Transcriptomic and protein expression analysis reveals clinicopathological significance of Bloom's syndrome helicase (BLM) in breast cancer

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ABSTRACT

BLM has key roles in homologous recombination repair, telomere maintenance and DNA replication. Germ-line mutation in the *BLM* gene causes Bloom's syndrome, a rare disorder characterised by premature aging and predisposition to multiple cancers including breast cancer. The clinicopathological significance of BLM in sporadic breast cancers is unknown. We investigated *BLM* mRNA expression in the Molecular Taxonomy of Breast Cancer International Consortium cohort (n=1950) and validated in an external dataset of 2413 tumours. BLM protein level was evaluated in the Nottingham Tenovus series comprising 1650 breast tumours. High *BLM* mRNA expression was highly significantly associated with high histological grade, larger tumour size, ER negative, PgR negative and triple negative phenotypes (ps<0.0001). High *BLM* mRNA expression was also linked to aggressive molecular phenotypes including PAM50.Her2 (p<0.0001), PAM50.Basal (p<0.0001) and PAM50.LumB (p<0.0001) and Genufu subtype (ER+/Her2-/High proliferation) (p<0.0001). PAM50.LumA tumours and Genufu subtype (ER+/Her2-/low proliferation) were more likely to express low levels of *BLM* mRNA (ps<0.0001). Integrative molecular clusters (intClust) intClust.1 (p<0.0001), intClust.5 (p<0.0001), intClust.9 (p<0.0001) and intClust.10 (p<0.0001) were also more likely in tumours with high *BLM* mRNA expression. High *BLM* mRNA expression was associated with poor breast cancer specific survival (BCSS) (ps<0.000001). At the protein level, altered sub-cellular localisation with high cytoplasmic BLM and low nuclear BLM was linked to aggressive phenotypes. In multivariate analysis, BLM mRNA and BLM protein levels independently influenced BCSS (p=0.03). This is the first and the largest study to provide evidence that BLM is a promising biomarker in breast cancer.

INTRODUCTION

Blooms syndrome helicase (BLM) is a key member of the RecQ family of DNA helicases and essential for the maintenance of genomic stability. BLM is an ATP-dependent 3'-5' DNA helicase involved in unwinding a variety of DNA substrates that can arise during DNA replication and repair (1-5). BLM has important roles in the initiation and regulation of homologous recombination (HR) repair of DSB (double-strand breaks). In addition, BLM is required for Holliday junction dissolution during the terminal stages of HR. To accomplish its various biological functions, BLM interacts with several DNA repair factors including topoisomerase III, hRMI1, hRMI1 and Rad51. BLM is also part of the BRCA1-associated genome surveillance complex (BASC), which contains BRCA1, MSH2, MSH6, MLH1, ATM, PMS2, the RAD50-MRE11-NBS1 protein complex and BLM (6). In addition to its DNA repair function, BLM is involved in the processing of stalled replication forks during replication and in telomere maintenance in cells (1-5).

Bloom's syndrome (BS) is a rare disorder caused by germ-line mutation in the *BLM* gene. BS is characterised by cancer predisposition, growth retardation, immunodeficiency, sunlight hypersensitivity and impaired fertility (7). BLM germ-line mutation results in dramatic reduction in *BLM* mRNA levels and BLM protein expression leading to extensive chromosomal instability manifested classically as excessive frequency of sister chromatid exchanges (SCEs) in BS cells (1-5). BS patients are prone to develop leukemia, lymphomas and to a variety of epithelial cancers including breast cancers (7). Interestingly, polymorphisms in the *BLM* gene has been associated with increased risk of development of sporadic breast cancers (8). In preclinical models, depletion of BLM by shRNA not only reduced proliferation in cells (9) but also sensitized to chemotherapeutic agents such as

camptothecins, cisplatin, 5-fluoruracil and hydroxyurea treatment (1-5, 7). BLM is an attractive anti-cancer drug target and small molecule inhibitors of BLM are currently under pre-clinical development (10). However, target validation studies including prognostic and/or predictive significance of BLM in human sporadic tumours have not been reported and therefore remain largely unknown. We hypothesised that BLM may be dysregulated in sporadic breast cancers and influence clinical outcomes in patient. Here in we present the first and the largest comprehensive study providing compelling evidence that altered BLM expression has prognostic and predictive significance in patients. Our data suggest that BLM is a rational target in breast cancer.

