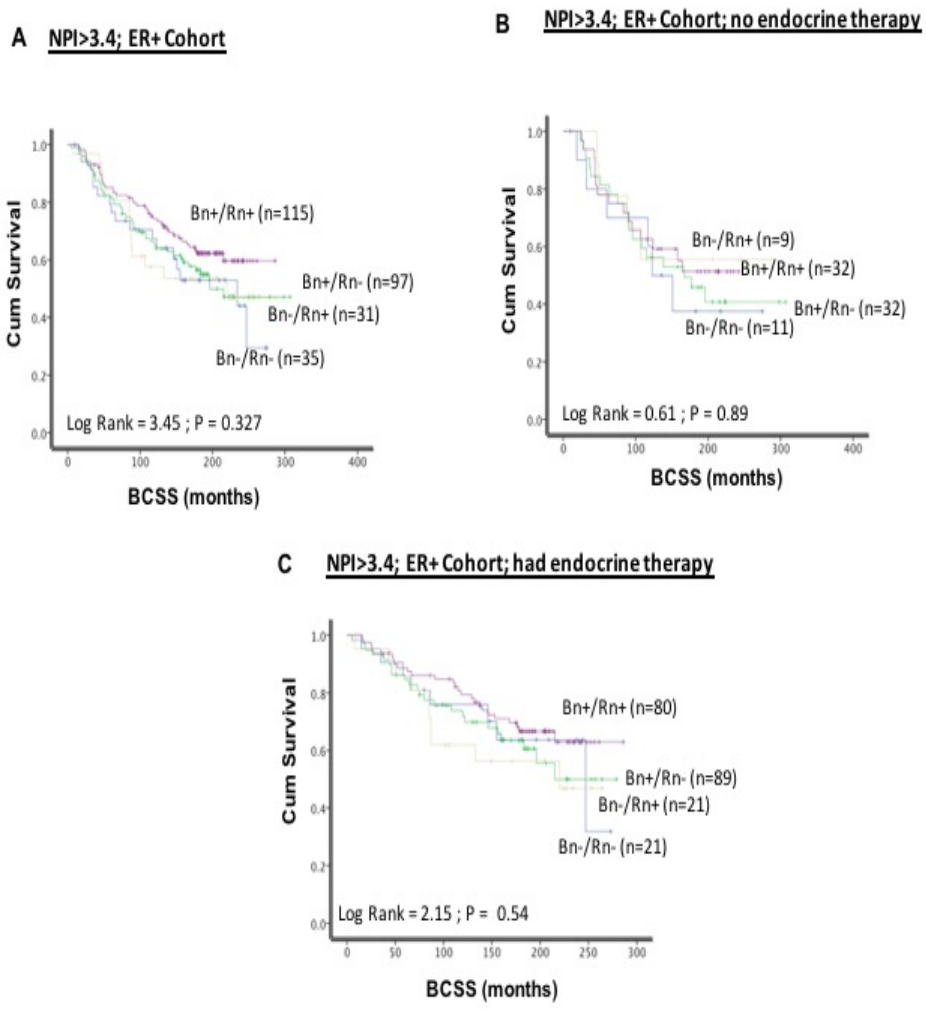


Figure 7.10 Kaplan Meier curves showing BCSS based on BLM/Rad51 protein co-expression in ER+ patients

A. ER+ cohort; **B.** ER+ patients with NPI >3.4, who received no endocrine therapy; **C.** ER+ patients, NPI >3.4, who had adjuvant endocrine therapy.

7.3.11 3.3.2 Breast cancer cell lines show robust BLM mRNA expression

We initially profiled a panel of breast cancer cell lines. MDA-MB-231, MDA-MB-436, and MDA-MB-468 showed reduced BLM mRNA expression as compared to control MCF-7 cell line. (Figure 7.11)

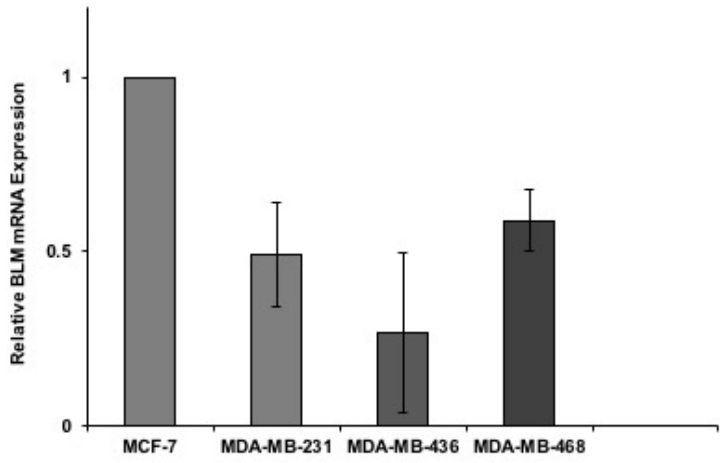


Figure 7.11 mRNA expression of BLM in breast cancer cell lines.

A graph of mRNA expression of BLM in MDA-MB-231, MDA-MB-436, and MDA-MB-468 breast cancer cell lines relative to MCF7 control breast cancer cell line. Data presented as mean ± SEM of two independent experiments, each performed in triplicate.

7.3.12 High BLM mRNA expression is significantly associated with aggressive breast cancer clinicopathological features

BLM mRNA level was investigated in a large series of 1977 tumours comprising the METABRIC (Molecular Taxonomy of Breast Cancer International Consortium) cohort. High BLM mRNA expression showed statistically significant association with aggressive clinicopathological features (Table 7.8), such as lymph node stage, high histological grade, larger tumour size, Nottingham prognostic index (NPI) >3.4, Her-2 overexpression, ER-negative, PR negative and triple negative phenotypes (p values<0.0001). High BLM mRNA expression was also found to be significantly linked to previously described molecular phenotypes in breast cancer; PAM50.Her2 (p<0.0001), PAM50.Basal (p<0.0001) and PAM50.LumB (p<0.0001), Genufu subtype (ER-/Her2-), Genufu subtype (ER+/Her2-/High proliferation) and Genufu subtype (Her2 positive) breast tumours. However, PAM50.LumA tumours and Genufu subtype (ER+/Her2-/low proliferation) were more likely to express low levels of BLM mRNA (p values<0.0001). Similarly, BLM mRNA level was significantly associated with the various biological subgroups [labelled integrative clusters (intClust) 1-10] described in the METABRIC study which was based on gene copy number changes and gene expression data (Curtis et al). High BLM mRNA expression was also significantly associated with intClust.1 (p<0.0001), intClust.5 (p<0.0001), intClust.9 (p<0.0001) and intClust.10 (p<0.0001), which had the worst clinical outcome in the METABRIC study (Curtis et al). Low BLM mRNA expression was also linked with intClust.3 (p<0.0001), intClust.4

($p<0.0001$), intClust.7 ($p=0.003$) and intClust.8 ($p<0.0001$), which had intermediate to good prognosis in the METABRIC study (Curtis et al).

