MIB1/Ki-67 labelling index can classify grade 2 breast cancer into two clinically distinct subgroups

Mohammed A. Aleskandarany, Emad A. Rakha, R. Douglas Macmillan, Desmond G. Powe, Ian O. Ellis, Andrew R. Green

MIB1/ Ki-67 Labelling Index Can Classify Grade 2 Breast Cancer into Two Clinically Distinct Subgroups

Mohammed A Aleskandarany[1,2], Emad A Rakha [3], R Douglas Macmillan[4], Desmond G Powe[2], Ian O Ellis [1,3] Andrew R Green[1],

[1]Division of Pathology, School of Molecular Medical Sciences, University of Nottingham, [2]Pathology Department, Faculty of Medicine, Menoufyia University, Egypt, [3]Department of Pathology Nottingham University Hospitals NHS Trust, [4]Breast Institute, Nottingham University Hospitals NHS Trust, Nottingham, United Kingdom.

Corresponding author:

Dr Andrew R Green
School of Molecular Medical Sciences, University of Nottingham
Queen's Medical Centre, Nottingham NG7 2UH
Tel: (44) 01158230786
Fax: (44) 0115- 9627768
Email: andrew.green@nottingham.ac.uk

Key Words:
Breast carcinoma, Growth fraction, Grade, Immunohistochemistry, Ki67

Abstract

Background:

Histological grade is recognized as one of the strongest prognostic factors in operable breast cancer (BC). Although grade 1 and grade 3 tumours are biologically and clinically distinct, grade 2 tumours bear considerable difficulty in outcome prediction and planning therapies. Several attempts such as genomic grade index have been performed to subclassify grade 2 into two subgroups with clinical relevance. Here we present evidence that the routinely evaluable immunohistochemical MIB1/Ki67 labelling index (MIB-LI) can classify grade 2 tumours into two clinically distinct subgroups.

Methods:

In this study, growth fraction of 1550 primary operable invasive breast carcinomas were immunohistochemically assayed on full-face tissue sections using the MIB1 clone of Ki-67. Growth fractions were assessed as number of MIB1 positive nuclei in 1,000 tumour nuclei at high-power magnification and expressed as MIB1-LI.

Results:

Using a 10% cut-point of MIB1-LI, grade 2 breast cancers were classified into low (49.8%) and high (50.2%) proliferative subgroups. Univariate and multivariate survival analysis revealed statistically significant differences between these subgroups regarding patients' breast cancer specific survival (p<0.001), and metastasis free survival (p< 0.001) which was independent of the well-established prognostic factors (HR=2.944, 95% CI= 1.634-5.303, p<0.001).

Conclusions:

Our results further demonstrate that grade 2 breast cancers may represent at least two biological or behaviourally different entities. Assay of growth fraction in breast cancer using MIB1/Ki67 immunohistochemistry is a robust cost-effective diagnostic tool that subdivides grade 2 tumours into low and high risk populations providing additional prognostic information in planning therapies and outcome prediction.

Introduction:

Histologic tumour grade is well recognized as one of the powerful prognostic factors in human breast cancer (BC) [1], which has been validated in multiple independent studies [2-4]. In current routine BC diagnostics, over half of all BC cases are assigned histologic grade 1 or 3 status, with low and high risk of early recurrence, respectively. However, the remaining substantial percentage of tumours (30%–60%) are classified as histologic grade 2, which have an intermediate risk of recurrence but remain less informative for clinical decision than grade 1 and 3 tumours [5].

One of the features common to breast carcinomas is the increased rate of proliferation over that seen in normal breast epithelia [6]. In the current practice, *m*itotic frequency *s*core (MS) is incorporated with tubule formation and nuclear pleomorphism into the designation of histological grade in the widely adopted Elston and Ellis modification of the Scarff-Bloom-Richardson histologic grading system [7]. However, the number of mitotic figures is not linearly correlated with the rate of proliferation, especially in aneuploid tumours due to wide variation in cell cycle duration, particularly the mitotic phase of the cell cycle [6]. Therefore, other methods of assessing proliferative activity in BC have been previously studied with their documented advantages and disadvantages [6,8], with some focus on histologic grade 2 refinement [9,10].

Over the recent years, gene expression profiling has resulted in a paradigm shift in researchers' understanding of BC biology and prognostication. Despite the fact that several gene signatures, developed using different microarray platforms, have been shown to correlate with patient outcome, many of these have a relatively small gene overlap. A feature common to these signatures is their ability to identify high-risk patients but generally identify a greater number of low-risk patients compared with current clinical guidelines [11]. Moreover these global gene expression analysis studies have demonstrated the prime role of proliferation signatures in BC prognosis and prediction of response to therapy [12,13]. For instance, a recent meta-analysis of publicly available BC gene expression studies showed that the key biological drivers in nine prognostic signatures were proliferation related genes, in addition to ER signalling and ERBB2 amplification [14]; results which have been reinforced by other authors [15]. As a consequence, attempts, such as genomic grade index (GGI) and molecular grade index (MGI), have been made to stratify grade 2 BC into low and high risk subgroups to improve clinical relevance [16,17]. In their study, Sotiriou et al concluded that the gene

signature characteristics displayed by grade 2 BC appears to be a mixture of signatures displayed by grade 1 and 3, rather than being independent or intermediate between the two. Moreover, the MGI appears to be as powerful prognostically as the GGI and relies only on five genes; all involved in different cell cycle phases, evaluated using quantitative PCR [17]. However, the complexity, quality assurance requirements and interpretation issues of expression array-based profiling and assays using nucleic acids (RNA and DNA) extracted from formalin-fixed paraffin embedded samples, coupled with its cost, potentially limit the use of this technology in the routine clinical setting [18,19].

