Ki-67 biomarker in breast cancer of Indian women

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Abstract

Background: Biological markers that reliably predict clinical or pathological response to primary systemic therapy early during a course of chemotherapy may have considerable clinical potential. Aims: Aims of study to evaluated changes in Ki-67 (MIB-1) labeling index and apoptotic index (AI) before, during, and after neoadjuvant anthracycline chemotherapy in breast cancer in Indian women. Materials and Methods: Breast cancer tissues were collected from Grant Medical College and Sir J.J. Group of Hospitals, Mumbai, India. Twenty-seven patients receiving neoadjuvant FEC (5-fluorouracil, epirubicin, and cyclophosphamide) chemotherapy for operable breast cancer underwent repeat core biopsy after 21 days of treatment. Results: The objective clinical response rate was 56%. Eight patients (31%) achieved a pathological response by histopathological criteria; two patients had a near-complete pathological response. Increased day-21 AI was a statistically significant predictor of pathological response (p = 0.049). A strong trend for predicting pathological response was seen with higher Ki-67 indices at day 21 and AI at surgery (p = 0.06 and 0.06, respectively). Conclusion: The clinical utility of early changes in biological marker expression during chemotherapy remains unclear. Until further prospectively validated evidence confirming the reliability of predictive biomarkers is available, clinical decision-making should not be based upon individual biological tumor biomarker profiles.

Keywords: Ki-67 (MIB-1), breast cancer, prognostic factor, proliferative labeling index, apoptotic index, chemotherapy, primary systemic therapy.

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Introduction

Primary breast cancer treated with neoadjuvant chemotherapy or primary systemic therapy (PST) provides an ideal model to evaluate the role of biological markers as predictive and prognostic factors. Many retrospective studies have identified patterns of biomarker expression before or after chemotherapy which have predictive or prognostic significance in relation to different clinical endpoints. However, no single pre-treatment biomarker that can accurately predict response to PST has been found to be of clinical utility to date. Despite high objective response rates to PST, a small proportion of patients will fail to respond or will progress during primary chemotherapy. The early identification of non-responders may spare these patients the unnecessary toxicity of ineffective chemotherapy and allow them to be offered alternative treatment strategies or non-cross-resistant regimens. Biological markers that can reliably predict clinical or pathological response early during a course of treatment therefore have considerable clinical potential.

PST confers equivalent survival and increased breast conservation rates compared with primary surgery and adjuvant cytotoxic chemotherapy [1, 2]. Complete pathological response (pCR) is a strong prognostic indicator for prolonged disease-free and overall survival [3]. Patients achieving a complete clinical response (cCR) also have a statistically superior disease-free and overall survival advantage over clinical non-responders [3, 4]. It should be acknowledged that in the smaller of these two studies [4], patients received some chemotherapy post-operatively. Clinical response is frequently used as a
surrogate intermediate endpoint for predicting disease-free survival and outcome after primary chemotherapy; pCR is a valid intermediate surrogate endpoint for predicting overall survival.

The ability to biopsy breast tumors in situ during primary chemotherapy provides a unique opportunity to evaluate molecular biomarkers in the tumor before and during treatment and to relate these changes to both clinical and pathological response. Immunohistochemical (IHC) analysis of tumor material from repeat biopsies during treatment may therefore help unravel the complex molecular mechanisms that ultimately determine clinical outcomes and thereby provide more useful and reliable intermediate predictive and prognostic factors.

The nuclear antigen Ki-67 (MIB-1) is a proliferation biomarker expressed only in cycling cells. A strong correlation between S-phase fraction and Ki-67 index has been demonstrated [5, 7]. Consequently, quantitative assessment of Ki-67 staining on paraffin-embedded tumor sections provides an accurate estimate of the proliferation index of individual tumors. Cytotoxic chemotherapy induces programmed cell death by apoptosis. The percentage of apoptotic cells in tumor sections may be measured by labeling fragmented DNA breaks and calculating the apoptotic index (AI) using the TUNEL (terminal transferases uridyl nick-end labeling) assay [8].

In our study, Ki-67 and apoptosis were assessed on histopathological material before, during, and after PST for operable breast cancer to evaluate whether early changes in proliferation or apoptosis predict clinical or pathological response to treatment in breast cancer of Indian women.