Table 7.7 Association between BLM mRNA expression and clinicopathologic variables in the METABRIC cohort

Variable	BLM mRNA Expression		P Values	
	Low	High	*Unadjusted*	*Adjusted**
	N (%)	N (%)		
A) Pathological Parameters				
Lymph node stage				
Negative	434(56.9%)	601(49.8%)	**0.003**	**0.0034**
Positive (1-3)	100(13.1%)	214(17.7%)		
Positive (>3)	229(30.0%)	393(32.5%)		
Grade				
G1	124(17.3%)	45(3.8%)	**$1.9X10^{-63}$**	**$1.0X10^{-5}$**
G2	404(56.3%)	366(31.3%)		
G3	190(26.5%)	760(64.9%)		
Tumour Size (cm)				
T 1a+b(1.0)	49(6.4%)	43(3.6%)	**$1.4X10^{-5}$**	**$1.0X10^{-5}$**
T 1c(>1.0-2.0)	334(43.9%)	432(36.1%)		
T2 (>2.0-5)	341(44.9%)	660(55.1%)		
T3 (>5)	36(4.7%)	62(5.2%)		
NPI				
≤ 3.4	385(50.3%)	295(24.3%)	**$2.2X10^{-32}$**	**$1.0X10^{-5}$**
>3.4	380(49.7%)	917(75.7%)		
Her2 overexpression (No)	733(95.8%)	999(82.4%)	**$1.3X10^{-18}$**	**$1.0X10^{-5}$**
(Yes)	32(4.2%)	213(17.6%)		
Triple negative (No)	731(95.6)	929 (76.7)	**$6.5X10^{-29}$**	**$1.0X10^{-5}$**
(Yes)	34(4.4)	283(23.3)		
ER (Negative)	55(7.2%)	415(34.2%)	**$4.3X10^{-43}$**	**$1.0X10^{-5}$**
(Positive)	710(92.8%)	797(65.8%)		
PR (Negative)	223(29.2%)	713(58.8%)	**$6.4X10^{-38}$**	**$1.0X10^{-5}$**

(Positive)	542(70.8%)	499(41.2%)		
Genefu subtype				
ER-/Her-2 negative	20(5.1%)	130(21.5%)	**$2.2X10^{-12}$**	**$1.0X10^{-5}$**
ER+/Her-2 negative/high proliferation	71(18.3%)	295(48.8%)	**$2.2X10^{-22}$**	**$1.0X10^{-5}$**
ER+/Her-2 negative/low proliferation	283(72.8%)	85(14.0%)	**$4.4X10^{-78}$**	**$1.0X10^{-5}$**
Her-2 positive	15(3.9%)	95(15.7%)	**$6.2X10^{-9}$**	**$1.0X10^{-5}$**
PAM50 subtype				
PAM50.Her2	33(5.2%)	205(18.0%)	**$3.8X10^{-14}$**	**$1.0X10^{-5}$**
PAM50.Basal	19(3.0%)	311(27.3%)	**$2.2X10^{-36}$**	**$1.0X10^{-5}$**
PAM50.LumA	483(76.2%)	232(20.4%)	**$8.1X10^{-117}$**	**$1.0X10^{-5}$**
PAM50.LumB	98(15.5%)	391(34.3%)	**$1.7X10^{-17}$**	**$1.0X10^{-5}$**
IntClust subgroups				
intClust.1	21(2.7%)	116(9.6%)	**$5.8X10^{-9}$**	**$1.0X10^{-5}$**
intClust.2	20(2.6%)	52(4.3%)	0.053	0.055
intClust.3	203(26.5%)	87(7.2%)	**$2.1X10^{-32}$**	**$1.0X10^{-5}$**
intClust.4	191(25.0%)	152(12.5%)	**$1.2X10^{-12}$**	**$1.0X10^{-5}$**
intClust.5	21(2.7%)	168(13.9%)	**$2.6X10^{-16}$**	**$1.0X10^{-5}$**
intClust.6	27(3.5%)	59(4.9%)	0.155	4.03
intClust.7	92(12.0%)	97(8.0%)	**0.003**	**0.003**
intClust.8	156(20.4)	144(11.9%)	**$2.7X10^{-7}$**	**$1.0X10^{-5}$**
intClust.9	28(3.7%)	118(9.7%)	**$4.8X10^{-7}$**	**$1.0X10^{-5}$**
intClust.10	6(0.8%)	219(18.1%)	**$4.5X10^{-32}$**	**$1.0X10^{-5}$**

Bold = Statistically significant;HER2: human epidermal growth factor receptor 2; ER: oestrogen receptor; PR: progesterone receptor; Triple negative: ER-/PR-/HER2- .

7.3.13 High BLM mRNA expression in tumours is associated with adverse breast cancer specific survival

In univariate survival analysis, high BLM mRNA expression in tumours was associated with adverse breast cancer-specific survival (BCSS) in the whole cohort ($p<0.0001$) (Figure 7.12 A). In ER+ sub-group, high BLM mRNA expression was associated with poor BCSS ($p<0.0001$) (Figure 7.12 B). In the

ER+ sub-group that received adjuvant endocrine therapy, high BLM mRNA expression remains associated with poor BCSS ($p<0.0001$) (Figure 7.12 D). In ER- sub-group, low BLM mRNA expression was associated with poor BCSS with borderline significance ($p=0.049$) (Figure 7.12 C). In the ER- sub-group that received adjuvant chemotherapy, BLM mRNA expression did not significantly influence the outcome ($p=0.062$) (Figure 7.12 E).

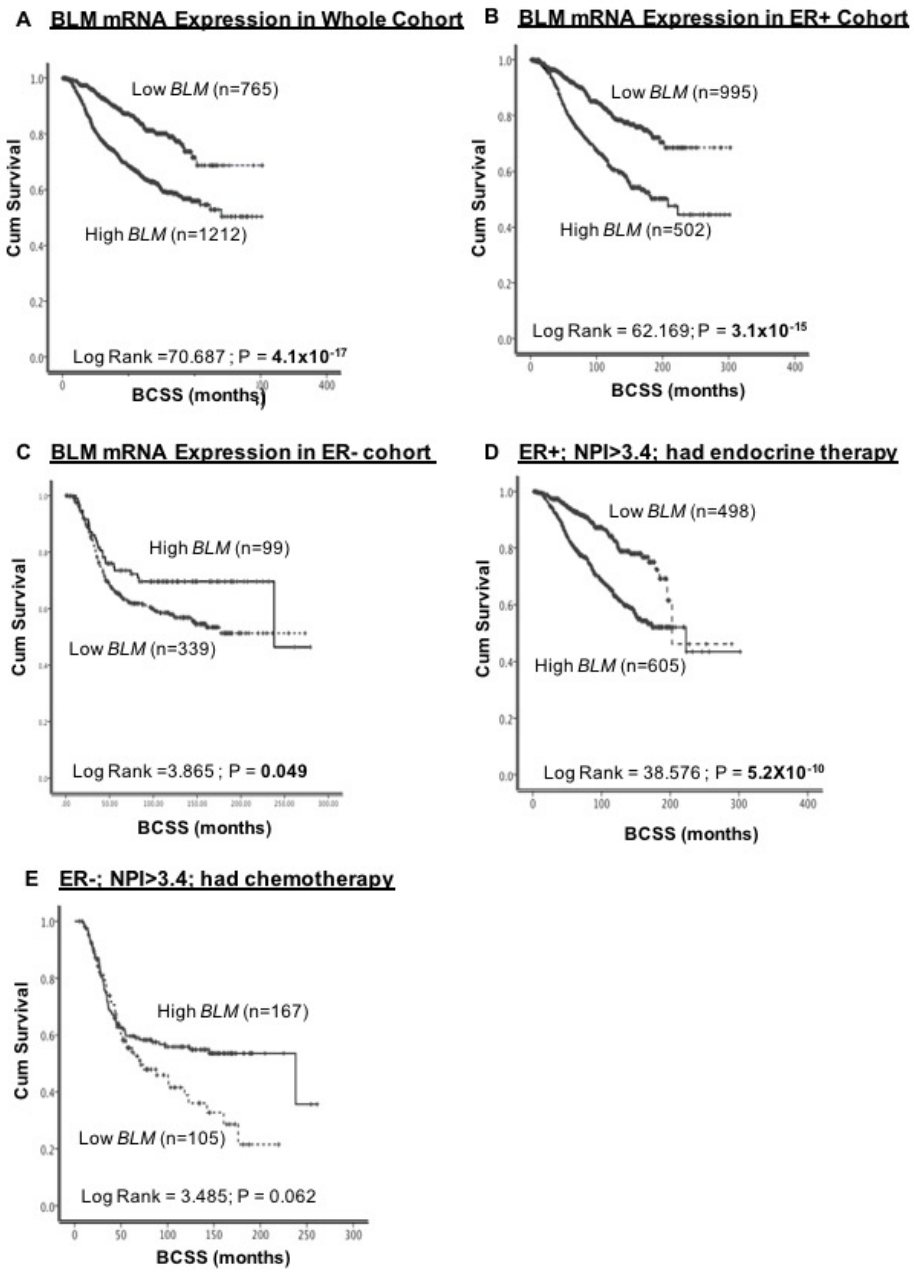


Figure 7.12 Kaplan Meier curves showing BCSS (Breast cancer-specific survival) based on BLM mRNA expression

