# Immunohistochemical phenotype of breast cancer during 25-year follow-up of the Royal Marsden Tamoxifen Prevention Trial

Simone I Detre[1], Susan Ashley[2], Kabir Mohammed[2], Ian E Smith[3], Trevor J Powles[4] and Mitch Dowsett[1]

*Correspondence to*: Professor Mitch Dowsett, Academic Department of Biochemistry, 4th Floor Wallace Wing, Royal Marsden Hospital NHS Trust, Fulham Road, London SW3 6JJ

E-mail: mitch.dowsett@icr.ac.uk

1   Academic Department of Biochemistry, 4th Floor Wallace Wing, Royal Marsden Hospital NHS Trust, Fulham Road, London, SW3 6JJ

2   RDSU – Research and Development, Royal Marsden Hospital NHS Trust, Sutton, SM2 5PT

3   Breast Unit, Royal Marsden Hospital NHS Trust, Fulham Road, London, SW3 6JJ

4   Cancer Centre London, Wimbledon, London, SW19 5NB

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**Abstract**

The randomized double-blinded Royal Marsden Tamoxifen Breast Cancer Prevention Trial in healthy high-risk women started in 1986 and is still blinded. 2471 eligible participants were randomly assigned to tamoxifen (20mg/day) or placebo for 8 years. Analysis in 2006 showed a 30% risk reduction of ER-positive invasive breast cancer mostly in the post treatment period. Biomarker analysis in this population may identify any sub-group specific preventive effects tamoxifen. After a median follow-up of 18.4 years, 242 patients had developed invasive cancer, 134 on placebo and 108 on tamoxifen. From these, 180 tissue blocks were available and ER, PgR, Ki67, HER2 and EGFR were immunohistochemically analysed. A 32% reduction in ER+ and PgR+ invasive cancers resulted after 8 years of treatment. Quantitative levels of ER and PgR were lower in the tamoxifen-treated group, significantly so for ER (p=0.001). These lower ER levels were restricted to the post-treatment period (p=0.018). Amongst the ER+ group there was a similar proportional decrease in PgR+ and PgR- tumours by tamoxifen. The median levels of Ki67 were similar in both arms. The numbers of HER2 positive and EGFR positive cancers were higher in the tamoxifen arm but not significantly so. In conclusion, tamoxifen's preventive effects result in reduced ER-positive but not ER-negative tumours and reduced ER expression in the ER-positive cases largely confined to the post-treatment period. Overall reductions in PgR expression are explained by lower frequency of ER-positive cases. Impact on Ki67, HER2 and EGFR was modest.

**Introduction**

In vivo laboratory evidence that the incidence of breast cancer could be reduced and an observed reduction in the risk of new contralateral breast cancer resulting from adjuvant treatment of women with primary breast cancer with the selective estrogen receptor modulator (SERM), tamoxifen[1, 2] indicated that this drug could be used to prevent breast cancer in healthy women. The first trial, the Royal Marsden Prevention Trial (RMPT), began recruitment in 1986[3]. Three other major randomised, placebo-controlled trials of tamoxifen given for between 5 and 8 years were conducted. Together with the RMPT these recruited a total of over 25,000 healthy women at increased risk of breast cancer. Overview of these trials confirmed a significant reduction in the risk of developing breast cancer of 33% that, among invasive tumours, was restricted to a reduction in the incidence of ER-positive disease (by 44%)[4]. A reduction in risk persisted for at least 15 years[4, 5]. These positive data alongside the low incidence of side effects with tamoxifen have led to it being recommended for use by regulatory bodies as a risk-reduction strategy in healthy women at increased risk of breast cancer.