Patients and Methods

Treatment protocol

A series of 39 female patients with operable (T2–T4, N0 or N1, M0) invasive primary breast cancer were identified between May 2007 to Dec 2010. Patients with metastatic disease (M1) or inflammatory breast cancer (T4d) were excluded. Core biopsy of the primary tumor was performed at diagnosis and repeated on day 21, immediately prior to the second cycle of chemotherapy. Six cycles of FEC chemotherapy (5-fluorouracil 600 mg/m², epirubicin 60 mg/m², and cyclophosphamide 600 mg/m²) were administered at 21-day intervals. Bi-dimensional clinical tumor measurements were recorded before every treatment. Four patients developed disease progression by clinical criteria during chemotherapy and proceeded to immediate surgery. The remaining women underwent breast-conserving surgery or mastectomy at the surgeon’s discretion approximately 1 month after the final cycle of chemotherapy. All patients who were treated by breast-conserving surgery received post-operative radiation to the residual breast (40 Gy in 15 daily fractions plus 10-Gy boost to tumor bed in five fractions; n = 12) plus or minus lymph nodes (50 Gy in 25 fractions for a period of 5 weeks; n = 2). Post-mastectomy chest wall radiation was delivered to 13 of 15 patients (11 chest wall only, 2 chest wall and nodes). No patient received post-operative chemotherapy. Women with estrogen receptor (ER)-positive tumors received 5 years of adjuvant tamoxifen (20 mg daily) starting after surgery.

Immunohistochemistry

The most representative tumor tissue block was chosen from each case and 5 μm sections were taken to poly-L-lysine coated slides for immunohistochemical staining. Standard streptavidin-biotin immunoperoxidase method was used for immunostaining with Ki-67 Clone: MIB-1; M7240; DakoCytomation, Denmark, dilution: 1:25. The tissue sections were deparaffinized in xylene, rehydrated in alcohol series, immersed in distillate water. The sections were then boiled in citrate buffer solution (10 mmol/L, pH=6.0) in a microwave oven, 3 times for ten minutes for epitope retrieval in staining with Ki-67. Endogenous peroxidase activity was blocked using a 0.3% solution of hydrogen peroxide in tris-buffered saline (TBS) at room temperature for 10 minutes and rinsed with TRIS buffer. Primary antibodies were applied for 30 minutes at room temperature and washed in TRIS buffer. Linking antibody and streptavidin peroxidase complex (DAKO LSAB Kit, K0355; DakoCytomation, Denmark) were added consecutively for ten minutes at room temperature and washed in TRIS buffer. Peroxidase activity was visualized with 0.03% 3, 3-diaminobenzidine tetra hydrochloride (DAB) (DAB kit; K3467; DakoCytomation, Denmark), applied for 5 minutes. The sections were then washed in deionizer water, counterstained with Mayer’s Hematoxylin and mounted.

The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay

Apoptotic cells were visualized using a commercial end labeling (TUNEL) assay previously described [8]. Briefly, endogenous peroxidase activity was inactivated with 1% hydrogen peroxide in phosphate-buffered saline (PBS) (pH 7.4) for 10 minutes. Nuclei of tissue sections were stripped of proteins by incubation with 0.5% pepsin (pH 2.0) (Sigma Chemical Co, Poole, Dorset, UK) for 30 minutes at 37°C. The sections were washed five times in distilled water to remove all traces of pepsin. Each section undergoing the TUNEL protocol was incubated for 5 minutes in Tris buffer (pH 7.6) and then for 1 hour at 37°C in 100 μl of reaction mixture consisting of 15 units TdT FPLC pure (Pharmacia, Windsor, Berkshire, UK), 0.5 nmol biotin-16-dUTP (Boehringer Mannheim, Mannheim, Germany), 5 mM cobalt chloride, 0.2 M sodium cacodylate, 25 mM Tris HCl (pH 6.6), and 0.25 mg/ml bovine serum albumin (BSA) dissolved in distilled water. After extensive washing in distilled water, the sections were incubated for 30 minutes at room temperature in 1:400 dilution of horseradish peroxidase conjugated to streptavidin (DakoCytomation, Denmark) in PBS supplemented with 1% BSA and 0.5% Tween 20. Color was developed for 10 minutes using 0.05% dianmonobenzidine plus 0.07% imidazole plus 0.1% hydrogen peroxide and further intensified in 0.5% copper sulphate with 0.9% sodium chloride for 1 minute. The sections were counterstained in Mayer’s haematoxylin,
dehydrated, cleared in xylene, and mounted in DPX.