For this study, we have assessed proliferative activity using the well characterized routinely available MIB1 antibody which binds to Ki-67 protein in grade 2 invasive breast carcinomas to determine whether it could improve the accuracy of predicting clinical outcome for such a group of patients with a varied and an unpredictable course of disease progression. This retrospective study adheres to REMARK criteria [20].

This study was approved by Nottingham Research Ethics Committee 2 under the title of "Development of a molecular genetic classification of breast cancer". None of the authors has any competing interests.

Materials and Methods:

This study was based on a well-characterized consecutive series of early stage (stage I-III, n=1550) primary operable invasive breast carcinoma from patients entered into the Nottingham Tenovus Primary Breast Carcinoma Series between 1990 and 1998 (n=1,550). Patients were under the age of 70 and managed in accordance to a uniform protocol. Patients' clinical history and tumour characteristics, information on therapies and outcomes were available and prospectively maintained. The method used for tumour grading is that described by Elston and Ellis [7] that involves assessment of three components of tumour morphology; tubule formation, nuclear pleomorphism and frequency of mitoses (each scored from 1 to 3). The mitosis was scored based on the number of mitoses per 10 high power fields (M1; ≤9, M2; 10-19, and M3≥20, using a microscopic field diameter of 0.59 mm) [21].

Outcome data include survival status, survival time, cause of death, disease free interval, time to loco-regional recurrence, and distant metastasis and was maintained on a prospective basis. The Breast Cancer Specific Survival (BCSS) is defined as the time (in months) from the date of primary surgery to the date of breast cancer-related death. Metastasis free interval (MFI) is defined as the duration (in months) from the date of primary surgery to the appearance of distant metastasis. Adjuvant treatment was scheduled on the basis of patient and tumour prognostic /predictive factor status including Nottingham Prognostic Index (NPI), estrogen receptor (ER) status, and menopausal status. Patients within the good prognostic group (NPI <3.4) did not receive adjuvant therapy. Hormonal therapy (HT) was prescribed to patients with ER positive tumours and NPI scores of >3.4 (moderate and poor prognostic groups). Premenopausal patients within the moderate and poor prognostic groups were candidates for CMF (Cyclophosphamide, Methotrexate, and 5-Flourouracil) chemotherapy; and those ER positive LN positive patients were offered HT in addition to CMF. Conversely, postmenopausal patients with moderate or poor NPI and ER positive were offered HT, while ER negative patients received CMF if fit.

Data on a wide range of biomarkers of known clinical and biological relevance to breast cancer were also available. These include, hormone receptors [estrogen receptor (ER), progesterone receptor (PR), androgen receptor (AR), epidermal growth factor receptor family members (HER1 [EGFR], and HER2), cytokeratins [CKs] (basal CKs; CK5/6 and CK14, and luminal CKs; CK18 and 19), P53, and cadherin family members [E-cadherin and P-cadherin] [22].

The patients had a median age of 54 years (range 18-70 years). Outcome data was available for 1513/1550 patients, with a median overall survival of 123 months (range 4 to 247 months) and the median time of metastasis free survival of 110 months (range 2-247 months). Distant recurrence occurred in 483 cases (31 %), 411 (27%) patients died from breast cancer, while 875 (58%) patients were alive at the end of follow-up. Figure 1 shows numbers of patients with available data through the study and different analyses.

Immunohistochemistry (IHC):

Formalin-fixed paraffin tissue sections (4µm) mounted on Superfrost slides (Surigpath) were immunohistochemically stained, employing the standard streptavidin-biotin complex method, as previously described [23]. Heat induced antigen (epitope) retrieval (HIER) was performed in citrate buffer (pH6.0), using microwave oven-assisted heating for 20 minutes. Primary mouse monoclonal anti-Ki-67 antibody (MIB1 clone, M7240, DAKO, Denmark), diluted 1:100 (optimum working dilution) in normal swine serum (NSS)/TBS, was applied to each slide and incubated for 60 minutes at room temperature. Strept AB complex (Dako) diluted in 1:100 in NSS/TBS was applied and incubated for 60 minutes. 3-3'Diaminobenzidine tetrahydrochloride (Dako liquid DAB Plus, K3468) was used as a chromogen. The sections were counterstained with Mayer's haematoxylin. Positive (FFPE tonsil section) and negative (primary antibody replaced by TBS) controls were included in each staining run.

Ki-67/MIB1 scoring

The MIB1-LI was quantitatively determined using human eye light microscopical assessment. The entire slide was scanned for immunostaining evaluation using a light microscope at low power magnification (x100). All tumour cell nuclei with homogenous granular staining, multiple speckled staining, or nucleolar staining were regarded as positively stained regardless of intensity, while any cytoplasmic immunoreactivity was considered non-specific, and hence not taken into consideration. Scoring was performed in the areas with highest number of positive nuclei (hot spot) within the invasive component of the tumour. The MIB1-LI (tumour growth fraction) was expressed as the percentage of MIB1 positive malignant cells in 1,000 malignant cells assessed under high power magnification (x400).

Statistical analysis

Statistical analysis was performed using SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA). Cut-off values for the different biomarkers included in this study were chosen before statistical analysis. Standard cut-offs were used for established prognostic factors and were the same as for previously published patient series [22,24]. Determination of the optimal MIB1-LI cut-off points was performed using X-tile bioinformatics software; version 3.6.1, 2003-2005, Yale University, USA as previously described [25]. Analysis of categorical variables was performed with the appropriate statistical test. Survival curves were analyzed by Kaplan-Meier method with significance determined by the Log Rank test. Multivariate analysis was performed by Cox hazard analysis. A p-value <0.05 (two sided) was considered significant.

Results

In the current study, the median patients' age at diagnosis of this cohort was 54 years (range 18-70 years), with 60% of patients were postmenopausal. Grade 2 cases constituted 506/1550 (33%), while 275 (18%) grade 1 and the remaining 764 cases (49%) were grade 3. As reported [7], a statistically significant difference between grades 1, 2 and 3 was found regarding BCSS and distant metastasis free survival (MFS) using Kaplan Meier survival analysis (p<0.001), with grade 2 tumours having intermediate risk (Figure 2A&B).