The phenotype of primary breast tumors is a major determinant of the medical treatment of patients and certain key features are associated with long-term prognosis. ER and HER2 are assessed in all tumours for treatment selection and progesterone receptor (PgR) and the proliferation marker Ki67 in most for the assessment of prognosis. Information on these biomarkers in patients developing breast cancer either during or after risk reduction therapy with tamoxifen is therefore important for better understanding of the likely

benefits of such treatment as well as for the identification of any sub-group specific preventive effects. Other than ER status there have been few reports on any differences in the phenotype of breast cancers developing during or after the tamoxifen treatment period. We previously reported that in 67 tumors from the RMPT (35 placebo, 32 tamoxifen arm) median ER levels were lower in the tumours developing in tamoxifen-treated patients(6). At that time when most patients were still on their randomized treatment (median follow-up 70 months) there was no reduction in breast cancer incidence.

The most recent report of the clinical outcome of the trial was at a median of 13 years 2 months (maximum 19 years 10months)(5). After that length of follow-up 186 patients had developed invasive cancer (104 placebo, 82 tamoxifen; hazard ratio [HR] 0.78 p=0.10) and 139 of these were ER-positive (86 placebo; 53 tamoxifen; HR 0.61, p=0.005). The HRs for the 8-year treatment period and the post-treatment period were 0.77 (p=0.3) and 0.48 (p=0.004), respectively.

In the meantime the NSABP have reported(7) the microarray gene expression analysis of 108 tumours from their P1 tamoxifen prevention trial, 69 placebo and 39 tamoxifen-treated. ER expression, whether measured by semi-quantitative immunohistochemistry or gene expression, was lower in the 27 ER-positive tumors on tamoxifen than those in the 57 ER-positive tumours in the placebo group. The only other gene to show substantial differential expression was *GFRA1*, which codes for the Glial cell line-derived neurotrophic receptor alpha-1 also known as Glial cell line-derived neurotrophic factor receptor (GDNFR). Our group has reported that activation

of this receptor in ER-positive breast cancer is associated with resistance to tamoxifen and aromatase inhibitors(8).

The primary objective of the current study was to determine whether randomisation to possible risk-reduction treatment with tamoxifen or placebo was associated with differences in the commonly measured phenotypic markers ER, PgR, HER2 and Ki67 as well as epidermal growth factor receptor (EGFR). The last of these is uncommonly expressed in ER-positive tumours and is associated with tamoxifen resistance when it is(9).   The study was conducted in tumors from the RMPT which at its most recent analysis had completed over 20 years of follow-up(5) and gave us the opportunity to fulfill a secondary objective, i.e. to determine whether any phenotypic differences varied according to whether the patients were in the on-treatment or post-treatment period at the time of tumor presentation.

**Materials and Methods**

The trial (I SRCTN07027313) was approved by the Royal Marsden Hospital Ethics Committee. Consent to use tissue for research was provided by all patients in whom tumours arose after 1st September 2006 when the Human Tissue Act became active. The study design and clinical outcome data have previously been published(5).   A total of 2494 healthy women were randomly assigned to oral tamoxifen (20mg/day) or placebo for a treatment period of 8 years. Participants, medical professionals and laboratory staff remain blind to the randomised treatment unless unblinding was specifically requested and the analyses are based on an intention to treat.

Immunohistochemical analyses for ER, PgR, Ki67, HER2 and EGFR were undertaken on sections from formalin-fixed paraffin wax-embedded blocks using reagents listed in Supplementary Table 1. The immunohistochemical staining was performed on a Dako Autostainer using REAL kits for all biomarkers except HER2. FISH analyses (PathVysion) were carried out on HER2 positive cases when scored as IHC2+. Haematoxylin & eosin stained slides were used to confirm presence of invasive breast carcinoma. In situ breast cancers were excluded. If patients received neoadjuvant therapy, core-cut biopsies taken at diagnosis were used; otherwise sections from the excision biopsy were taken.

ER and PgR were scored as H-scores (range 0-300)(10). The positivity cut-off for ER and for PgR H-score was >1 to equate closely to that recommended in ASCO/CAP guidelines(11). Ki67 was assessed as % positivity of nuclear stained cells and had no designated cut-off. EGFR was scored as percentage positive membrane staining and deemed positive if the score was greater than 1. HER-2 was considered positive if the IHC score was assessed as 3+ by ASCO/CAP criteria, (12) or if assessed as 2+ and FISH analysis showed HER2/CEP17 ratio of >2.0.