IHC scoring was performed without prior knowledge of the clinical response. Ki-67 score was counted on a minimum of 10 randomly selected X40 objective magnification high-power fields containing representative sections of tumor and calculated as the percentage of positively stained cells to total cells. The AI was assessed by counting at least 3,000 malignant cells at X400 objective magnification. Stained apoptotic cells were recorded, and cells displaying classic apoptotic morphology but not staining were also incorporated in the AI. Non-staining apoptotic cells were recognized in the midst of cells with normal morphology by having either condensed, irregular nuclei frequently with a crescent-shaped appearance or fragmented nuclei within cells showing cytoplasm withdrawal. Areas with extensive necrosis were avoided.

Statistical analysis
Statistical analysis was carried out using SPSS-16 procedure (SPSS Analytical Software Inc, Chicago, IL) and SAS 9.1 (SAS Institute Inc., Cary, USA). Associations between ordinal variables were assessed using χ² analyses or the Fisher exact test in the case of two-by-two variables. Analyses involving Ki-67 and AI as continuous variables were investigated using analysis of variance. A logistic regression analysis was performed.

Results
In our study immunohistochemical staining results of invasive duct cancer of the breast in Indian women was observed, the entire slide was scanned for immunostaining evaluation by light microscope. Tissue sections exhibiting nuclear immunoreactivity for Ki-67 in tumor cells were identified as dark brown nuclei shows high proliferative labeling index in Figure 1a. Low proliferative labeling index shown in Figure 1b. of invasive duct breast cancer.

Day -21 biopsy
Sufficient invasive cancer suitable for immunohistochemical analysis was present in 27 of the 39 day-21 biopsies. The remaining 12 patients were excluded from the analysis: eight yielded no demonstrable invasive tumor on day-21 biopsy, two comprised high-grade DCIS (ductal carcinoma in situ) only (presumably due to geographical miss), and two contained tiny foci of invasive tumor deemed too small to reliably interpret immunohistochemical staining.

Patient demographics
Of the 27 evaluable patients, 52% were pre-menopausal. Most tumors were grade 2 (33%) or grade 3 (41%), stage T2 (44%) or T3 (42%), and clinically node-negative (63%) before treatment. 56% were ER-positive and 41% HER-2/neu (human epidermal growth factor-2)-positive. The patient characteristics are shown in (Table 1).

Response rates
All 27 patients were evaluable for clinical response on completion of chemotherapy. Surgical blocks were retrieved for pathological scoring in 26 cases. The objective clinical response rate (CR + PR) was 56% (15/27). Four patients (15%) progressed by clinical criteria after two, two, four, and six cycles of chemotherapy, respectively, and proceeded to immediate surgery. The remaining eight patients (30%) had clinically stable disease on completion. Eight patients (30%) achieved a pathological response by histopathological criteria. There were no complete pathological responders, although two women had a 'near-pCR' with residual foci of invasive cancer measuring 1 and 2 mm in maximum dimension, respectively.
Table 1 Patient characteristics