MATERIALS AND METHODS

BLM gene expression: METABRIC (Molecular Taxonomy of Breast Cancer International Consortium) cohort was evaluated for *BLM* gene expression. The METABRIC study protocol, detailing the molecular profiling methodology in a cohort of 1980 breast cancer samples is described by Curtis et al (11). Patient demographics are summarized in supplementary Table S1 of supporting information. ER positive and/or lymphnode negative patients did not receive adjuvant chemotherapy. ER negative and/or lymphnode positive patients received adjuvant chemotherapy. RNA was extracted from fresh frozen tumours and subjected to transcriptional profiling on the Illumina HT-12 v3 platform. The data was pre-processed and normalized as described previously (11). *BLM* expression was investigated in this data set (BLM probe id: ILM_1709484). 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 was used to identify a cut-off in gene expression values such that the resulting subgroups have significantly different survival courses.

The external validation was done using bc-GenExMiner v3.0 (Breast Cancer Gene-Expression Miner v3.0) online dataset (http://bcgenex.centregauducheau.fr) comprising previously published gene expression datasets from fifteen independent breast cancer studies totalling 2413 tumours and summarized in supplementary Table S2. The bioinformatics tool is composed of two statistical mining modules. The first module is a "prognostic module", which offers the possibility to evaluate the in vivo prognostic informativity of genes of interest in breast cancer, and the second module is a "correlation module", which permits to compute correlation coefficients between gene expressions or to find lists of correlated genes in breast cancer. We used the prognostic module in this external validation. Statistical analyses were performed by means of survival statistical tests (Cox model, Kaplan–Meier and Forest plots). Supplementary Table S2 summarizes individual cohorts where BLM mRNA expression was investigated.

BLM protein expression in breast cancer: The study was performed in a consecutive series of 1650 patients with primary invasive breast carcinomas who were diagnosed between 1986 and 1999 and entered into the Nottingham Tenovus Primary Breast Carcinoma series. Patient demographics are summarised in Supplementary Table S3. This is a well-characterized series of patients with long-term follow-up that have been investigated in a wide range of biomarker studies (12-20). All patients were treated in a uniform 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 scheduled based on prognostic and predictive factor status, including Nottingham Prognostic Index (NPI), oestrogen receptor-α (ER-α) status, and menopausal status. Patients with NPI scores of <3.4 (low risk) did not receive AT. In pre-menopausal patients with NPI scores of ≥3.4 (high risk), classical Cyclophosphamide, Methotrexate, and 5-Flurouracil (CMF) chemotherapy was given; patients with ER-α positive tumours were also offered endocrine therapy.

Postmenopausal patients with NPI scores of ≥3.4 and ER-α positivity were offered endocrine therapy, while ER-α negative patients received classical CMF chemotherapy. Median follow up was 111 months (range 1 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 relapse. Breast cancer specific survival (BCSS) was defined as the number of months from diagnosis to the occurrence of BC related-death. Local recurrence free survival (LRS) was defined the number of months from diagnosis to the occurrence of local recurrence. DM-free survival was defined as the number of months from diagnosis to the occurrence of DM relapse. Survival was censored if the patient was still alive at the time of analysis, lost to follow-up, or died from other causes.

Tumor Marker Prognostic Studies (REMARK) criteria, recommended by McShane et al (21), were followed throughout this study. Ethical approval was obtained from the Nottingham Research Ethics Committee (C202313).