A. whole cohort; B. ER+ cohort; C. ER- cohort; D. ER+ patients with NPI >3.4, who received endocrine therapy and E. ER- patients with NPI >3.4, who received chemotherapy.

7.3.14 BLM mRNA expression was is an independent predictor for breast cancer specific survival

In multivariate Cox regression analysis that included other validated prognostic factors, such as lymph node stage, histological grade and tumour size, BLM mRNA expression was a powerful independent predictor for breast cancer-specific survival ($p<0.00001$) (Table 7.9).

Table 7.8 Multivariate analysis in the METABRIC cohort confirms that BLM mRNA overexpression is a powerful independent prognostic factor

	P-Value	**HR**	**95% CI for HR**	
			Lower	**Upper**
Breast Cancer Specific Survival				
BLM *mRNA* expression	**$2.0x10^{-6}$**	1.523	1.278	1.815
Size	**$1.0x10^{-6}$**	1.112	1.068	1.158
Grade				
G1		1.0		
G2	0.121	1.782	1.094	2.903
G3	**0.0044**	2.03	1.241	3.321
LN Status				
LN (1-3)	0.21	1.697	1.367	2.108
LN(>3)	**$1.0x10^{-6}$**	3.646	2.890	4.601

Bold: Statistically significant; HR: Hazard Ratio; CI: Confidence interval; LN: Lymph node

Taken together, this data provides evidence that the BLM mRNA level has prognostic significance in breast cancer.

7.4 BLM cell line study

The aim of this chapter was to investigate the cell line characterisation of BLM-KD and control HeLa cells. We also did functional studies to ascertain the functional consequences of cisplatin chemotherapy on BLM-KD and control HeLa cells.

7.4.1 Western blot and RT (real-time) PCR confirms the silencing of BLM in BLM-KD HeLa cells

Silencing validation of knock-down BLM HeLa SilenciX cells was done at protein and mRNA level. We initially done western blot analysis using BLM antibody as described in section 2.3.2.2. The primary BLM antibody dilution used was 1:1500 (overnight incubation). In western blot analysis, we did not see any BLM protein expression in BLM knock-down HeLa cells , however BLM protein expression was seen in HeLa control cells (Figure 7.13 A). As Tebu bio, recommended silencing validation by RT-PCR method, so we then proceeded to RT-PCR and confirmed 75% silencing of BLM gene in BLM knock-down HeLa cells as compared to HeLa control cells (Figure 7.13 B)

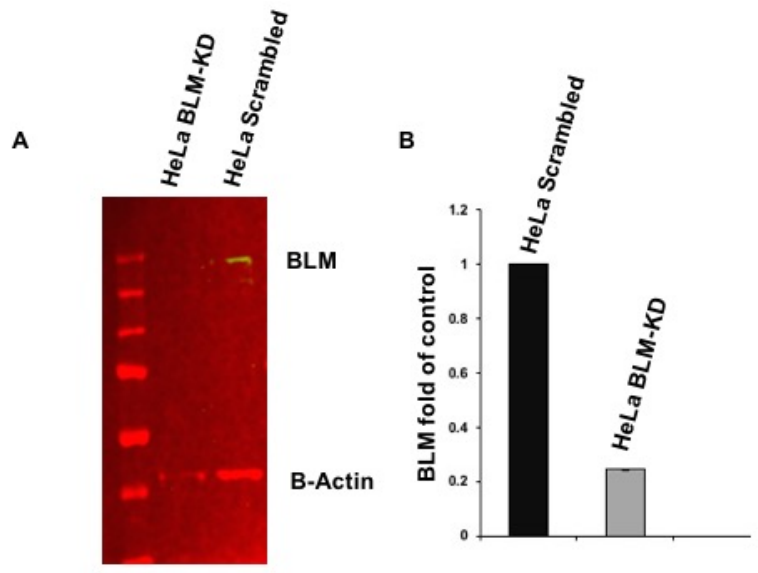


Figure 7.13 A. Western blot showing no BLM protein expression in BLM-KD HeLa cells B. Graph represents BLM mRNA expression in BLM-KD-HeLa and HeLa control cells. BLM-KD HeLa cells showing 75% silencing as compared to HeLa control cells.

7.4.2 BLM-KD HeLa cells grow slower as compared to control HeLa cells

To define the growth characteristics for BLM KD HeLa cells in comparison to control HeLa cells, we carried out cell survival assay (Growth curve and MTS assay). The method discussed in detail in section 2.3.2.3. The results showed that the BLM-KD HeLa cells had the slower growth rate as compared to HeLa control cells (Figure 7.14 A,B).