| Characteristic                        | n   | Percentage |
|--------------------------------------|-----|------------|
| Age: median                          | 51  | years      |
| Age: range                           | 29-65 |
| Menstrual status                     |     |            |
| Pre                                  | 14  | 52         |
| Peri                                 | 6   | 22         |
| Post                                 | 7   | 26         |
| Clinical TNM stage at diagnosis      |     |            |
| T2                                   | 12  | 44         |
| T3                                   | 13  | 48         |
| T4                                   | 2   | 7          |
| Clinical node status                 |     |            |
| N0                                   | 17  | 63         |
| N1                                   | 10  | 37         |
| Breast                               |     |            |
| Right                                | 15  | 56         |
| Left                                 | 12  | 44         |
| Breast surgery                       |     |            |
| Wide excision                        | 12  | 44         |
| Mastectomy                           | 15  | 56         |
| Postoperative radiotherapy           |     |            |
| Nil                                  | 2   | 7          |
| Breast                               | 12  | 44         |
| Chest wall                           | 13  | 48         |
| ER status (biopsy)                   |     |            |
| ER-Positive                          | 15  | 56         |
| ER-Negative                          | 12  | 44         |
| HER-2/neu status (biopsy)            |     |            |
| HER-2/neu-positive                   | 11  | 41         |
| HER-2/neu-negative                   | 16  | 59         |
| Tumor grade                          |     |            |
| Unknown*                             | 5   | 19         |
| G1                                   | 2   | 7          |
| G2                                   | 9   | 33         |
| G3                                   | 11  | 41         |
| Pathological T stage                 |     |            |
| pCR                                  | 0   | 0          |
| PT1                                  | 8   | 30         |
| PT2                                  | 17  | 63         |
| PT3                                  | 2   | 7          |
| Pathological N stage                 |     |            |
| pN0                                  | 11  | 41         |
| pN1                                  | 12  | 44         |
| pNX                                  | 4   | 15         |

*Grading not possible due to chemotherapy artifact. ER, estrogen receptor; HER-2/neu, human epidermal growth factor 2; TNM, tumor, node, metastasis.

Biomarkers before, during and after chemotherapy

The median and range of Ki-67 indices before chemotherapy, at day 21, and after treatment were 27.9% (4.1%–43.9%), 17.3% (4.1%–44.8%), and 21.7% (2.4%–50.4%), respectively. The apoptotic indices at baseline, day 21, and surgery were 1.92% (0.23%–5.4%), 1.69% (0.33%–11.2%), and 2.19% (0.9%–4.9%), respectively. At each time point, there was a significant positive relationship between these two parameters: the correlation coefficients were 0.47 (p = 0.026), 0.65 (p = 0.0005), and 0.66 (p = 0.0014) in the biopsy, day-21 and surgery samples, respectively.

Changes in biomarkers during and after chemotherapy

A reduction in Ki-67 index from pre-treatment values was observed in 63% (17/27) of patients at day 21 and 69% (18/26) at surgery shown in Figure 2; there was no tumor material available for one patient at surgery. Eleven patients demonstrated sequential reductions in Ki-67 LI throughout the two study periods, and four patients showed sequential increases during therapy. Four of the 17 tumors that showed a reduction in LI between biopsy and day 21 showed increases in proliferation between day 21 and surgery. Of the 10 tumors that showed no change or an increase in Ki-67 LI during the first 3 weeks of chemotherapy, half displayed a subsequent reduction between day 21 and surgery.

AI decreased in 50% (10/20), increased in 45% (9/20), and was unchanged in one patient shown in Figure 3. Overall, between initial biopsy and surgery, a similar pattern was seen with eight (47%) out of 17 patients, with successful staining showing a reduction in AI. Between day 21 and surgery, the majority of tumors (10 of 18) increased in apoptotic activity shown in Figure 3. Unlike in the Ki-67 LI data, there was no consistent pattern in apoptosis throughout treatment.

Ki-67(MIB-1), clinical and pathological response

Neither pre-treatment nor post-chemotherapy median Ki-67 index differed significantly between clinical or pathological responders and non-responders. Clinical responders (CR+PR) had significantly lower median Ki-67 indices at day 21 than did non-responders (11.4% versus 27.0%, p = 0.02). A similar trend for lower day-21 Ki-67 in
patients achieving a cCR was also recorded \((p = 0.10)\). Clinical responders exhibited significantly greater percentage reductions in Ki-67 at day 21 than did non-responders \((-50.6\% \text{ versus } -5.3\%, p = 0.04)\). A decrease or no change in day-21 Ki-67 was observed in 80% \((12/15)\) of clinical responders compared with 58% \((7/12)\) of non-responders shown in Figure 2. In the 11 patients who showed sequential reductions in Ki-67 throughout the study period, 9 \((82\%)\) achieved a clinical response \((p = 0.019)\) shown in Figure 3.

Paradoxically, the median day-21 Ki-67 was higher in pathological responders \((30.3\% \text{ versus } 14.1\%, p = 0.046)\). There were no association between pathological response and changes in Ki-67 throughout the study period and no correlation between clinical and pathological responses shown in Figure 3.