Tissue Microarrays (TMAs) and immunohistochemistry (IHC): Tumours were arrayed in tissue microarrays (TMAs) constructed with 2 replicate 0.6mm cores from the centre and periphery of the tumours. The TMAs were immunohistochemically profiled for BLM and other biological antibodies (Supplementary Table S4) as previously described (12-20). Immunohistochemical staining was performed using the Thermo Scientific Shandon Sequenza chamber system (REF: 72110017), in combination with the Novolink Max Polymer Detection System (RE7280-K: 1250 tests), and the Leica Bond Primary Antibody Diluent (AR9352), each used according to the manufacturer's instructions (Leica Microsystems). The tissue slides were deparaffinised with xylene and then rehydrated through five decreasing

concentrations of alcohol (100%, 90%, 70%, 50% and 30%) for two minutes each. Pre-treatment antigen retrieval was performed on the TMA sections using sodium citrate buffer (pH 6.0) and heated for 20 minutes at 950C in a microwave (Whirpool JT359 Jet Chef 1000W). A set of slides were incubated for 18 hours with the primary anti-BLM antibody (NBP1-89929, Novus Biologicals, UK), at a dilution of 1:100. Negative and positive (by omission of the primary antibody and IgG-matched serum) controls were included in each run. The negative control ensured that all the staining was produced from the specific interaction between antibody and antigen.

Evaluation of immune staining: The tumour cores were evaluated by two scorers (TAF and AA) and the concordance between the two scorer was excellent (k = 0.79). Whole field inspection of the core was scored and intensities of nuclear staining were grouped as follows: 0 = no staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining. The percentage of each category was estimated (0-100%). H-score (range 0-300) was calculated by multiplying intensity of staining and percentage staining. A median H score of $\geq$ 50 was taken as the cut-off for high BLM nuclear and cytoplasm expression. Not all cores within the TMA were suitable for IHC analysis as some cores were missing or lacked tumour (<15% tumour).

Statistical analysis: Data analysis was performed using SPSS (SPSS, version 17 Chicago, IL). Where appropriate, Pearson's Chi-square, Fisher's exact, Student's t and ANOVA one way tests were used. Cumulative survival probabilities were estimated using the Kaplan–Meier method, and differences between survival rates were tested for significance using the log-rank test. Multivariate analysis for survival was performed using the Cox proportional hazard model. The proportional hazards assumption was tested using standard log-log plots. Hazard ratios (HR) and 95% confidence intervals (95% CI) were estimated for each variable.

All tests were two-sided with a 95% CI and a p value < 0.05 considered significant. For multiple comparisons, p values were adjusted according to Benjamini-Hochberg method (22).

Breast cancer cell lines and culture: MCF-7 (ER+/PR+/HER2-, BRCA1 proficient), MDA-MB-231 (ER-/PR-/HER2-, BRCA1 proficient), MDA-MB-468 (ER-/PR-/HER2-, BRCA1 proficient) and MDA-MB-436 (ER-/PR-/HER2-, BRCA1 deficient) were purchased from ATCC and were grown in RPMI (MCF-7, MDA-MB-231) or DMEM (MDA-MB-468 and MDA-MB-436) medium with the addition of 10% foetal bovine serum and 1% penicillin/streptomycin. Cell lysates were prepared and Western blot analysis performed. Primary anti-BLM antibody (NBP1-89929, Novus Biologicals, and UK) was incubated over night at room temperature at a dilution of 1:1500. Primary anti-β actin antibody (1:10000 dilution [Abcam]) was used as a loading control. Infrared dye-labelled secondary antibodies (Li-Cor) [IRDye 800CW Mouse Anti-Rabbit IgG and IRDye 680CW Rabbit Anti-Mouse IgG] were incubated at a dilution of 1:10000 for 1 hour. Membranes were scanned with a Li-Cor Odyssey machine (700 and 800nm) to determine protein expression.