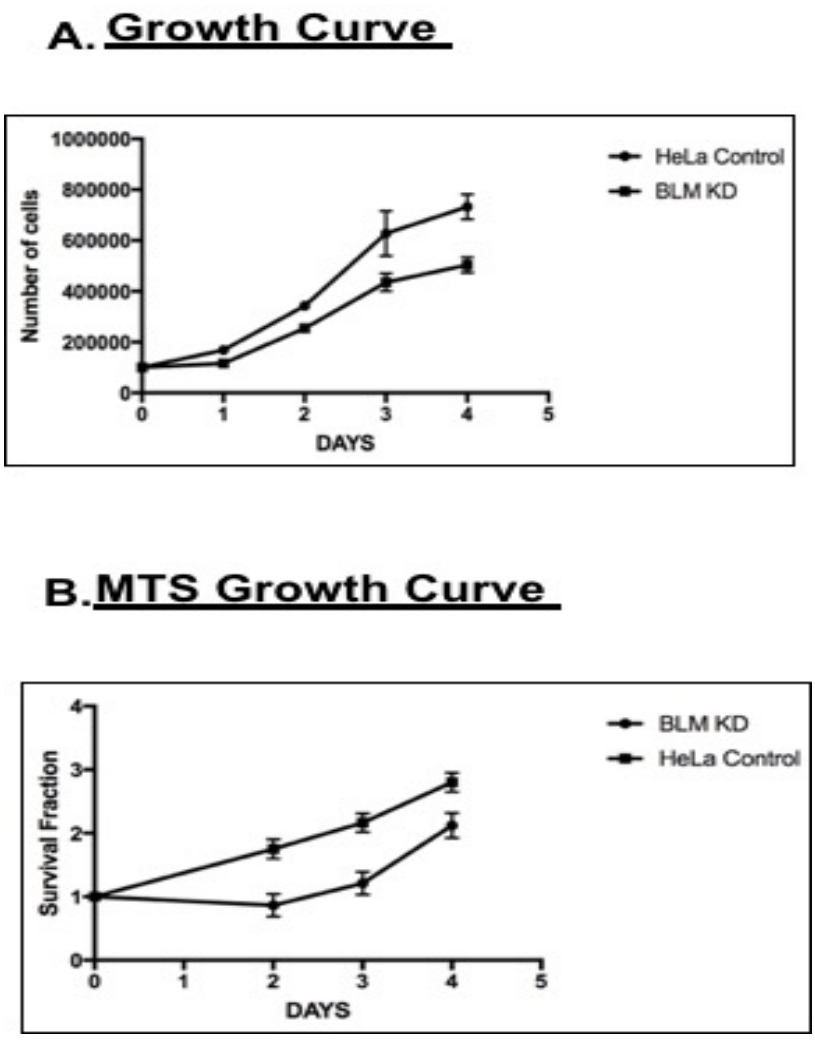


Figure 7.14 Cell growth in BLM-KD and control HeLa cells

A. Growth curve of BLM-KD-HeLa and HeLa- Control cells showing the slower growth rate of BLM-KD-HeLa cells compared to HeLa control. **B.** MTS Growth curve of BLM-KD-HeLa and HeLa- Control cells showing the slower growth rate of BLM-KD-HeLa cells compared to HeLa control. Data presented as mean ± SEM of two independent experiments, each performed in triplicate.

7.4.3 Cell survival assays showing potential platinum sensitivity in BLM-KD and control HeLa cells

We then assessed the effects of platinum chemotherapy on BLM-KD HeLa and BLM control HeLa cells. We noticed that BLM-KD Hela cells were more sensitive to cisplatin chemotherapy as shown in Figure 7.15 A and B.

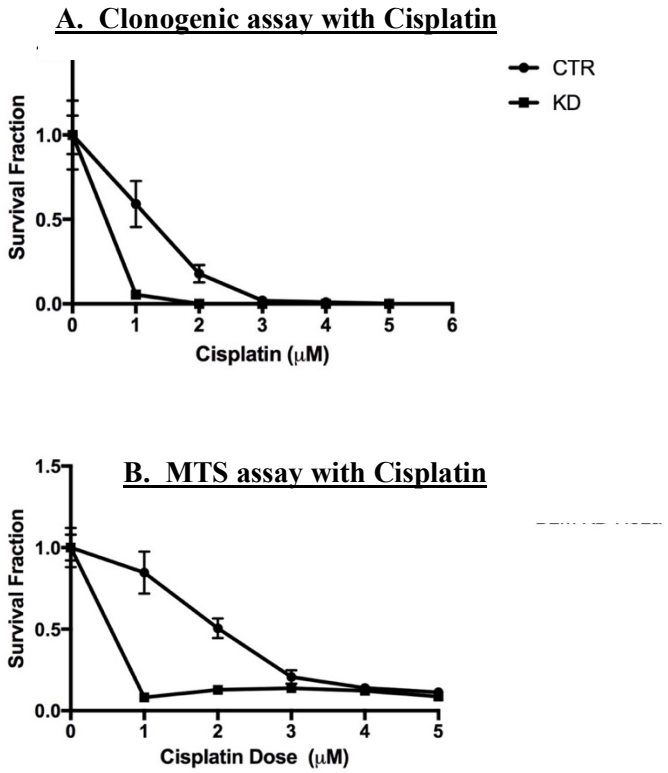


Figure 7.15 Clonogenic and MTS assay

A. Clonogenic assay showing platinum sensitivity in both BLM-KD and control HeLa control cells. **B.** MTS assay showing platinum sensitivity in both BLM-KD and control HeLa control cells. Data presented as mean ± SEM of two independent experiments, each performed in triplicate.

7.4.4 BLM-KD untreated cells results in cell cycle arrest in G1 phase and cisplatin treated BLM-KD cells result in cell cycle arrest in S phase

Given the known elevation of BLM in mitosis process we speculated that BLM knock down might affect cell cycle progression. We investigated the effect of cisplatin treatment on the cell cycle progression by flow cytometry analysis as described in section 2.3.2.4.1. As shown in Figure 7.17, BLM-KD untreated cells result in cell cycle arrest in G1 phase and cisplatin treated BLM-KD cells result in cell cycle arrest in S phase.

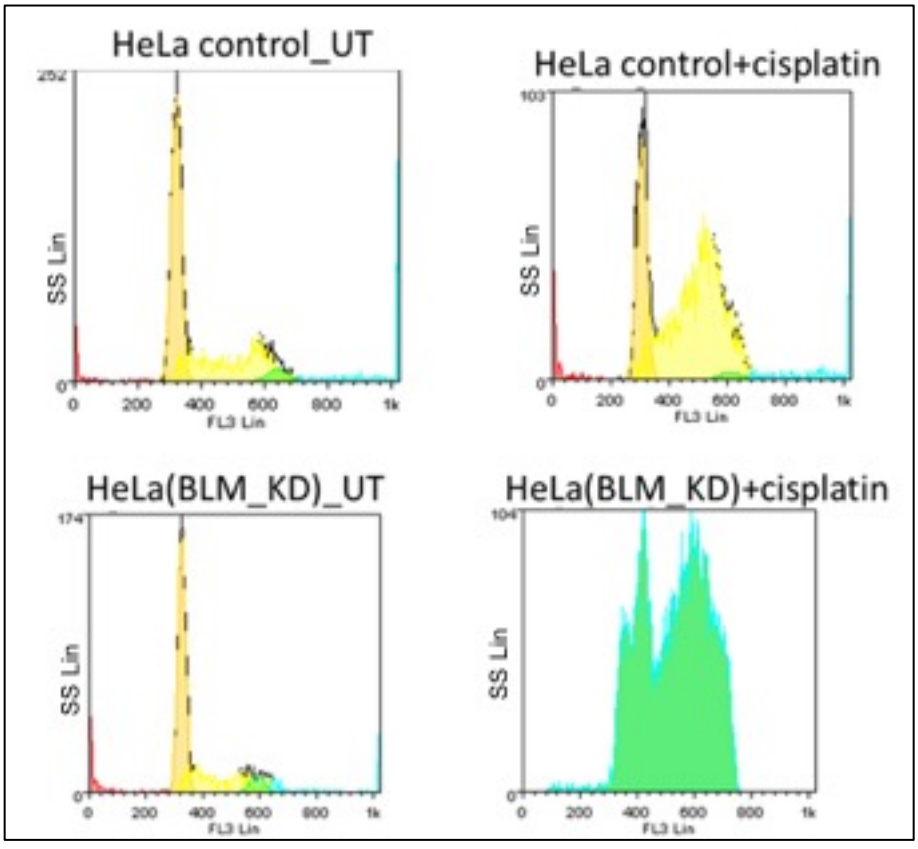


Figure 7.16 Representation of cell cycle analysis by weasel in HeLa control and BLM-KD HeLa cells treated with cisplatin

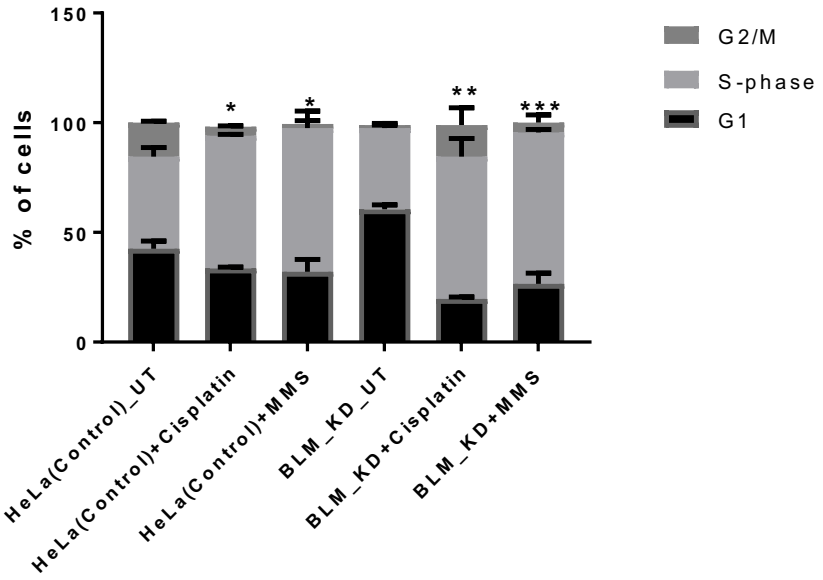


Figure 7.17 Cell cycle analysis by flow cytometry in HeLa control and BLM-KD cells treated with cisplatin (1µM concentration at 24 hours)