Of all tumours, 885 cases (58%) were ductal carcinoma of no special type (duct/NST), with the remainder consisting of tumours of other histologic types including medullary, tubular mixed, lobular mixed, and mixed NST and lobular. ER positive tumours constituted (69.9%), while 177 cases (11.4%) were HER2 positive (based on IHC and HER2 gene amplification detection using CISH). The NPI in the studied cases ranged from 2.04-5.00 (mean, 4.40). Regarding the adjuvant systemic therapy used, 285 patients (20%) received classic CMF adjuvant chemotherapy, while 481 (32%) received tamoxifen as hormonal therapy. Table 1 summarizes the clinico-pathological characteristics of the study series.

The MIB1-LI for this series ranged from (0-100%, median 15%). Using X-tile bioinformatics software package, a MIB1-LI of 10% was found to distinguish low from highly proliferative tumours. In this study histological grade 1, 2 and 3 BCs were stratified into either a) low (MIB1-LI<10%, or b) high proliferative (MIB1-LI ≥10%, groups: G1a, G1b; G2a, G2b; and G3a, and G3b, respectively, Table 2. Figure 3 shows two different grade 2 cases with low (A) and high (B) MIB1-LI.

Association of MIB1-LI and mitotic scores with patients' outcome in grade 2 tumours:

Using the 10% MIB1-LI cut-point, grade 2 tumours were split into low proliferative G2a (252, 49.8%) and high proliferative G2b (254, 50.8%) groups. Univariate survival analysis showed that Grade 2 tumours with high proliferation (G2b) had significantly shorter BCSS (p < 0.001, Hazard ratio (HR) =3.116, 95% CI= 1.994-4.971, Figure 4A) relative to those grade 2 tumours with low proliferation (G2a). G2b tumours had a 76% 10 year BCSS compared to 92% in the G2a cases. Moreover, a statistically significant difference was observed between G2a and G2b cases regarding

MFS (p<0.001, HR=2.152, 95%CI= 1.505-3.079, Figure 4B). In addition, un-treated and tamoxifen treated patients had the same pattern of survival with respect to tumour proliferation, where patients with G2b tumours experienced shorter BCSS and MFS than those with G2a tumours (Figure 4 C-F). Similarly, using the mitotic frequency scores (M1, M2, and M3), grade 2 cases were divided into three distinct proliferative subgroups with significant survival differences between M1 and M2 subgroups regarding their BCSS (p =0.002, HR=1.502, 95%CI=1.163-1.940) and MFS (p =0.006, HR=1.476, 95%CI=1.173-1.856). However, no significant difference was observed between the M2 and M3 groups regarding BSCC and MFS (p>0.05).

Comparison of outcome between grades using proliferative subgroups:

Similarly, we subdivided grade 1 into low (G1a) and high (G1b) proliferative subgroups using 10% MIB1-LI. A significant difference was observed between grade 1 and grade 2 tumours subdivisions (G1a/b, G2a/b) regarding their BCSS and DFS (p<0.001, LR=69.3, p<0.001, respectively; Figure 5A&B). A significant difference in patient outcome between G1a and G1b was observed regarding BCSS (p=0.007, HR=3.031, 95% CI=1.295-7.095) and MFS (p<0.001, HR=1.311, 95% CI=1.237-3.389). Moreover, there was significant difference between G2b and G3a regarding BCSS (p=0.01). However, no significant differences were noted between G2a and either G1b or G1a (p>0.05). Importantly, patient outcome remained similar between the low and high proliferative grade 3 tumours (G3a&b) regarding their BCSS and DFS (p=0.982& 0.766, respectively).

Multivariate analysis

Multivariate Cox proportional hazard model analysis of predictors of BCSS grade 2 tumours was performed including MIB1-LI, mitotic scores M1, M2 and M3, nodal stage, tumour size, molecular subtype [luminal (ER^+ and or PR^+), $HER2^+$, basal-like BC (BLBC, ER^-, PR^-, $HER2^-$, and positive for CK5/6, and/or CK14 and/or EGFR), and triple negative non-basal BC (TNnon-B, all negative)] [24], and adjuvant therapy. This analysis demonstrated that MIBLI is the strongest independent predictor of BCSS (HR = 3.251, 95 CI = 1.796– 5.886, p<0.001) and MFS (HR =2.188, 95 CI = 1.364– 3.509, p= 0.001) in grade 2 BC, (Table 3). Interestingly, the mitotic score which was able to subdivide BC grade 2 cases in univariate analysis lost its significance in multivariate model analysis (p=0.692 and 0.376 for BCSS and MFS, respectively).

Discussion

Breast cancer is regarded as a heterogeneous group of tumours with diverse behaviour, outcome, and response to therapy [26] and patients with apparently similar clinical and pathological features can show distinct outcomes and varied response to therapy [27]. With the increasing focus on personalization of treatment there is an increasing need for additional refinement of prognostic factors to improve patients' risk stratification and the targeting of treatment for those who will truly benefit, thereby avoiding iatrogenic morbidity in those who will not [18,28]. Patients with intermediate risk of recurrence , for example as exhibited by patients with histologic grade 2 tumours pose significant difficulty in the management in balancing risk of relapse with potential to experience side effects [29]. Further sub-classification of histologic grade 2 tumours, possibly into low and high risk categories, would be beneficial to increase the prognostic stratification of these patients [16].