Quantitative real –time PCR: Total RNA was extracted from MCF-7, MDA-MB-231, MDA-MB-468 and MDA-MB-436 cells using RNeasy Mini kit (QIAGEN, UK). The quantification of the extracted RNA was done using a NanoDrop 2000c Spectrophotometer (Thermo Scientific, UK). The cDNA was synthesized from 0.5 μg of total RNA using RT^2 first strand kit (QIAGEN, UK). qPCR was performed using SYBR Green PCR Master mix (applied biosystems,Warrington,UK) with primer set (BLM QuantiTect Prier Assay,Cat. No. QT00027671, QIAGEN) targeting BLM gene. RECQL5 mRNA level was also quantified. The glyceraldehyde-3-phosphate dehydrogenase housekeeper gene was used as an internal control (GAPDH QuantiTect Prier Assay, Cat. No. QT00079247, QIAGEN). The real-time

PCR for each RNA sample was performed in triplicate. NTC (No Template Control) was used to rule out cross contamination of reagents and surfaces. NTC included all the RT-PCR reagents except the RNA template. Minus reverse transcriptase (- RT) control was used to rule out genomic DNA contamination.

RESULTS

High *BLM* transcript levels correlate to aggressive breast cancer

BLM mRNA level was investigated in the METABRIC (Molecular Taxonomy of Breast Cancer International Consortium) cohort comprising 1980 breast tumours. High *BLM* mRNA expression was highly significantly associated with aggressive clinicopathological features (Table 1) including high histological grade, larger tumour size, high-risk Nottingham prognostic index (NPI >3.4), Her-2 over expression, ER negative, PgR negative and triple negative phenotypes (ps<0.0001). High *BLM* mRNA expression was also found to be significantly associated with 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 (ps<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 (11). High *BLM* mRNA expression was 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 (11). Low *BLM* mRNA expression was associated 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 (11).

We then proceeded to 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 1A). In ER+ sub-group, high *BLM* mRNA expression was associated with poor BCSS ($p<0.0001$) (Figure 1B). In the ER+ sub-group that received adjuvant endocrine therapy, high *BLM* mRNA expression remains associated with poor BCSS ($p<0.0001$) (Figure 1D). In ER- sub-group, low *BLM* mRNA expression was associated with poor BCSS with borderline significance ($p=0.049$) (Figure 1C). In the ER- sub-group that received adjuvant chemotherapy, *BLM* mRNA expression did not significantly influence outcome ($p=0.062$) (Figure 1E). 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 2). External validation was performed using bc-GenExMiner v3.0 (Breast Cancer Gene-Expression Miner v3.0) online dataset (http://bcgenex.centregauducheau.fr) comprising previously published gene expression datasets from fifteen independent breast cancer studies totalling 2413 tumours and summarized in supplementary materials and Table S2. The dataset provides information on metastasis relapse (MR) free survival data. As shown in the Forest plot (Supplementary Figure S1) low *BLM* mRNA expression was significantly associated with better MR free survival Supplementary Figure S1A and S1B). Taken together, the data provides the first compelling evidence that high BLM *mRNA* expression has prognostic and/or predictive significance in breast cancer.

Altered sub-cellular localisation of BLM protein is associated with aggressive breast cancer