All analyses were carried out blind to randomisation with the trial statistician supplying pathology numbers and case details. All statistical analyses were performed by the trial statistician. The cut-off point for the current analysis was 1st October 2010. The primary endpoint was the occurrence of invasive breast cancer. Invasive breast cancer-free survival was calculated using the Kaplan Meier method. Non-invasive breast cancers were censored. The Cox proportional hazards model was used to check for the

treatment effect and hazard ratio with 95% confidence interval reported.  A secondary planned analysis of ER-positive invasive breast cancer was also done. Biomarker data were summarised and compared between treatment arms in the overall patient population and in subgroups according to ER-status and diagnosis of cancer during treatment or post-treatment.  The continuous biomarker variables were summarised using mean and 95% confidence interval and median and interquartile range.  The scores were then compared using the non-parametric Mann-Whitney U-test.  Categorical variables were summarised using number of observations and percentages according to treatment arms and compared using Chi-square test.

**Results**

The current IHC analysis was conducted in all available tumours that arose by 1st October 2010 (median follow-up 18.4 years, maximum 23.7 years). By that time 242 patients had developed invasive cancer, 134 on placebo and 108 on tamoxifen (hazard ratio [HR] 0.80 95% CI 0.62-1.02, p=0.076) (Table 1).  Of these 187 were ER-positive, 108 on placebo and 79 on tamoxifen (HR 0.72 95%CI 0.54-0.97, p=0.028). The HR in the post-treatment period for all patients was 0.74 (95%CI 0.53-1.02, p=0.067) and for ER-positive cases was 0.68 (95% CI 0.47- 0.996, p=0.048). A complete updated clinical report of the trial will be published separately. The efficacy end-points included in this report are sufficient to allow full interpretation of the tumor-based biomarker data.

Immunohistochemical (IHC) data was available from 179 patients. Reasons for non-availability of data were: 38 tumour blocks could not be

retrieved from sites; 12 subjects either had no written consent for biomarker analysis recorded or declined; 11 samples had too little tumour to assess biomarkers.  A similar proportion of tumours were available in the IHC cohort for each of the two arms: placebo, 75% (100/134); tamoxifen 73% (79/108). The major demographics of the population are shown in Table 2. In the tamoxifen arm in the IHC cohort there were 54 ER-positive and 25 ER-negative tumours [1 ER-negative/PgR-positive]) compared with 86 ER-positive, 14 ER-negative in the placebo arm.  The difference in the proportions of ER-positive and ER-negative tumours between the arms was statistically significant (chi-squared p=0.008).

In the overall follow-up period PgR-positive status was also lower in the tamoxifen arm than in the placebo arm (63% vs 76%, p=0.06) (Table 3). This was only statistically significant beyond 8 years (p=0.039) but the proportions were little different from those in the first 8 years (Table 4). Overall 76 (76%) tumours were ER-positive/PgR-positive in the placebo arm compared with 49 (62%) in the tamoxifen arm.

As in the earlier 13-year median follow-up clinical report (6) there was a greater preventive effect of tamoxifen in the post-treatment period in this IHC cohort (0-8 years: 40 tamoxifen, 44 placebo; beyond 8 years: 39 tamoxifen, 56 placebo) (Table 3). There was no significant difference in the proportions of ER-positive versus ER-negative tumours between the treatment arms in the first 8 years but only 64% were ER-positive in the tamoxifen arm after 8 years compared with 86% in the placebo arm (p=0.014).

Among the ER-positive tumours the ER level as estimated by H-score was somewhat lower in tamoxifen-treated tumours but this was not statistically

significant either overall (p=0.053) or in the separate time periods (Tables 3 and 4). PgR levels also showed non-significant trends to being lower in the ER-positive cases.

There were 12 HER2-positive cases in the tamoxifen arm and 10 in the placebo. Fifteen cases were EGFR positive in the tamoxifen arm and 12 in the placebo arm (Table 3; p=NS for HER2 and EGFR).