In this study, the cell cycle associated protein Ki-67, as assessed by IHC using the MIB1 antibody, was tested as a marker of growth fraction to determine its potential to refine early invasive grade 2 BC. In our series, grade 2 BC constituted 33% of the whole cohort, confirming previous observations that this forms a substantive proportion of cases in routine practice. Our results demonstrated that Ki-67/MIB1-LI categorized, using a 10% cut-point, BC grade 2 into two distinct subgroups which exhibited significantly different outcome, which was independent from other factors. Similar methodology applied to grades 1 and 3 cases also stratified into proliferative subgroups, however the proportion of cases were different with the majority of the grade 1 being low proliferative and the majority of grade 3 being high proliferative. Significant BCSS and MFS differences were revealed between the subdivisions of the grade 1, while no differences were noticed between grade 3 subdivisions. However, the number of cases in the G1b subgroup was limited (40 patients, 2.5% of whole series), and probably might not be a reflection of biological difference. Therefore further studies are required in order to determine the clinical relevance of proliferation assessment within low grade BC in routine practice.

It is noteworthy that there was no difference in outcomes of G2 low proliferative (G2a) and the proliferative subdivisions of grade 1 (G1a & G1b). On the contrary, the outcome of grade 2 highly proliferative subgroup (G2b) was significantly different from those of grade 3 low proliferative (G3a). Therefore, G2b (16% of the whole cohort) showed a distinct outcome that was intermediate between grade 1 and grade 3. In other words, grade 2 BC was not, using MIB1-LI, totally re-distributed between either grade 1 or 3, but a substantial cohort of grade 2 cases were shown to have an intermediate risk

when compared to grade 1 and grade 3 BC. Interestingly, in the study of Sotiriou and colleagues, the GGI reclassified patients with histologic grade 2 BC into two groups with distinct clinical outcomes similar to those of histologic grades 1 & 3 [16]. Although their observation challenges the existence and clinical relevance of an intermediate-grade, they have characterized a group of grade 2 cases with GGI intermediate between grades 1 and 3. The existence of an intermediate-risk group, based on the study of tumour growth fraction was demonstrated in our series, but its frequency is lower than that seen using the histologic grading systems alone. Furthermore, our assay is more applicable to the routine histologic practice because MIB1-LI assay is accepted to be a low cost, simple method which is perfectly suitable for standardization in clinical laboratory practice, in contrast to array-based profiling and assays using nucleic acids extracted from FFPE tissues [18,19], which require quality assurance and cost/effectiveness appraisal prior to their adoption into routine clinical practice.

The mitotic frequency score, which is incorporated into the Nottingham grading system, also stratified grade 2 BC into three risk groups in this study. However, MIB-LI not only had a higher hazard ratios (~ 2 fold) in predicting the BCSS and MFS of grade 2 BC, but also it eliminated mitotic scores from the multivariate model rendering it insignificant.

In addition to GGI, other attempts to refine grade 2 BC have been reported, and are thus noteworthy. Le Doussal et al. were able to separate grade 2 node negative BC into three significantly different subgroups according to their MFS, through excluding tubule formation from the Scarf-Bloom Richardson histologic grading system [30]. In addition, Baak et al. reported that Mitotic Activity Index (MAI) was the most important prognostic factor in node negative BC among the 3 morphologic features of the Nottingham combined histologic grade, with no and limited additional prognostic value of tubule formation and nuclear atypia respectively [31]. Moreover, Lynch et al. in their pilot study highlighted the importance of mitotic counting in refining grade 2 cases to improve prognostic accuracy, which was similar to S-phase fraction assessment using flow cytometry, while, no additional significance of Ki-67 evaluation using IHC was noted [10]. From these findings, it could be concluded that proliferation assessment is the most useful and is the prime determinant of outcome in BC patients relative to other grade components. However, these studies were restricted to either a particular stage [30,31], or carried out on a relatively small number of cases [10].

In conclusion, our results further demonstrate that grade 2 breast cancers may represent combination of at least two biological or behaviourally different entities. Assay of growth fraction in breast cancer,

using the routinely available anti-Ki-67 MIB1 antibody by IHC, is a robust cost-effective method and potential routine diagnostic tool which can subdivide grade 2 tumours into low and high risk populations, providing additional prognostic information and patient stratification when planning therapy and predicting outcome.

Acknowledgements

MAA is funded by the Ministry of High Education (Egypt). Part of this work has been partially funded by the Breast Cancer Campaign.