BLM is a 1417 amino acid protein with a highly conserved centrally located helicase domain. In addition, BLM has multiple domains involved in DNA- binding, ATPase activity and interaction with other binding partners. The nuclear localisation signal is present in the C-terminal region of the protein (1-5). BLM is primarily expressed in late S/G2 phase of the cell cycle. Upon DNA damage BLM localises to the nucleus where it interacts with Rad51 and is intimately involved in HR repair that is operational during the S-phase of the cell cycle (23). In addition, BLM undergoes post translational modifications such as phosphorylation and SUMOylation that can affect intracellular localisation and biochemical activity (1-5). We proceeded to evaluation of BLM protein expression in breast cancers. We initially profiled a panel of breast cancer cell lines. As shown in Supplementary Figure S2A; MDA-MB-231, MDA-MB-436 and MDA-MB-468 breast cancer cells have robust expression of BLM protein. In contrast, MCF-7 has low BLM expression. At the mRNA level, *BLM* is highly expressed in MDA-MB-231 cells compared to MCF-7, MDA-MB-436 and MDA-MB-468 cells. The data demonstrates differential BLM expression across different breast cancer cell lines. We then conducted immunohistochemical evaluation of BLM protein expression in the Nottingham Tenovus series comprising 1650 breast tumours. Surprisingly, we observed complex sub-cellular localization of BLM protein in breast cancers including tumours exhibiting nuclear staining only, cytoplasmic staining only, nuclear-cytoplasmic co-expression or negative staining.

Nuclear BLM protein level and breast cancer: Low nuclear BLM level was seen in 54% of tumours (n= 682/1253) and high nuclear BLM level was observed in 46% of tumours (n=

571/1253) (Supplementary Figure S2B). As shown in supplementary table S5, low nuclear BLM 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 more common in tumours with low nuclear BLM protein level ($p<0.01$). BRCA1 negative, low XRCC1, low FEN1, low SMUG1, low APE1, low Polβ, low ATR 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 pCHEK1 and low nuclear Chk2 were more common in tumours with low nuclear BLM protein level ($p<0.05$).

Cytoplasmic BLM protein level and breast cancer: High cytoplasmic BLM level was seen in 53% of tumours (n= 642/1212) and low cytoplasmic BLM level was seen in 47% of tumours (n= 570/1212) (Supplementary Figure S2B). As shown in supplementary table S6, high cytoplasmic BLM level was significantly associated with pleomorphism, tumour type, high XRCC1, high FEN1, high APE1, high ATR, high DNA-PKcs, high MIB1, high Chk2, high Bax levels.

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 (Supplementary Figure S2B). As shown in Table 3, 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.

BLM and Rad51 protein co-expression in breast cancer: A key interacting partner of BLM is Rad51 (24). Together BLM-Rad51 play an essential role in HR repair (1-5). We therefore conducted exploratory nuclear co-expression studies in breast cancer. As shown in supplementary Table S7, we observed significant association between BLM-/Rad51- tumours and NPI>3.4, high grade, high mitotic index, pleomorphism, tumour type, ER- and PR- tumours.

Survival analyses: In univariate analysis, in high risk ER positive tumours that received no endocrine therapy, patients whose tumours had high nuclear/low cytoplasmic BLM had poor breast cancer specific survival (p=0.036) implying that altered expression has prognostic significance (Supplementary Figure S3). In patients who received endocrine therapy, although low nuclear/high cytoplasmic BLM tumours have the worst survival, there was no statistical significance. Similarly in ER- tumours, BLM level did not significantly influence survival. When BLM (nuclear) and Rad51 (nuclear) were investigated together, BLM-/Rad51- tumours have poor survival in the whole cohort and in the ER- sub-group that received adjuvant chemotherapy (Supplementary Figure S4). BLM/Rad51 expression did not influence survival in ER + tumours (Supplementary Figure S5). In multivariate analysis (Supplementary Table S8), nuclear BLM level independently influenced survival (p=0.026). Tumour stage, grade and HER-2 expression were other factors independently associated with breast cancer specific survival.