The median level of Ki67 was 10.2% in both the tamoxifen and placebo arms overall and was also little different between the arms in the two time periods (Tables 3 and 4). The mean levels were, however, higher among the tamoxifen treated patients indicating a skewed distribution that was particularly apparent in the post-8 year period. These higher values of Ki67 in the overall time period are apparent in both the ER-positive and ER-negative treated groups (Figure 1).

**Discussion**

Tamoxifen was the first SERM to be shown to be effective at breast cancer risk reduction in healthy women. The benefit-harm ratio is sufficiently favourable for its use to be approved by regulatory agencies in defined high-risk groups in Europe and the USA.  This position has been established as a result of 4 large randomized trials, including the RMPT reported here(13-15). The RMPT results on breast cancer incidence are largely consistent with those in the other trials although the reduced incidence of invasive breast cancer did not emerge until later than in other trials. This may be partly a matter of chance or may be affected by the greater familial risk for the RMPT population than that of the other trials with possible differences in the

phenotypical profile of the familial cancers(5, 6).   There is, therefore, interest in important phenotypic features of the tumours presenting in the RMPT trial on or after their preventive treatment, such as ER, PgR, HER2 and Ki67.

To provide maximum statistical power for the analyses we collected and analysed as many invasive breast cancers that occurred prior to 2010 in the trial as possible. The data therefore provide an update on breast cancer incidence beyond the most recent full publication of the trial(5) and from the most recent overview analysis(4).   As expected the data are no different for the first 8 years of follow-up during which treatment was given. But with the longer follow up the total number of invasive breast cancers increased from 186 to 242, for the most part after 8 years post-randomisation.  The significantly reduced incidence of invasive breast cancer after tamoxifen treatment occurred exclusively in ER-positive disease.

Nearly three-quarters of the 242 breast cancers were collected and had sufficient tissue for analysis.  While the absence of the whole cohort may lead to some bias, the proportions of patients with tumours available for IHC were very similar in each of the two arms such that the conclusions from the cohort are likely to be representative of the whole trial.

Given that it is estimated that many years are required for a breast carcinoma to develop from an initiation event to clinical presentation, reductions in breast cancer incidence in the first few years of prophylactic tamoxifen are likely to be due predominantly to an impact on pre-existing occult disease(16).   However, it seems likely that the majority of invasive breast cancers presenting in the post-8 year period in this trial would be due to

a primary preventive mechanism on either initiation or early promotional events.

Tamoxifen is known to impact on PgR and Ki67 expression in established ER-positive breast cancer(17, 18).   In our studies of neoadjuvant use of tamoxifen the early increase seen in PgR after a few weeks of tamoxifen due to an early agonist effect of tamoxifen fell back to levels that are no different to the overall population after 12 weeks. Ki67 levels on the other hand were initially suppressed and remained suppressed. The observations on phenotype for tumours that present during treatment may therefore be affected by regulatory effects of treatment rather than being representative of the intrinsic tumour phenotype. In contrast in the post 8-year period of follow-up any differences would be expected to be representative of the intrinsic phenotype of cancers that survived initiation or promotion during treatment or were initiated post treatment

The major findings in this study are that the reduction in the incidence of breast cancer continues to be only in ER-positive disease even beyond 8 years and those ER-positive tumours tend to also have lower ER levels than that in the placebo population. These effects are similar to those reported by Kim et al(7) although in that paper the data were from women having received a median of only 4.5 years of randomised therapy(15).   Our findings provide evidence against ER -negative cancers arising from ER positive precursors because by now we should be seeing a reduction in ER negative cancers. In fact there appears to be a consistent increase in ER negative cancers in the tamoxifen trials(4) including in this study. We and others have observed that breast cancer patients with ER-positive primary breast cancers that relapse on

tamoxifen therapy in a minority of cases exhibit ER-negative recurrences(19).
Thus at least some of these ER-negative tumours in the treated women in
RMPT may have presented as ER-positive disease if they had not been
treated with tamoxifen. A substantial switch of ER-positive to ER-negative
status in a subclinical tumour under the influence of tamoxifen could mask any
preventive effect of tamoxifen on ER-negative subclinical tumours in this and
other tamoxifen prevention studies(20).   The lower frequency of PgR-positive
disease in the tamoxifen arm appears to relate largely to the reduced
incidence of ER-positive disease.