References

1. Soerjomataram, I., Louwman, M.W., Ribot, J.G., Roukema, J.A. and Coebergh, J.W. (2008) An overview of prognostic factors for long-term survivors of breast cancer. Breast Cancer Res Treat, 107, 309-30.
2. Pereira, H., Pinder, S.E., Sibbering, D.M., Galea, M.H., Elston, C.W., Blamey, R.W., Robertson, J.F. and Ellis, I.O. (1995) Pathological prognostic factors in breast cancer. IV: Should you be a typer or a grader? A comparative study of two histological prognostic features in operable breast carcinoma. Histopathology, 27, 219-26.
3. Dalton, L.W., Page, D.L. and Dupont, W.D. (1994) Histologic grading of breast carcinoma. A reproducibility study. Cancer, 73, 2765-70.
4. Frierson, H.F., Jr., Wolber, R.A., Berean, K.W., Franquemont, D.W., Gaffey, M.J., Boyd, J.C. and Wilbur, D.C. (1995) Interobserver reproducibility of the Nottingham modification of the Bloom and Richardson histologic grading scheme for infiltrating ductal carcinoma. Am J Clin Pathol, 103, 195-8.
5. Ignatiadis, M. and Sotiriou, C. (2008) Understanding the molecular basis of histologic grade. Pathobiology, 75, 104-11.
6. Whitfield, M.L., George, L.K., Grant, G.D. and Perou, C.M. (2006) Common markers of proliferation. Nat Rev Cancer, 6, 99-106.
7. Elston, C.W. and Ellis, I.O. (1991) Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology, 19, 403-10.
8. Hall, P.A. and Levison, D.A. (1990) Review: assessment of cell proliferation in histological material. J Clin Pathol, 43, 184-92.
9. Meyer, J.S., Alvarez, C., Milikowski, C., Olson, N., Russo, I., Russo, J., Glass, A., Zehnbauer, B.A., Lister, K. and Parwaresch, R. (2005) Breast carcinoma malignancy grading by Bloom-Richardson system vs proliferation index: reproducibility of grade and advantages of proliferation index. Mod Pathol, 18, 1067-78.
10. Lynch, J., Pattekar, R., Barnes, D.M., Hanby, A.M., Camplejohn, R.S., Ryder, K. and Gillett, C.E. (2002) Mitotic counts provide additional prognostic information in grade II mammary carcinoma. J Pathol, 196, 275-9.
11. Haibe-Kains, B., Desmedt, C., Piette, F., Buyse, M., Cardoso, F., Van't Veer, L., Piccart, M., Bontempi, G. and Sotiriou, C. (2008) Comparison of prognostic gene expression signatures for breast cancer. BMC Genomics, 9, 394.
12. Dai, H., van't Veer, L., Lamb, J., He, Y.D., Mao, M., Fine, B.M., Bernards, R., van de Vijver, M., Deutsch, P., Sachs, A., Stoughton, R. and Friend, S. (2005) A Cell Proliferation Signature Is a Marker of Extremely Poor Outcome in a Subpopulation of Breast Cancer Patients. vol. 65, pp. 4059-4066.
13. Bonnefoi, H., Underhill, C., Iggo, R. and Cameron, D. (2009) Predictive signatures for chemotherapy sensitivity in breast cancer: are they ready for use in the clinic? Eur J Cancer, 45, 1733-43.
14. Wirapati, P., Sotiriou, C., Kunkel, S., Farmer, P., Pradervand, S., Haibe-Kains, B., Desmedt, C., Ignatiadis, M., Sengstag, T., Schutz, F., Goldstein, D.R., Piccart, M. and Delorenzi, M. (2008) Meta-analysis of gene expression profiles in breast cancer: toward a unified understanding of breast cancer subtyping and prognosis signatures. Breast Cancer Res, 10, R65.
15. Cheang, M.C., Chia, S.K., Voduc, D., Gao, D., Leung, S., Snider, J., Watson, M., Davies, S., Bernard, P.S., Parker, J.S., Perou, C.M., Ellis, M.J. and Nielsen, T.O. (2009) Ki67 index, HER2 status, and prognosis of patients with luminal B breast cancer. J Natl Cancer Inst, 101, 736-50.

16. Sotiriou, C., Wirapati, P., Loi, S., Harris, A., Fox, S., Smeds, J., Nordgren, H., Farmer, P., Praz, V., Haibe-Kains, B., Desmedt, C., Larsimont, D., Cardoso, F., Peterse, H., Nuyten, D., Buyse, M., Van de Vijver, M.J., Bergh, J., Piccart, M. and Delorenzi, M. (2006) Gene expression profiling in breast cancer: understanding the molecular basis of histologic grade to improve prognosis. J Natl Cancer Inst, 98, 262-72.
17. Ma, X.J., Salunga, R., Dahiya, S., Wang, W., Carney, E., Durbecq, V., Harris, A., Goss, P., Sotiriou, C., Erlander, M. and Sgroi, D. (2008) A five-gene molecular grade index and HOXB13:IL17BR are complementary prognostic factors in early stage breast cancer. Clin Cancer Res, 14, 2601-8.
18. Rakha, E.A., El-Sayed, M.E., Reis-Filho, J.S. and Ellis, I.O. (2008) Expression profiling technology: its contribution to our understanding of breast cancer. Histopathology, 52, 67-81.
19. Srinivasan, M., Sedmak, D. and Jewell, S. (2002) Effect of Fixatives and Tissue Processing on the Content and Integrity of Nucleic Acids. vol. 161, pp. 1961-1971.
20. McShane, L.M., Altman, D.G., Sauerbrei, W., Taube, S.E., Gion, M. and Clark, G.M. (2005) REporting recommendations for tumor MARKer prognostic studies (REMARK). Nat Clin Pract Oncol, 2, 416-22.
21. Ellis, I.O., Programmes, N.H.S.C.S., Royal College of, P. and The Science, C. (2005) *Pathology reporting of breast disease : a joint document incorporating the third edition of the NHS Breast Screening Programme's Guidelines for pathology reporting in breast cancer screening and the second edition of the Royal College of Pathologists' Minimum dataset for breast cancer histopathology*. NHS Cancer Screening Programmes ; Royal College of Pathologists.
22. Abd El-Rehim, D.M., Ball, G., Pinder, S.E., Rakha, E., Paish, C., Robertson, J.F.R., Macmillan, D., Blamey, R.W. and Ellis, I.O. (2005) High-throughput protein expression analysis using tissue microarray technology of a large well-characterised series identifies biologically distinct classes of breast cancer confirming recent cDNA expression analyses. International Journal of Cancer, 116, 340-350.
23. Aleskandarany, M.A., Green, A.R., Rakha, E.A., Mohammed, R.A., Elsheikh, S.E., Powe, D.G., Paish, E.C., Macmillan, R.D., Chan, S., Ahmed, S.I. and Ellis, I.O. (2009) Growth fraction as a predictor of response to chemotherapy in node negative breast cancer. Int J Cancer.
24. Rakha, E.A., Elsheikh, S.E., Aleskandarany, M.A., Habashi, H.O., Green, A.R., Powe, D.G., El-Sayed, M.E., Benhasouna, A., Brunet, J.S., Akslen, L.A., Evans, A.J., Blamey, R., Reis-Filho, J.S., Foulkes, W.D. and Ellis, I.O. (2009) Triple-negative breast cancer: distinguishing between basal and nonbasal subtypes. Clin Cancer Res, 15, 2302-10.
25. Camp, R.L., Dolled-Filhart, M. and Rimm, D.L. (2004) X-Tile: A New Bio-Informatics Tool for Biomarker Assessment and Outcome-Based Cut-Point Optimization. vol. 10, pp. 7252-7259.
26. Simpson, P.T., Reis-Filho, J.S., Gale, T. and Lakhani, S.R. (2005) Molecular evolution of breast cancer. Journal of Pathology, 205, 248-254.
27. Elston, C.W., Ellis, I.O. and Pinder, S.E. (1999) Pathological prognostic factors in breast cancer. Crit Rev Oncol Hematol, 31, 209-23.
28. Pusztai, L. and Hortobagyi, G.N. (1998) High-dose chemotherapy: how resistant is breast cancer? Drug Resistance Updates, 1, 62-72.