DISCUSSION

This is the first and the largest study to evaluate BLM in sporadic breast cancers. We provide compelling evidence that high *BLM* mRNA expression is a strong prognostic and predictive biomarker in breast cancer. High *BLM* mRNA was linked to aggressive clinicopathological phenotypes. High *BLM* mRNA was associated with aggressive molecular phenotypes including PAM50. Luminal B, PAM50. Her2 and PAM50. basal molecular phenotypes. Given the role of BLM during replication and proliferation (25), it is perhaps not surprising that high *BLM* mRNA was more frequent in aggressive breast cancers. To further support this hypothesis we also observed that low *BLM* mRNA expression was more common in PAM50. Lumina A and ER+/Her-2 negative/low proliferation Genefu subtype tumours. Interestingly, *BLM* mRNA levels are also linked to biologically distinct integrative clusters reported in the METABRIC study (11). High *BLM* mRNA level was frequent in intClust 10 subgroup which is the most highly genomically instable sub group with basal-like features. Low *BLM* mRNA level was seen in intClust 3 subgroup that is characterised by low genomic instability. Together the data suggest that *BLM* mRNA level may also inform genomic stability status in breast. 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 manifest 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), intClust 7 (16p gain/16q loss with higher frequencies of 8q amplification luminal A subgroup) and intClust 8 subgroups (classical 1q gain/16q loss luminal A subgroup) (11). Of note, the data presented here is strikingly similar to the clinicopathological associations we recently reported for

FEN1 (flap endonuclease 1), a key player in long-patch base excision repair and DNA replication, in the METABRIC cohort. Interestingly, BLM has been shown to stimulate FEN1 activity in a preclinical study (26). The functional interaction appeared to be independent of BLM helicase activity in that study (26).

At the protein level, low nuclear and/or high cytoplasmic expression was associated with aggressive phenotypes. Association with high cytoplasmic expression was surprising. As cytoplasmic function of BLM has not been described previously, we speculate that cytoplasmic accumulation in a proportion of breast tumours probably reflects dysregulation of mechanisms involved in nuclear localization of BLM. Cytoplasmic accumulation along with low nuclear BLM expression could then increase genomic instability in tumours and promote a mutator phenotype characterised by aggressive biology. To support this hypothesis we also observed that 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. In addition, low nuclear BLM was associated with 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. Moreover, in multivariate analysis, nuclear BLM level independently influenced survival. As BLM and Rad51 are known to interact with each other for efficient HR repair (24), we also performed BLM-Rad51 co-expression studies. As expected, low nuclear BLM/low nuclear RAD51 tumours exhibited aggressive phenotype and 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 (27). Similar to our study, the authors observed a positive correlation between BLM and Ki67 but did not report any clinicopathological associations (27). Another

interesting observation in the current study was that although *BLM* mRNA overexpression was categorically associated with aggressive tumours and poor outcomes, at the protein level, the association appeared more complex with low nuclear BLM protein level or low nuclear/high cytoplasmic BLM protein level being associated with adverse features. We speculate that either BLM mRNA is subjected to post-transcriptional regulation or post translational dysregulation of BLM protein expression/sub-cellular localization could in turn affect *BLM* mRNA expression through feedback loops. Detailed mechanistic studies are therefore required to understand the regulation of BLM in vivo. Data presented in the current study also suggest that BLM could be a promising marker for personalization of therapy. As low BLM is a marker of impaired HR repair, we would argue that low BLM tumours could be targeted by synthetic lethality using inhibitors of base excision repair such as those targeting PARP (28). Alternatively high BLM tumours could be targeted by small molecular inhibitors of BLM that are currently under development (10). In conclusion we provide the first clinical evidence that BLM is a promising biomarker and a rational drug target in breast cancer.

COMPETING FINANCIAL INTERESTS: The authors declare no competing financial interests

AUTHORS' CONTRIBUTIONS

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Table 1: Association between *BLM* mRNA expression and clinico-pathologic variables in METABRIC cohort (N=1950).

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%)		
PgR (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; PgR: progesterone receptor;Triple negative: ER-/PgR-/HER2-. *Adjusted p values were calculated using Benjamini-Hochberg method to adjust for multiple testing.