**AI and Clinical and Pathological Response**
Median AI at all three time points and relative changes at day 21 and surgery did not differ significantly between clinical and pathological responders or non-responders shown in Figure 3. However, there was a trend toward higher pre-treatment AI in pathological responders \((2.72 \text{ versus } 1.65, p = 0.10)\). A non-significant trend toward increased apoptosis at day 21 in pathological responders was also observed \((5.30 \text{ versus } 1.68, p = 0.12)\). No pattern in the distribution of changes in day-21 AI emerges between clinical and pathological responders when the data are represented graphically.

**Biological Characteristics of Complete or 'Near-Complete' Pathological Responders**
The ability to predict pCR, arguably the most useful endpoint of all, could not be assessed in this cohort, because no patient achieved a pCR. However, two patients had only tiny foci of residual invasive carcinoma demonstrable after chemotherapy. Both these 'near-pCR' patients had a very high AI at operation \((3.96 \text{ and } 3.61)\), significantly greater than patients not achieving a 'near-pCR' \((p = 0.04)\). One of these two patients was evaluable for day-21 AI; a large increase in AI was seen \((5.3 \text{ versus } 3.86)\) after the first cycle of chemotherapy. No clear trend in changes in Ki-67 during or after treatment was seen in the two patients with excellent pathological tumor regression.

**Logistic Regression Analysis for Prediction of Response by Different Modalities of Assessment**
Logistic regression analyses were performed to establish which, if any, of the biological marker variables measured at different time points could predict response outcomes by clinical, radiological, or pathological criteria (Table 2). Increased AI at day 21 was a statistically significant predictor of pathological response \((p = 0.049)\). Similarly, greater Ki-67 indices at day 21 and higher AI at surgery displayed a strong trend for predicting pathological response \((p = 0.06 \text{ and } 0.06, \text{ respectively})\). Reductions in Ki-67 and AI at day 21 were strongly predictive of better clinical response by UICC category \((p = 0.01 \text{ and } 0.02, \text{ respectively})\). No significant associations were observed between the various biological markers and clinical CR or radiological response assessed by mammography and/or ultrasound. Low baseline AI was associated with poor worst radiological response \((p = 0.04)\).

**Table 2** Logistic regression analysis showing significant associations for prediction of response by different modalities of assessment and response classifications

| Response variable | p Value |
|-------------------|---------|
| Pathological response (R/NR) |         |
| Ki-67 D21 | 0.0616 |
| AI D21 | 0.0497 |
| AI Sx | 0.0620 |
| Pathological CR |         |
| Not assessable | - |
| 'Near' pathological CR |         |
| No significant associations | - |
| Clinical response (CR/PR/SD/PD) |         |
| [Path T stage T1 versus T3 | 0.0028 |
| Ki-67 D21 | 0.0097 |
| AI D21 | 0.0224 |
| Clinical response (CR/PR/NR) |         |
| [Path T stage T1 versus T3 | 0.0066 |
| Ki-67 D21 | 0.0326 |
| AI D21 | 0.0224 |
| Clinical response (R/NR) |         |
| Ki-67 D21 | 0.0323 |
| Clinical CR |         |
| No significant associations | - |
| Radiological response (R/NR) |         |
| No significant associations | - |
| Mammmographic response (CR/PR/SD/PD) |         |
| No significant associations | - |
| USS response (CR/PR/SD/PD) |         |
| No significant associations | - |
| Worst radiological response (CR/PR/SD/PD) |         |
| AI biopsy | 0.0418 |
| Worst radiological response (CR/PR/NR) |         |
| No significant associations | - |

AI: apoptotic index, CR: complete response, D21: day-21, NR: non-responder, PD: progressive disease, PR: partial response, R: responder, SD: stable disease, USS: ultrasound scan.

**Discussion**
Standard UICC (International Union against Cancer) criteria were used to define objective clinical response [9]. Changes in the calculated product of bi-dimensional tumor measurements on two successive evaluations were recorded at each visit. Complete response (CR) was defined as no residual palpable abnormality, partial response (PR) as greater than 50% tumor shrinkage, stable disease (SD) as less than 50% tumor shrinkage or no change, and progressive disease as an increase of at least 25%.