29. Elston, C.W. and Ellis, I.O. (2002) Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology, 41, 154-61.
30. Le Doussal, V., Tubiana-Hulin, M., Friedman, S., Hacene, K., Spyratos, F. and Brunet, M. (1989) Prognostic value of histologic grade nuclear components of Scarff-Bloom-Richardson (SBR). An improved score modification based on a multivariate analysis of 1262 invasive ductal breast carcinomas. Cancer, 64, 1914-21.
31. Baak, J.P., van Diest, P.J., Voorhorst, F.J., van der Wall, E., Beex, L.V., Vermorken, J.B. and Janssen, E.A. (2005) Prospective multicenter validation of the independent prognostic value of the mitotic activity index in lymph node-negative breast cancer patients younger than 55 years. J Clin Oncol, 23, 5993-6001.

Figure 1: Diagram showing flow of patients through the study

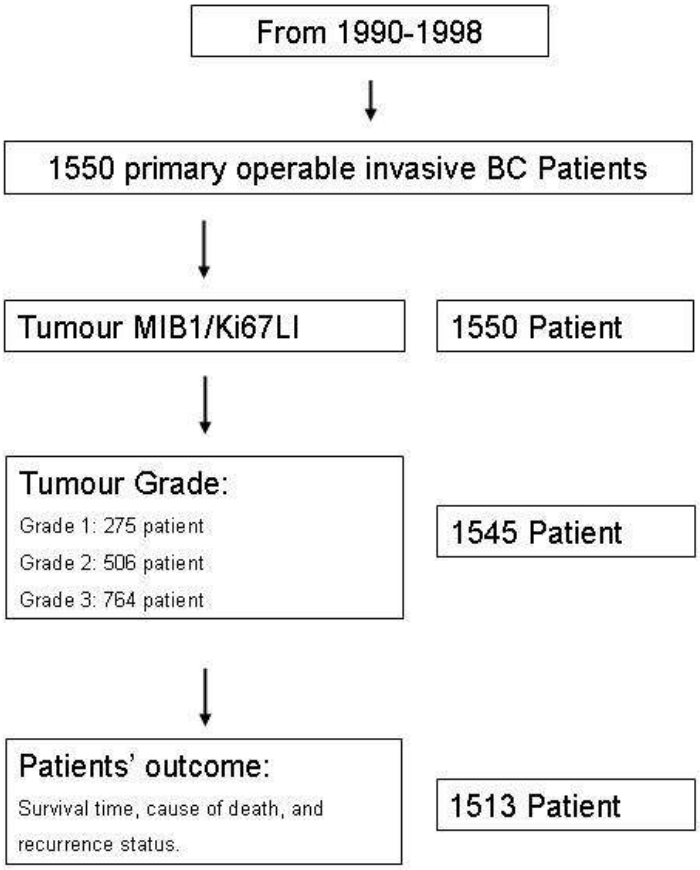

Figure 2: Kaplan-Meier survival plot in grades 1- 3 invasive breast carcinomas: A: Breast cancer specific survival (BCSS); B: Metastasis free survival

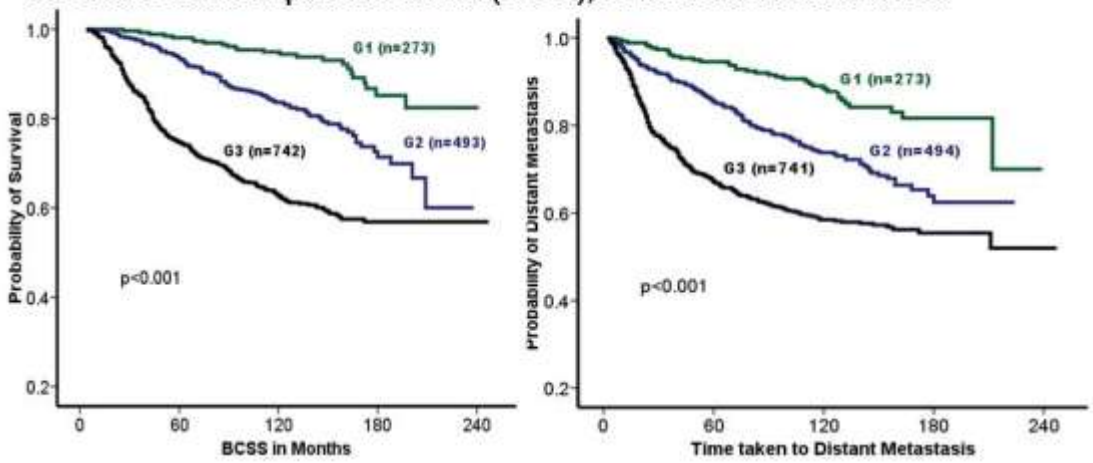

Figure 3: Grade 2 invasive ductal carcinoma showing positive nuclear staining using MIB1 IHC: (A) Low proliferative case MIB1-LI = 7), (B) High proliferative case (MIB1-LI = 50), [original magnification x200].