Graph showing BLM-KD untreated cells resulting in cell cycle arrest in G1 phase and cisplatin treated BLM-KD cells resulting in cell cycle arrest in S phase. Data presented as mean ± SEM of two independent experiments, each performed in triplicate. *p<0.05, **p<0.01, ***p<0.001, calculated by one-way Anova.

7.4.5 BLM-KD HeLa cells treated with cisplatin show accumulation of γ-H2AX foci

An isotype control was included for both BLM-KD and control HeLa cell lines in all γ-H2AX flowcytometry experiments using a normal mouse IgG conjugate (Merck Millipore). No difference was seen between unstained and normal mouse IgG FITC conjugate, which ruled out non-specific staining (example from BLM-KD cell line is shown in Figure 7.18.

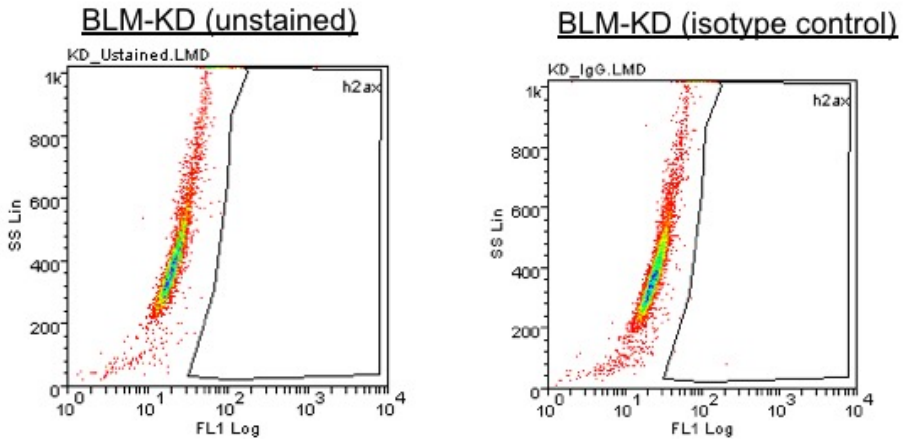


Figure 7.18 Example of isotype control staining in the BLM-KD cell line by flow cytometry

Cisplatin treatment can lead to double strand breaks in platinum sensitive cells. To study the effect of BLM-KD HeLa cells on platinum sensitivity, we stained the cells with anti phospho-histone H2AX ser139 which is a marker of double strands breaks formation. Then quantification of γ-H2AX positive cells was performed by flow cytometry as detailed in section 2.3.2.4.1.

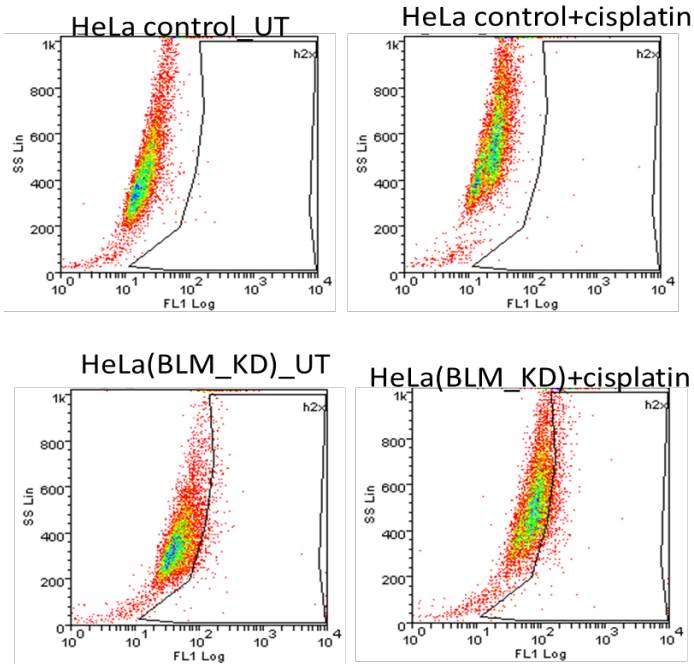


Figure 7.19 Representative images of γ-H2AX analysis by weasel

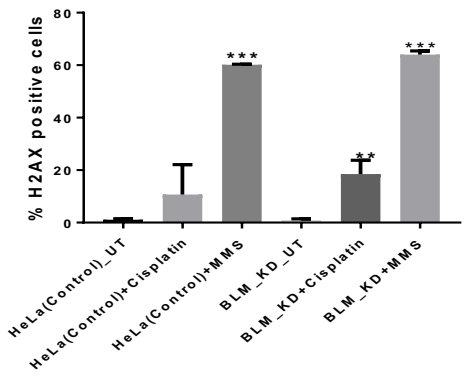


Figure 7.20 γ-H2AX quantification by flow cytometry in HeLa control and BLM_KD treated with cisplatin (1µM concentration at 24 hours)

Graph showing that Cisplatin treated BLM-KD-HeLa cells demonstrate more double strand breaks as compared to BLM-KD untreated cells. Data presented as mean ± SEM of two independent experiments, each performed in triplicate. **p<0.01, ***p<0.001, calculated by one-way Anova.

7.4.6 BLM-KD HeLa cells undergo apoptosis in response to platinum chemotherapy

Cell cycle arrest as well as double strands breaks if left unrepaired, cells then eventually undergo apoptosis. We confirmed this by doing Annexin V analysis of apoptotic cells.