Although many different systems for grading pathological response have been proposed [10, 15], no standard method for pathological assessment after chemotherapy has been adopted. A previously described simple scoring system that can be applied in clinical practice was therefore employed [16]. A consultant histopathologist (P.I.
Richman) blinded to clinical outcome reviewed all paired biopsy and surgical specimens. We defined 'histological tumor response' by both (a) an apparent reduction in tumor cell/stroma ratio and (b) one or more chemotherapy-induced cytological changes (that is, enlarged cells with finely vacuolated cytoplasm, an enlarged vesicular nucleus with a prominent single eosinophilic nucleolus, or an enlarged hyper chromatic dense nucleus with an irregular outline). The following classification was used to score surgical specimens for pathological response: CR, no residual invasive carcinoma; PR, residual invasive cancer with histopathological tumor response; and SD, residual invasive cancer with no histopathological tumor response.

The prognostic significance of pre-treatment Ki-67 index in breast tumors varies. Intuitively, rapidly proliferating tumors confer a poor prognosis, and the majority of studies confirm this association [17, 26]. In some series, breast tumors with a high proliferative index have a worse prognosis despite endocrine treatment [27, 28] or chemotherapy [29]. However, other authors report no significant difference in outcome after chemotherapy or hormone treatment in patients with rapidly proliferating tumors compared with those with more slowly growing tumors [30, 33].

Changes in tumor cell proliferation before and after pre-operative treatment have also been evaluated. A reduction in Ki-67 index has been demonstrated after chemotherapy [30, 34, 35], tamoxifen therapy [31, 36], and chemoendocrine therapy [37, 38]. More recently, studies have focused on the evaluation of early changes in cell proliferation during treatment by analyzing Ki-67 index in repeat tumor samples taken at varying intervals during chemotherapy. Two studies at the Royal Marsden Hospital (London, UK) performed on cytological material obtained from fine needle aspiration cytology (FNAC) during chemoendocrine treatment showed that reductions in Ki-67 proliferation index after 10, 14, or 21 days significantly predict clinical response [37, 38]. However, Billgren and colleagues demonstrated that a decrease of more than 25% in proliferating fraction after the first course of chemotherapy significantly predicted a reduced risk of disease recurrence (p = 0.033) but showed no correlation with local objective response [39]. Multivariate analysis revealed that the decrease in proliferating fraction significantly added prognostic information to lymph node status. In a similar study, patients who responded to neoadjuvant chemotherapy and concurrent tamoxifen were found to be more likely to have a reduction in Ki-67 ten days after chemotherapy than were non-responders [40]. Post-treatment Ki-67 index is also of prognostic importance: In a series of 42 patients treated with primary chemotherapy, high proliferative index in residual tumor was associated with a worse disease-free survival [41].

In our study, there was no significant difference in baseline Ki-67 or AI between responders and non-responders assessed by clinical, pathological, or radiological criteria. Both pre-treatment and post-chemotherapy cell proliferation and apoptosis failed to predict response by any modality of assessment.

More than two thirds of tumors exhibited a decrease from baseline Ki-67 index at day 21 and at surgery. There was no significant difference in the magnitude of the decrease in Ki-67 from baseline to surgery between different groups. The degree of cell proliferation measured before or after chemotherapy was not able to discriminate clinical or pathological responders from non-responders. Clinical responders were more likely to exhibit a reduction in Ki-67 index after one cycle of chemotherapy. This group also displayed larger relative decreases in cell proliferation after the first cycle of treatment (median -50.6, range -73.0 to 93.3) than did non-responders (median -5.3, range -43.4 to 57.7) (p = 0.04). Paradoxically, Ki-67 expression at day 21 was greater in pathological responders compared with non-responders, despite the fact that the distribution of pre-treatment Ki-67 LI was similar in both groups. This observation seems counterintuitive because tumor regression would be expected to be accompanied by a reduction in cell proliferation. However, there was no association between clinical response and those patients who achieved a partial pathological response. These findings underline the uncertainty surrounding the optimum method of assessment of response in biomarker studies and raise concerns that one (or perhaps both) of the classifications of response used in this study may not be a reliable surrogate endpoint. Of the 27 evaluable patients, 52% were pre-menopausal. Most tumors were grade 2 (33%) or grade 3 (41%), stage T2 (44%) or T3 (42%), and clinically node-negative (63%) before treatment. 56% were ER+, 44% ER- and 41% HER-2/neu (human epidermal growth factor-2)-positive.