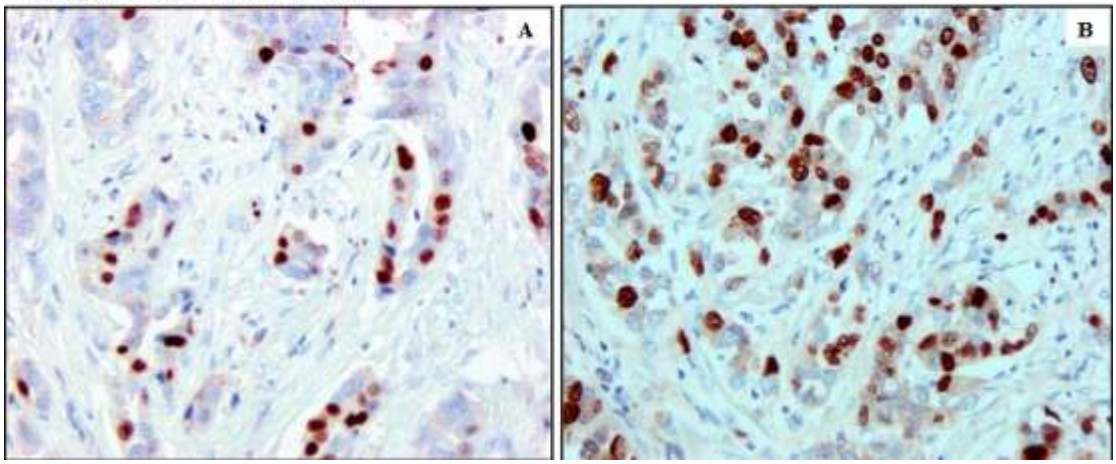

Figure 4: Kaplan-Meier survival plot of BCSS and Metastasis free survival in grade 2 breast tumours at 10% MIB1-LI cut point: A&B; in all grade 2 patients. C&D: Non-treated patients. E&F: Tamoxifen-treated patients.

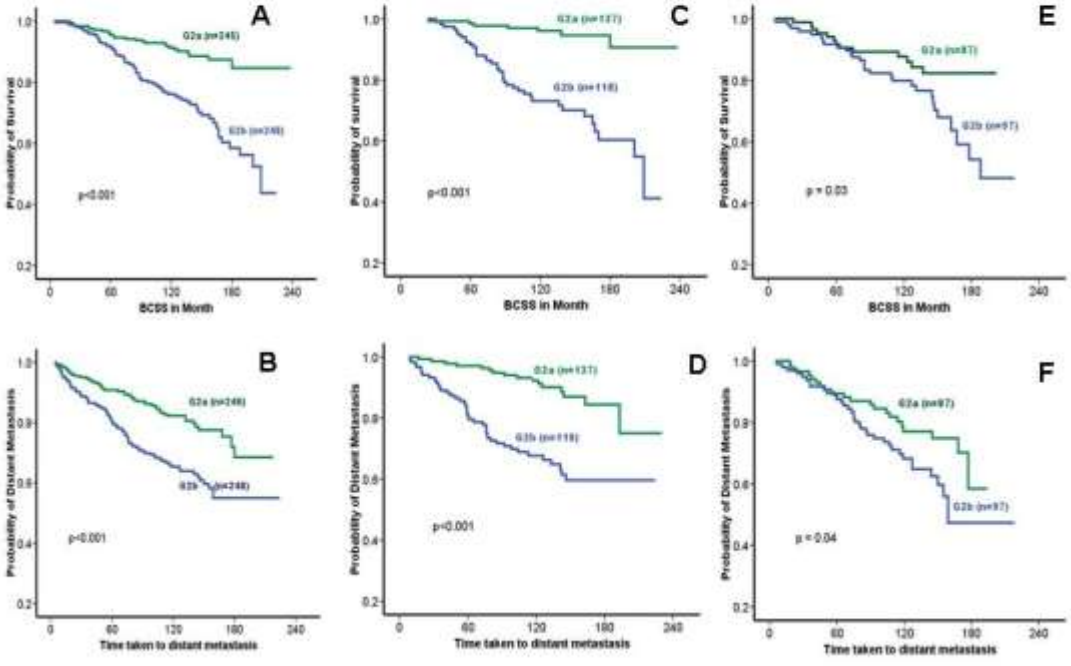

Figure 5: Kaplan-Meier survival plot for BCSS; A, and MFS; B, for tumour grades 1-3 combined with MIB1-LI at 10% cut-point