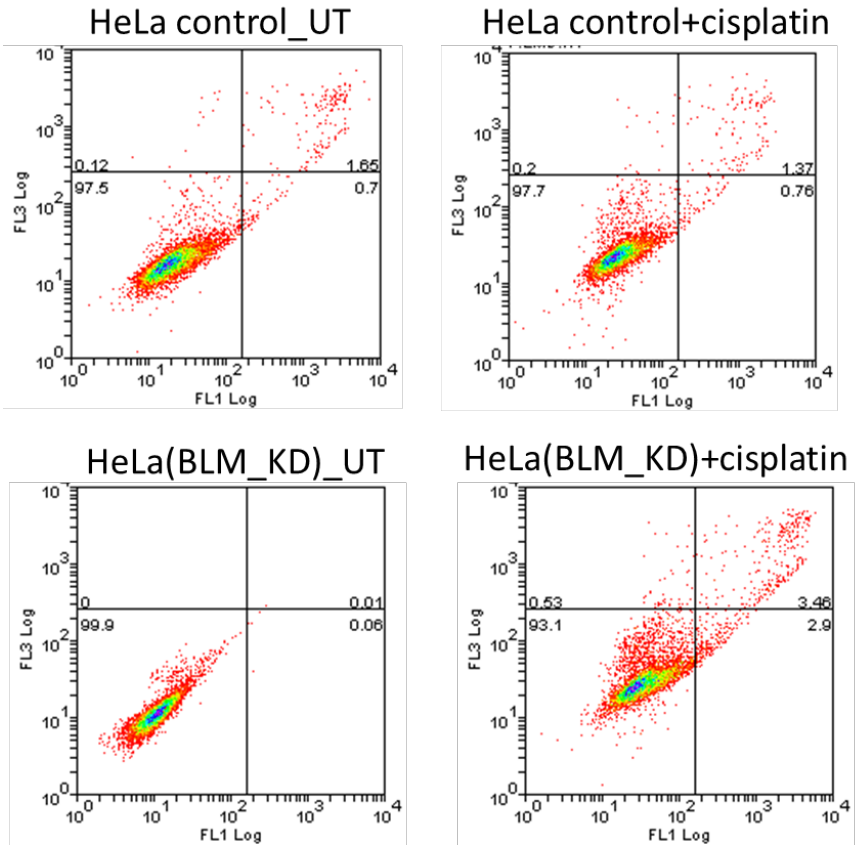


Figure 7.21 Representative images of annexin V analysis by flow cytometry

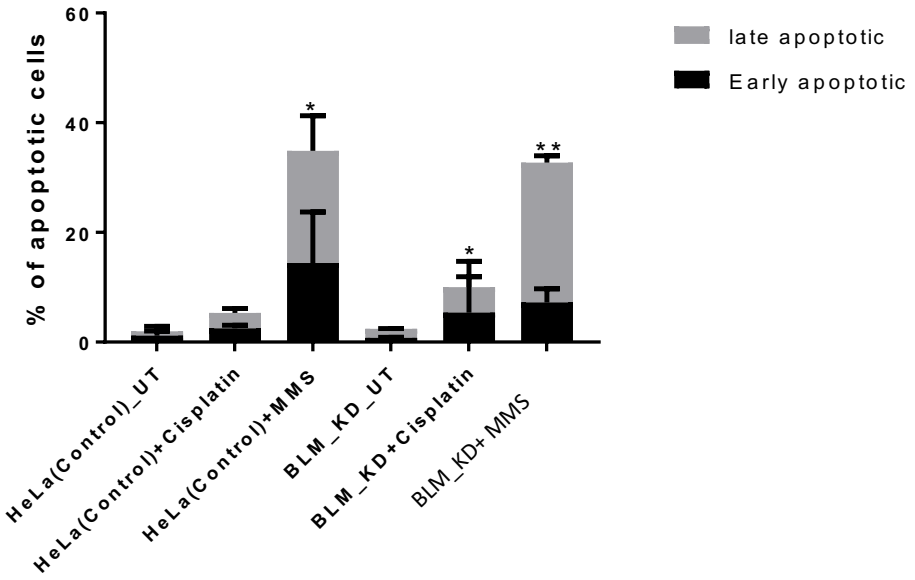


Figure 7.22 Quantification of apoptotic cells by flow cytometry in HeLa control and BLM-KD HeLa cells treated with cisplatin (1μM concentration at 24 hours)

Graph showing that Cisplatin treated BLM-KD-HeLa cells demonstrate more apoptosis as compared to BLM-KD untreated cells. Data presented as mean ± SEM of two independent experiments, each performed in triplicate. *p<0.05, **p<0.01, calculated by one-way Anova.

7.5 Discussion

In this chapter, we evaluated BLM protein and mRNA expression in sporadic breast cancers. We also investigated cell survival and functional studies on BLM-KD HeLa cells. Our data provide strong evidence that high BLM mRNA expression is a prognostic and predictive biomarker in breast cancer. High BLM

mRNA was correlated with aggressive breast cancer phenotypes. High BLM mRNA was also linked to aggressive molecular phenotypes including PAM50. Luminal B, PAM50.Her2 and PAM50.Basal molecular phenotypes. As discussed previously that BLM plays a vital role during replication and proliferation, so it was no surprise that high BLM mRNA was more common in aggressive breast cancers. We also noticed that low BLM mRNA expression was more frequent in PAM50. Lumina A and ER+/Her-2 negative/low proliferation Genefu subtype tumours, which further supported our hypothesis. In this study, BLM mRNA levels are also linked to biologically distinct integrative clusters as described in detail in the METABRIC study (Curtis et al, 2012a). High BLM mRNA level was frequent in intClust 10 subgroup which is the most highly genomically unstable subgroup with basal-like features. Low BLM mRNA level was noticed in intClust 3 subgroup which has low genomic instability. This data indicate that the BLM mRNA level may also give information regarding genomic stability status in breast cancer. In addition, high BLM mRNA level is also frequently seen in intClust 5 (HER-2 enriched with worst survival), intClust 9 (8q cis-acting/20qamplified mixed subgroup), and intClust 1 (17q23/20q cis-acting luminal B subgroup) subgroups that also show an aggressive phenotype. On the other hand, low BLM mRNA level is linked to intClust 4 (includes both ER-positive and ER-negative cases with a flat copy number landscape and termed the 'CNA-devoid' subgroup with extensive lymphocytic infiltration) (Curtis et al, 2012a), intClust 7 [16p gain/16q loss with higher frequencies of 8q amplification luminal A subgroup (Curtis et al, 2012a)] and intClust 8 subgroups

[classical 1q gain/16q loss luminal A subgroup (Curtis et al, 2012a)]. In the univariate analysis and in multivariate analysis, BLM mRNA expression significantly influenced survival. Although the precise molecular mechanisms regulating BLM mRNA expression remains largely unknown, current evidence indicates that BLM is preferentially up-regulated in transformed and proliferating cells (Kawabe et al, 2000). Our study data supports this preclinical observation. Our results are also consistent with the results of recent study published by Zhu et al. This study showed that high BLM mRNA expression in breast cancer patients was associated with reduced distant metastasis free survival (Zhu et al, 2018)

BLM is a 1417 amino acid protein. It has a highly conserved centrally located helicase domain, in addition to multiple domains which are involved in DNA-binding, ATPase activity and interaction with other binding partners. The nuclear localization signal is located in the C-terminal region of the BLM protein. BLM is mainly expressed in the late S/G2 phase of the cell cycle. In response to DNA damage, BLM localizes to the nucleus interacting directly with Rad51 and is involved in Homologous recombination repair that is functional during the S-phase of the cell cycle (Arora et al, 2015a; Bischof et al, 2001).

Studies have shown that BLM undergoes post-translational modifications such as phosphorylation and SUMOylation which can alter intracellular localization and biochemical activity (Brosh Jr, 2013; Chu & Hickson, 2009; Croteau et al, 2014a; Dautin & Bernstein, 2007; Payne & Hickson, 2009). After making an observation that BLM mRNA up-regulation was associated with aggressive

breast cancer phenotypes, we proceeded to evaluate BLM protein expression by immunohistochemistry technique. We made an interesting observation that BLM protein shows complex sub-cellular localization in breast tumours. Low nuclear and high cytoplasmic BLM co-expression was significantly correlated to aggressive phenotypes. We postulate that cytoplasmic accumulation in a proportion of breast tumours likely indicate an abnormality in mechanisms involved in nuclear localization of BLM. Cytoplasmic accumulation in conjunction with low nuclear BLM expression could potentially lead to genomic instability in tumours, hence promoting a mutator phenotype characterized by aggressive cancer biology. Our hypothesis was also supported by the fact that we noticed that tumours with high cytoplasmic/low nuclear BLM levels were more likely to be high grade, high mitotic index, pleomorphism, tumour type, PR-negative, triple negative and basal-like phenotype. Furthermore, low nuclear BLM was also linked to the impaired expression of other DNA repair factors including BRCA1 negativity, low XRCC1, low FEN1, low SMUG1, low APE1, low Polβ, low ATR, and low DNA-PKcs.