Some groups [47-49] investigating the modulation of steroid receptor status by PST reported no significant changes in ER or PgR after primary chemotherapy. The data presented here concur with two earlier small studies in which 10% [50] and 33% [51] of breast cancers expressed altered steroid receptor status after PST. A recently published comprehensive analysis of hormone receptor immunochemistry in 450 breast cancer patients confirmed these observations in a larger cohort and speculated on a possible hypothesis for the mechanism of changes in ER and PgR status after PST [52].

Neither pre-treatment nor post-chemotherapy median Ki-67 index differed significantly between clinical or pathological responders and non-responders. Clinical responders (CR+PR) had significantly lower median Ki-67 indices at day 21 than did non-responders (11.4% versus 27.0%, p = 0.02). A similar trend for lower day-21 Ki-67 in patients achieving a cCR was also recorded (p = 0.10). Clinical responders exhibited significantly greater percentage reductions in Ki-67 at day 21 than did non-responders (-50.6% versus -5.3%, p = 0.04). A decrease or no change in day-21 Ki-67 was observed in 80% (12/15) of clinical responders compared with 58%
(7/12) of non-responders. In the 11 patients who showed sequential reductions in Ki-67 throughout the study period, 9 (82%) achieved a clinical response ($p = 0.019$).

Paradoxically, the median day-21 Ki-67 was higher in pathological responders (30.3% versus 14.1%, $p = 0.046$). There were no association between pathological response and changes in Ki-67 throughout the study period and no correlation between clinical and pathological responses.

More than half the patients showed an increase in measured cell proliferation between day 21 and surgery. In responding patients, the reduction in Ki-67 index after one cycle of treatment was not sustained and was often followed by a rebound increase in cell proliferation by the time of surgery (responders 26.8, range 2.4 to 48.0; non-responders 18.9, range 6.8 to 50.4).

The observed changes in proliferation during treatment may have implications for determining the optimum duration of neoadjuvant chemotherapy prior to surgery for operable primary breast cancer. Recently published randomised clinical trials suggest that the addition of sequential taxane chemotherapy after four cycles of anthracycline PST increases clinical and pathological response rates and translates into improved overall survival [15]. The rebound increases in cell proliferation noted after six cycles of anthracycline treatment in this study may partly explain the superior clinical results achieved when patients are switched to non-cross-resistant chemotherapy regimens midway through neoadjuvant treatment. This phenomenon warrants further investigation to establish whether changes in tumor cell kinetics during treatment can identify which patients are most likely to benefit from sequential chemotherapy schedules.

Wide variations in AI were seen both during and after chemotherapy. The non-significant trend toward increased AI in pathological responders at day 21 was confirmed by the logistic regression analysis showing that increased day-21 AI is a statistically significant predictor of pathological response. This observation suggests that tumors exhibiting high levels of cell death after one cycle of chemotherapy are more likely to achieve pathological regression. The high AI seen in the two near-pCR patients at operation indicates that increased apoptosis after chemotherapy may also predict which patients will have a good pathological response. Analysis of a larger cohort is required to explore this hypothesis further. The magnitude of changes in AI during treatment did not predict clinical, radiological, or pathological response to treatment.

The optimum time point for detecting early cell kinetic changes that may predict clinical and pathological outcomes is unknown. Other groups have repeated FNA cytology 10 days after chemoendocrine treatment [37, 38]. Day 21 was arbitrarily chosen as a convenient time in this study, to coincide with patients' return to hospital for their second cycle of chemotherapy, although there are no convincing data that it is the most appropriate time to test biomarkers. It is possible that 21 days after chemotherapy is too late to observe the peaks of apoptotic response and suppression of proliferation induced by cytotoxic treatment; there may be earlier times when the biologic response to treatment is more critically related to therapeutic outcome. Indeed, there is some evidence that apoptotic response after chemotherapy lasts for several days only [43, 45]. Ideally, serial biopsies may help to chart the precise pattern of changes in biological markers during treatment; realistically, however, large studies of this type are impractical, because few patients are likely to agree to repeated invasive tumor biopsies.