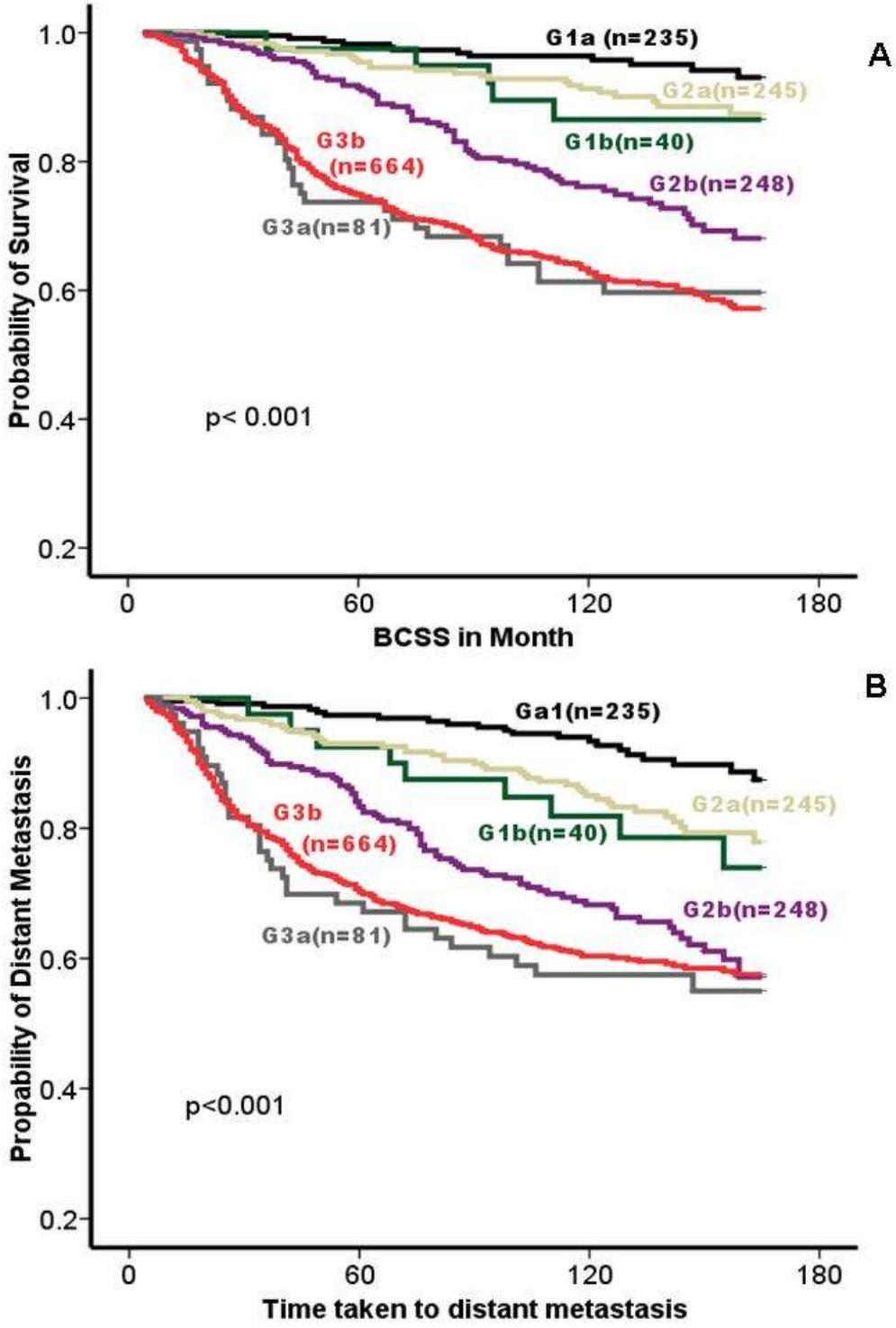

Table 1: Summary of the characteristics of the study cohort and their tumour tissue specimens

Characteristics	No (%)
Age:	
Median (Range)	54 (18-70)
Menopausal Status	
Premenopausal	612 (39.5)
Postmenopausal	936 (60.5)

Tumour Grade	
Grade 1	275 (17.8)
Grade 2	506 (32.8)
Grade 3	764 (49.4)
Nodal stage	
1: Node negative	946 (61.3)
2: 1-3 [positive lymph nodes	457 (29.6)
3: ≥ 4 positive lymph nodes	141 (9.1)
Tumour Size	
≤ 2 cm	956 (61.8)
> 2 cm	590 (38.2)
Histologic tumour type	
Ductal No Special Type	885 (58)
Other Histologic types	642 (42)
Nottingham Prognostic Index	
Good Prognostic Group (<3.4)	487 (31.5)
Moderate Prognostic Group (3.41-5.4)	801 (51.8)
Poor Prognostic Group (>5.41-?)	258 (16.7)
ER status	
Negative	391 (30.1)
Positive	906 (69.9)
HER2 status	
Negative	1289 (83.2)
Positive	177 (11.4)
Loco-Regional recurrence	
Local	183 (11.8)
Regional	153 (9.9)
Distant Metastasis	
No	1059 (68.7)
Yes	483(31.3)
Survival (month)	
BCSS: Median (Range)	123 (4-247)
DFI: Median (Range)	110 (2-247)

Table 2: Breast cancer grades 1, 2 and 3 and their subdivisions by MIB1-LI:

Grade	Low proliferative (MIB1-LI<10%)	High Proliferative (MIB1-LI≥10%)
Grade 1	235 (85.5)	40 (14.5)
Grade 2	252 (49.8)	254 (50.2)
Grade 3	81 (10.6)	683 (89.4)

Table 3: Cox proportional hazards analysis for predictors of BCSS and distant metastasis free survival (MFS): effect of MIB1-LI, mitotic scores, tumour size, nodal stage, molecular subtype and adjuvant therapy in grade 2 BC cases:

	BCSS	**MFS**

Variable (No.)	P value	HR	95% CI		P value	HR	95% CI	
MIB1-LI								
Low: (245)	< 0.001	3.251	1.796	5.886	0.001	2.188	1.364	3.509
High: (248)								
Mitotic score								
M1: (291)	0.569				0.246			
M2: (180)	0.876	0.957	0.554	1.655	0.965	1.011	0.635	1.608
M3: (22)	0.358	1.501	0.632	3.564	0.111	1.836	0.870	3.876
Tumour size (in centimetres)	0.041	1.338	1.016	1.813	0.001	1.541	1.187	2.002
Nodal stage								
1: (295)	0.001				0.009			
2: (160)	0.080	1.636	0.943	2.838	0.061	1.560	0.980	2.482
3: (38)	< 0.001	4.476	2.005	9.994	0,003	2.957	1.448	6.039
Molecular subtype								
Luminal: (422)	0.143				0.028			
HER2 Positive: (34)	0.064	1,828	0.965	3.461	0.010	2.127	1.199	3.772
Triple Negative: (13)	0.585	0.671	0.161	2.806	0.589	0.723	0.223	2.346
Endocrine therapy								
No : 307	0.07	0.803	0.633	1.018	0.076	0.840	0.693	1.018
Yes: 186								
Chemotherapy								
No: (453)	0.138	0.530	0.229	1.227	0.056	0.490	0.236	1.018
Yes: (40)								