As discussed previously, BLM and Rad51 are known to interact with each other for effective HR repair, hence we performed BLM-Rad51 co-expression studies. As per our expectation, we observed that low nuclear BLM/low nuclear RAD51 tumours revealed aggressive phenotype and were associated with poor survival. In a previous small study in normal and neoplastic human cells, BLM protein expression was shown to be overexpressed in a panel of tumour tissue compared to normal tissue including a cohort of nine breast tumours (Turley et al, 2001).

Similar to our study, the authors observed a positive correlation between BLM and Ki67 but did not report any clinicopathological associations (Turley et al, 2001)

In this study, BLM mRNA overexpression was associated with aggressive tumours and poor outcomes but at the protein level, the association appeared more complex with low nuclear/high cytoplasmic BLM protein level being linked to adverse features. We postulate that this could either be due to the fact that BLM mRNA is subjected to post-transcriptional regulation or another possibility is that post-translational dysregulation of BLM protein expression/sub-cellular localization could affect BLM mRNA expression through feedback loops. Further mechanistic studies are therefore required to understand the mechanism of regulation of BLM in vivo (Arora et al, 2015a).

Our study also indicates that BLM could be a promising marker for personalization breast cancer therapy. As low BLM is a marker of impaired HR repair, we would argue that low BLM tumours could be exploited by synthetic lethality approach using base excision repair inhibitors such as those targeting PARP or APE1. Alternatively, high BLM tumours could be targeted by small molecular inhibitors of BLM that are currently under development (Banerjee et al, 2013; Rosenthal et al, 2010).

BLM has previously shown to play a role in cell cycle regulation. BLM is abundantly expressed in mitosis and gets phosphorylated by CDC2 kinase in the mitotic phase (Bayart et al, 2006). We investigated cell cycle pattern in BLM-

KD HeLa cells. We observed differential cell cycle pattern in BLM-KD HeLa cells as compared to control HeLa cells. BLM-KD resulted in cell cycle arrest in G1 phase. It has been reported previously that BLM is down regulated in G1 phase, accumulates in S-phase and persists through G2/M. Thus our finding confirms the role of BLM for S-phase entry and cell cycle progression.

Additionally, the growth curve data for BLM deficient cells illustrate a slower growth rate for BLM knock down cells which could indicate longer doubling time and slower cell cycle progression in comparison to HeLa control cells. When treated with platinum drug, both HeLa control and BLM-KD HeLa cells showed S-phase arrest, however, this S-phase arrest was more profound in BLM-KD HeLa cells. As majority of BLM-KD HeLa cells were arrested in G1 phase ,cisplatin treatment led to substantial S-phase arrest in BLM-KD Hela cells as compared to the control HeLa cells. We observed more γ-H2AX accumulation in BLM-KD HeLa cells treated with cisplatin, which reflects double-strand breaks (DSBs) accumulation in BLM deficient cells and defective DNA repair and replication on platinum treatment in BLM deficient cells.

MTS and Clonogenic data showed more platinum sensitivity in BLM-KD HeLa cells. The cisplatin doses tested showed that HeLa cell line is platinum sensitive as well. Therefore, further study will be needed to confirm this finding in a platinum resistance cell line. In this chapter, our cell line data results show a potential platinum sensitivity in low BLM expressing tumours which emphasize

that BLM could be a potential predictive marker for platinum response in aggressive tumours.

In summary, this chapter provides clinical evidence that BLM is a promising predictive and prognostic biomarker in sporadic breast cancers.

Chapter 8

General discussions and conclusions

8 General discussion and conclusions

8.1 General Discussion

In this study we did comprehensive evaluation of five DNA RECQ helicases (RECQL1, RECQL4, RECQL5, WRN and BLM) at protein and mRNA level. At mRNA level, we observed that breast cancer patients with low levels of RECQL1 and WRN mRNA had poor survival. In contrast to our results, Zhu et al. recently showed that high expression of RECQL1 mRNA expression was significantly associated with reduced relapse free survival and post progression survival in all breast cancers. However in this study high RECQL1 expressing patients who were basal-like and p53 mutant had better survival outcome (Zhu et al, 2018). Furthermore, RECQL1 has been shown to be overexpressed in glioblastoma (Mendoza-Maldonado et al, 2011b), hepatocellular carcinoma (Futami et al, 2010a), ovarian cancers (Sanada et al, 2013), melanoma (Jewell et al, 2010), non-small cell lung cancer (Li et al, 2016), multiple myeloma (Viziteu et al, 2017) and head & neck cancer models (Zhang et al, 2006). In these studies high expression was associated with poor outcome. For WRN, our results are consistent with recently published study by Zhu et al. which showed that high expression of WRN mRNA was associated with improved overall and relapse free survival in breast cancer patients (Zhu et al, 2018). The exact mechanism of low mRNA expression of these RECQ helicase is unknown. All these helicases play important vital role in genomic stability (Sami & Sharma, 2013b; Sharma, 2014b; Sharma et al, 2012; Wu & Brosh, 2010), so low levels of RECQL1, and

WRN might be promoting genomic instability and mutator phenotype, hence leading to poor breast cancer phenotype and survival outcome. Two recent studies have shown that germ-line RECQL1 mutations are associated with increased breast cancer susceptibility (Cybulski et al, 2015a; Sun et al, 2015a). Moreover, whole exome sequencing has identified distinct populations of breast cancer susceptibility mutations in RECQL1 (Banerjee & Brosh, 2015). Mutations in the human WRN gene leading to the loss of WRN gene product are associated with Werner syndrome (WS) (Goto et al, 1992). We speculate that low expressions of RECQL1 and WRN could be due to epigenetic silencing of their promoter similar to previously reported epigenetic silencing of the BRCA1 promoter in approximately 11%-14% of breast tumours (Turner et al, 2004a). This suggest that breast tumours with low RECQL1 and low WRN might have oncogenic potential.

For RECQL5, RECQL4 and BLM, high mRNA expression was associated with poor outcome in whole cohort and ER+ cohort (including those who received endocrine therapy) suggesting their prognostic and predictive significance. For BLM, our results are consistent with the results of recent study published by Zhu et al. This study showed that high BLM mRNA expression in breast cancer patients was associated with reduced distant metastasis free survival (Zhu et al, 2018). This study also looked at RECQL5 mRNA expression in breast cancer and showed that increased expression of RECQL5 mRNA expression was significantly associated with poor overall survival in HER2 negative patients. Similar to our RECQL4 mRNA results, recent studies have shown that RECQL4

is overexpressed in gastric cancer, hepatocellular carcinoma and breast cancer and high RECQL4 mRNA expression is associated with poor outcome (Chen et al, 2018; Li et al, 2018; Zhu et al, 2018).

As discussed previously, RECQL5, RECQL4 and BLM play vital role in dealing with replication stress which is common occurrence in proliferating tumours. We postulate that high expression of RECQL5 , RECQL4 and BLM might be a secondary event that may allow rapidly progressive cancer cells to maintain high proliferative rate essential for cancer cell survival. Moreover, the prognostic significance was mainly observed in ER+ sub-group, supporting the evidence that high RECQL5 , high RECQL4 and high BLM mRNA expressions were more likely in ER+/HER2 -/high proliferation Genefu molecular sub-types of METABRIC cohort.