It is important to recognize the potential limitations of this study. Like most published series in this field, the number of patients reported is small. The use of tumor biopsies to assess molecular marker expression before and after treatment has become increasingly widespread as the search for predictive markers for neoadjuvant chemotherapy continues. Critics initially argued that this approach was subject to sampling error and intra-tumor variability. However, the widely quoted validation study by Ellis and colleagues [35] demonstrated that core biopsies accurately reflect the expression of biological markers in whole tumor sections.

In addition to clinical response, a novel descriptive histological response analysis was used to grade pathological response in this study. Although this system has not been prospectively validated or proven to relate directly to survival, the strong body of evidence that pCR is a good prognostic indicator for long-term survival justifies its use. Unfortunately, the analysis was hampered by the absence of complete pathological responders in this small series, forcing the authors to adopt the more widely used assessment of clinical response as an endpoint.

Various methods have been validated as measures of proliferation, including mitotic body counting, immunohistochemical staining of antigens associated with proliferation or the estimation of the fraction of cells in S-phase by flow cytometry or the incorporation of thymidine or BrdU. Each of these methods has been shown to have prognostic value in breast cancer, but all require biopsy or surgical samples of tumor tissue. This does lead to several limitations: biopsies are invasive and involve a degree of patient discomfort; deepseated tumors may not be amenable to biopsy; the biopsy may not be representative of the whole tumor, as tumor heterogeneity is well described; and the scoring methods are partly subjective and, therefore, variable.

Proliferation is to be used as a prognostic or predictive factor, it is important for pathology reports to use a standardized technique. Until the reliability of these new methods is confirmed, the current standard proliferation assay should be Ki-67 immunohistochemistry, given its relative simplicity and wide availability [46].

Good correlation is seen between Proliferating Cell
Nuclear Antigen (PCNA) and flow cytometrically determined cell cycle distributions based on DNA content, and Bromodeoxyuridine (BrdU) and the proliferation-associated Ki-67 antigen. PCNA and Ki-67 expression are positively correlated with p53 overexpression, high S-phase fraction, aneuploidy, high mitotic index, and high histopathologic grade in human breast cancer specimens, and are negatively correlated with estrogen receptor content. Individual proliferation biomarkers are associated with slightly different phases of the cell cycle and are not equivalent. Ki-67 is easily available and is cheaper biomarker available in India. Compared with these other markers like PCNA and BrdU, Ki-67 staining is easy to perform, economical, and more reproducible.

Novelty biomarkers that relate to the actions of chemotherapy drugs are needed if reliable predictive biomarkers are to be identified. One such example is topoisomerase IIa, a molecular target for anthracyclines. A recent study showed that strong topoisomerase IIa staining is an independent predictor of clinical tumor regression [53]; a confirmatory study is underway on this data set. In the meantime, it is imperative that large ongoing randomised clinical trials of new PST regimens encourage recruitment into parallel biological marker studies so that more powerful data sets can continue the search for favorable and unfavorable biological profiles that may ultimately help clinicians individualize treatments. Meanwhile, the ability to study the patterns of expression of thousands of candidate genes simultaneously using new micro-array technologies [54, 55] may rapidly surpass retrospective analyses using immunohistochemistry in the continuing search for predictive and prognostic factors.

**Conclusion**

In this small study, pre-treatment or post-chemotherapy median Ki-67 (MIB-1) index, median AI at all three time points, and relative changes at day 21 and surgery did not differ significantly between clinical or pathological responders and non-responders. Clinical responders achieved significantly greater percentage reductions in Ki-67 and lower median Ki-67 indices at day 21 than did non-responders. Pathological responders displayed higher median day-21 Ki-67 expression. Increased day-21 AI was a statistically significant predictor of pathological response. A strong trend for predicting pathological response was seen with higher Ki-67 indices at day 21 and AI at surgery.

The clinical utility of early changes in biological marker expression during chemotherapy remains unclear. For the time being, clinical decision-making should not be based upon individual biological tumor biomarker profiles until further prospectively validated evidence confirming the reliability of predictive markers is available. In the meantime, large prospective clinical trials of neoadjuvant chemotherapy should include parallel biological marker studies to facilitate immunohistochemistry and microarray analysis on histopathological tissue taken at various time points before, during, and after neoadjuvant chemotherapy to continue the search for clinically useful predictive biomarkers.

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