Table 2: Multivariate analysis in the METABRIC cohort confirms that BLM mRNA over expression 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

Table 3. BLM (nuclear and cytoplasmic protein co-expression) in breast cancer (n=1253)

VARIABLE	BLM Protein Expression				P- value	*P -Value (Adjusted)
	Nuc-/Cyto- N (%)	Nuc+/Cyto- N (%)	Nuc/Cyto+ N (%)	Nuc+/Cyto+ N (%)		
A) Pathological Parametersic						
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	207 (62.3)	153 (64.0)	215 (61.3)	203 (61.0)	0.946	39.73
2	92 (27.7)	66 (27.6)	106 (30.2)	102 (30.6)		
3	33 (9.9)	20 (8.4)	30 (8.5)	28 (8.4)		
Tumour Grade						
G1	53 (16.0)	52 (21.8)	45 (12.9)	59 (17.7)	**$3.0X10^{-6}$**	**$1.0X10^{-5}$**
G2	87 (26.2)	208 (42.0)	102 (29.1)	108 (32.4)		
G3	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	170 (59.2)	105 (53.3)	204 (65.2)	170 (58.2)	**$6.6X10^{-5}$**	**$1.0X10^{-4}$**
Tubular Carcinoma	55 (19.2)	39 (19.8)	59 (18.8)	66 (22.6)		
Medullary Carcinoma	12 (4.2)	0 (0.0)	12 (3.8)	3 (1.0)		
ILC	28 (9.8)	30 (15.2)	17 (5.4)	18 (6.2)		
Others	22 (7.7)	23 (11.7)	21 (6.7)	35 (12.0)		
Lymphovascular Invasion						
No	219 (67.2)	144 (62.1)	235 (67.9)	218 (65.3)	0.486	0.551
Yes	107 (32.8)	88 (37.9)	111 (32.1)	116 (34.7)		
B) Aggressive phenotype						

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)	**$1.0X10^{-6}$**	**$1.0X10^{-5}$**
PgR 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)	**$1.0X10^{-6}$**	**$1.0X10^{-5}$**
Polβ 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-PKcs 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)	**$1.0X10^{-6}$**	**$1.0X10^{-5}$**
E) Cell cycle/apoptosis regulators						
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 expression High expression	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 Overexpression	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 (Cytoplasmic) 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 High	145 (59.7) 98 (40.3)	72 (45.6) 86 (54.4)	113 (42.2) 155 (57.8)	92 (38.0) 150 (62.0)	**$9.0X10^{-6}$**	**$1.0X10^{-5}$**
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 Overexpression	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; PgR: progesterone receptor; CK: cytokeratin; Basal-like: ER-, HER2 and positive expression of either CK5/6, CK14 or EGFR; Triple negative: ER-/PgR-/HER2- . Adjusted p values were calculated using Benjamini-Hochberg false discovery rate method to adjust for multple testing. *Fischer test was used to obtain p values where one or more of cells has an expected frequency of five or less.

FIGURE LEGENDS

Figure 1: Kaplan Meier curves showing BCSS (Breast cancer specific survival) based on BLM mRNA expression in **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.