At protein level, similar to our RECQL1 and WRN mRNA results, low levels of RECQL1 and WRN protein levels were significantly associated with poor breast cancer survival in whole cohort suggesting prognostic significance. For RECQL4, BLM and RECQL5, we saw discordance between mRNA and protein results. Low RECQL5 protein expression was associated with aggressive phenotype but RECQL5 protein expression did not influence survival outcomes. Our observation indicates that RECQL5 may be subjected to complex post-transcriptional regulation. For RECQL4, we observed that tumours with low nuclear/high cytoplasmic expression had aggressive features and were associated with poor breast cancer-specific survival. We speculate that cytoplasmic accumulation of RECQL4 may reflect altered sub-cellular

localisation due to additional defects such as post-translational acetylation mediated mechanisms (Dietschy et al, 2009). An additional possibility is that cytoplasmic accumulation may reflect a mitochondrial role of RECQL4 (Chi et al, 2012; Croteau et al, 2012a; Gupta et al, 2014). For BLM, high BLM mRNA expression was associated with aggressive tumours and poor outcomes but at the protein level, the association appeared complex with low nuclear/high cytoplasmic BLM protein level being linked to adverse features. We speculate that this could either be due to the fact that BLM mRNA is subjected to post-transcriptional regulation or another possibility is that post-translational dysregulation of BLM protein expression/sub-cellular localization could affect BLM mRNA expression through feedback loops.

There is evidence to suggest that RECQ helicases co-operate with each other to perform their vital biological roles so there may be functional redundancy between them (Hu et al, 2005; Otsuki et al, 2008; Popuri et al, 2017b; Singh et al, 2012b). Hence, we looked at correlation between five helicases (Table 8.1). We saw significant correlation between low RECQL1 nuclear and high nuclear BLM ($p<0.05$); low nuclear RECQL4 and high nuclear WRN & BLM ($ps<0.05$) and low nuclear RECQL5 and high nuclear BLM ($p<0.05$). 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 other helicase.

Table 8.1: Correlation between five RECQ helicases

RECQL1			**Adjusted P-Value**
	Low N (%)	High N (%)	
RECQL5 Low High	201 (52.6) 181 (47.4)	140 (37.3) 235 (62.7)	**$6.0X10^{-6}$**
RECQL4 Low High	245 (65.9) 127 (34.1)	173 (50.6) 169 (49.4)	**$3.5X10^{-5}$**
BLM Low High	120 (30.7) 271 (69.3)	71 (19.5) 293 (80.5)	**$4.1X10^{-4}$**
WRN Low High	137 (51.7) 128 (48.3)	113 (42.6) 152 (57.4)	**0.037**
RECQL4			**Adjusted P-Values**
	Low N (%)	High N (%)	
RECQL1 Low High	245 (58.6) 173 (41.4)	127 (42.9) 169 (57.1)	**$3.5X10^{-5}$**
RECQL5 Low High	279 (57.2) 204 (42.8)	113 (32.7) 233 (67.3)	**$2.7X10^{-12}$**
BLM Low High	167 (32.9) 341 (67.1)	58 (16.6) 291 (83.4)	**0.010**
WRN Low High	177 (49.7) 179 (50.3)	89 (38.9) 140 (61.1)	**$2.4X10^{-5}$**
RECQL5			**Adjusted P-Values**

	Low N (%)	High N (%)	
RECQL1 Low High	201 (58.9) 140 (41.1)	181 (43.5) 235 (56.5)	**$2.4X10^{-5}$**
RECQL4 Low High	279 (71.2) 113 (28.8)	209 (47.3) 233 (52.7)	**$2.7X10^{-12}$**
BLM Low High	170 (37.0) 290 (63.0)	85 (16.5) 429 (83.5)	**$4.5X10^{-13}$**
WRN Low High	175 (55.2) 142 (44.8)	134 (37.7) 221 (62.3)	**$6.0X10^{-6}$**
BLM			**Adjusted P-Values**
	Low N (%)	High N (%)	
RECQL1 Low High	120 (62.8) 71 (37.2)	271 (48.0) 293 (52.0)	**$4.1X10^{-4}$**
RECQL4 Low High	167 (74.2) 58 (25.8)	341 (54.0) 291 (46.0)	**$1.07X10^{-7}$**
RECQL5 Low High	170 (66.7) 85 (33.3)	290 (40.3) 429 (59.7)	**$4.5X10^{-13}$**
WRN Low High	100 (52.6) 90 (47.4)	236 (46.0) 233 (54.0)	**0.118**
WRN			**Adjusted P-Values**

	Low N (%)	High N (%)	
RECQL1 Low High	 137 (54.8) 113 (45.2)	 128 (45.7) 152 (54.3)	**0.037**
RECQL4 Low High	 177 (66.5) 89 (33.5)	 179 (56.1) 140 (43.9)	**0.010**
RECQL5 Low High	 175 (56.6) 134 (43.4)	 142 (39.1) 221 (60.9)	**$6.0X10^{-6}$**
BLM Low High	 100 (29.8) 236 (70.2)	 90 (24.5) 277 (75.5)	0.118

Bold = Statistically significant; Adjusted p values were calculated using Benjamini-Hochberg false discovery rate method to adjust for multiple testing.

In multivariate Cox regression analysis that included all five RECQ helicases and validated prognostic factors, such as lymph node, stage, and NPI, none of the helicases came out as powerful independent predictor for breast cancer-specific survival.

It will be useful to look at all helicases in future studies. Our observation suggest that RECQL1 and WRN deficient tumours can be targeted with PARP or APE1 inhibitors using synthetic lethality approach. Moreover, high BLM expressing tumours can be targeted with BLM small molecular inhibitors in combination with chemo as we have shown that BLM deficient cell lines are sensitive to platinum based chemo.

8.2 Summary of Key findings

1) Low RECQL1 expression is associated with aggressive phenotypes and poor breast cancer survival, suggesting that these tumours may be amenable to targeting with synthetic lethality approach.

2) High RECQL5 mRNA expression and high RECQL5/low RAD51 protein expression is associated with aggressive breast cancer phenotypes and poor survival which is more pronounced in ER-positive subgroup.

3) RECQL4 amplification/gain in copy numbers, high RECQL4 mRNA, expression and low nuclear/high cytoplasmic RECQL4 protein expression is associated with aggressive breast cancer phenotypes, and poor survival. In tumours with high nuclear RECQL4 protein expression, c-MYC can stratify patients into prognostic groups.

4) Low WRN mRNA levels and low cytoplasmic/low nuclear WRN protein levels are associated with poor breast cancer-specific survival. ER-positive tumours with high TOPO1/high WRN expression show poor breast cancer-specific survival.

5) High BLM mRNA levels and low nuclear BLM/low nuclear RAD51 protein levels are associated with aggressive breast cancer phenotypes and poor breast cancer-specific survival. BLM-KD cell line shows platinum sensitivity.

8.3 Future directions

8.3.1 Assessment of RECQ helicase protein and mRNA expression in same patient cohort

One of the limitation of our study was that we studied mRNA and protein expression in two different patient cohorts. It will be important to study both protein and mRNA expression in the same patient cohort.

8.3.2 Synthetic Lethality

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 HR-defective BRCA1/2-deficient tumours. Given the role of RECQ helicase 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 under wide investigation in clinical trials.

8.3.3 Evaluation of helicase inhibitors as personalized cancer therapy

As discussed earlier, BLM and WRN small molecular inhibitors are currently being developed. As we have shown that tumours with high BLM expression have poor survival, so these tumours can be targeted with small molecule inhibitors on its own or in combination with chemotherapy or BER protein inhibitors.

8.3.4 Further cell line work

The preliminary cell line experiments not only confirm how BLM interaction with cell cycle regulators can play a vital role in drug response but also provide an early evidence that BLM is an important prognostic marker for tumour aggressiveness as shown earlier. It is important to investigate these findings further in breast cancer cell lines through BLM knockdown with siRNA and Knock out with CRISPR cas-9.

Chapter 9

References

9 References

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