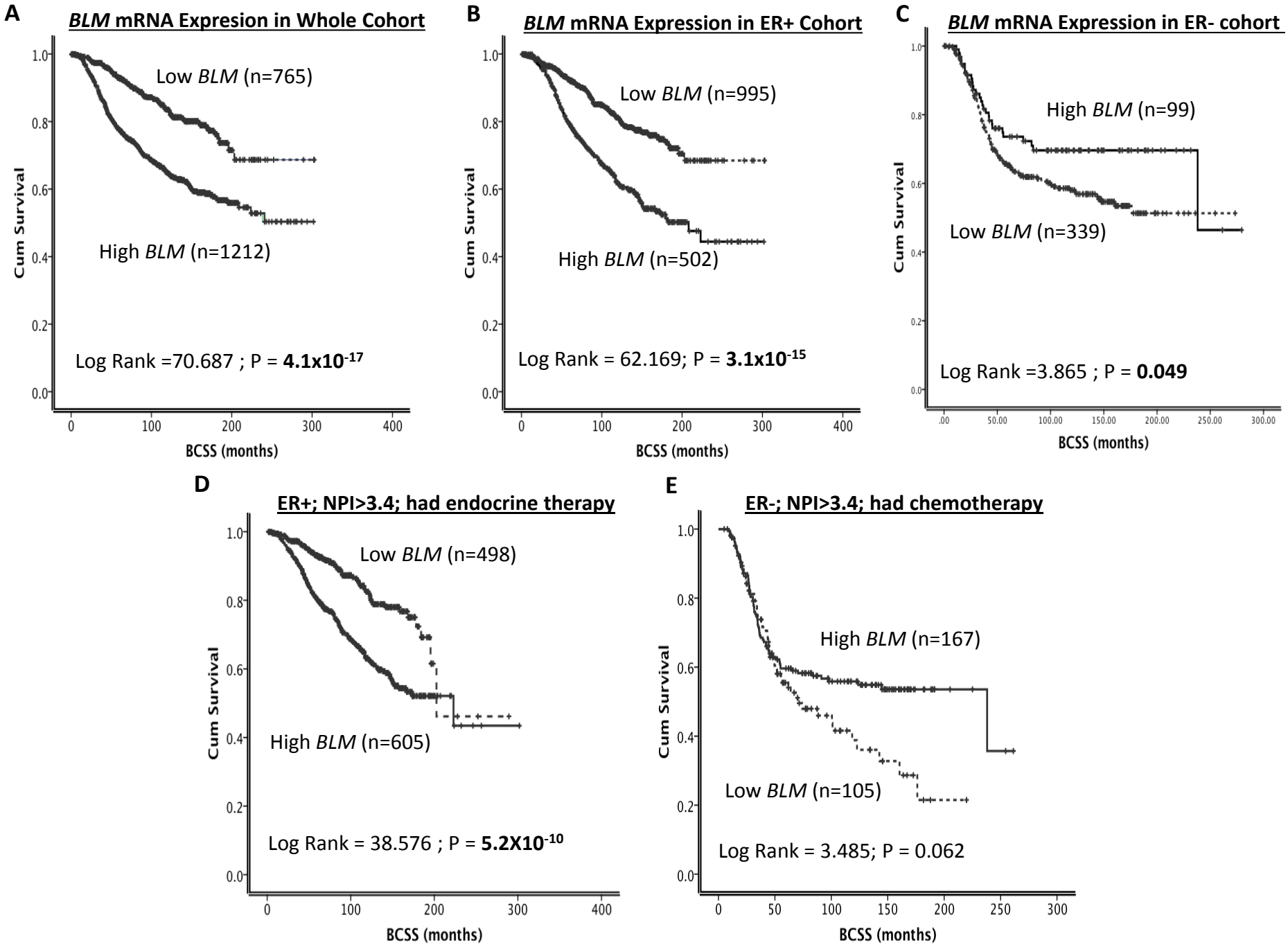
A
BLM mRNA Expresion in Whole Cohort
Low BLM (n=765)
High BLM (n=1212)
Log Rank =70.687 ; P = 4.1x10-17
Cum Survival
BCSS (months)
B
BLM mRNA Expression in ER+ Cohort
Low BLM (n=995)
High BLM (n=502)
Log Rank = 62.169; P = 3.1x10-15
Cum Survival
BCSS (months)
C
BLM mRNA Expression in ER- cohort
High BLM (n=99)
Low BLM (n=339)
Log Rank =3.865 ; P = 0.049
Cum Survival
BCSS (months)
D
ER+; NPI>3.4; had endocrine therapy
Low BLM (n=498)
High BLM (n=605)
Log Rank = 38.576 ; P = 5.2X10-10
Cum Survival
BCSS (months)
E
ER-; NPI>3.4; had chemotherapy
High BLM (n=167)
Low BLM (n=105)
Log Rank = 3.485; P = 0.062
Cum Survival
BCSS (months)

Figure 1