Ki67 is a frequently used marker of proliferation and is associated with
poorer prognosis in breast cancer overall and in patients with ER-positive
disease treated with endocrine therapy(21).   The median levels of Ki67 did
not differ between the cancers in the tamoxifen and placebo arms in RMPT
but there was an excess of patients with particularly high levels in the
tamoxifen treated patients.  Given that Ki67 is well known to be more highly
expressed in ER-negative tumours the excess of such tumours contributed to
but did not appear to be completely responsible for the higher Ki67 levels.

The incidence of HER2-positivity and EGFR-positivity both of which are
features of poor prognosis disease was numerically higher in the cancers in
the tamoxifen-arm but the differences did not approach statistical significance.

In summary, in the RMPT trial with prolonged follow-up there was an
overall decrease in breast cancer incidence. The marked decrease in the
incidence of ER-positive disease was partly offset by an increase in ER-
negative disease. Decreased PgR and increased Ki67 levels in the cancers in
the tamoxifen arm were explained by the greater numbers of ER-negative

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cancers.  The minor increases in Ki67, HER2 and EGFR and decreases in ER and PgR proximity are each generally associated with poorer outcome in primary disease presenting in the absence of endocrine therapy, but these differences were relatively minor and the majority of breast cancers that developed during or in the decade after tamoxifen preventive therapy presented as ER-positive/PgR-positive disease.

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**Legend to Figure:**

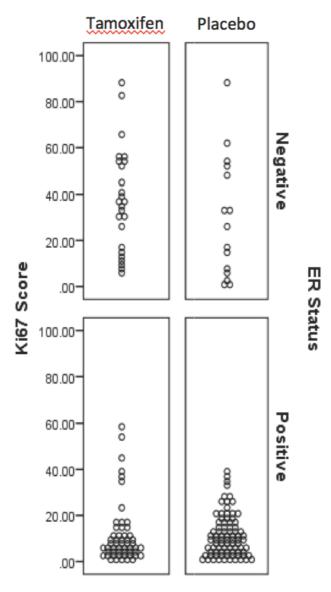**Figure 1:** Expression of Ki67 according to treatment arm and ER status

**Table 1:**

Summary of invasive breast cancer occurrence in tamoxifen and placebo arms in the BCPT by 1st Oct 2010 for all and ER+ patients during and after the treatment period. A: complete trial population; B: the IHC subset.

A

| Invasive cancers | HR (95% CI) | Cox (p-value) |
|---|---|---|
| **All patients** | | |
| Overall follow-up (events: n=242) Placebo (n=134) Tamoxifen (n=108) | 1 0.80 (0.62 – 1.02) | 0.076 |
| During treatment period (events: n=93) Placebo (n=49) Tamoxifen (n=44) | 1 0.89 (0.60 – 1.34) | 0.589 |
| Post treatment period (events: n=149) Placebo (n=85) Tamoxifen (n=64) | 1 0.74 (0.53 – 1.02) | 0.067 |
| **ER Positive** | | |
| Overall follow-up (events: n=187) Placebo (n=108) Tamoxifen (n=79) | 1 0.72 (0.54 – 0.97) | 0.028 |
| During treatment period (events: n=75) Placebo (n=42) Tamoxifen (n=33) | 1 0.78 (0.50 – 1.23) | 0.290 |
| Post treatment period (events: n=112) Placebo (n=66) Tamoxifen (n=46) | 1 0.68 (0.47 – 0.996) | 0.048 |

B

| Patients with IHC data | HR (95% CI) | Cox (p-value) |
|---|---|---|
| Overall follow-up (n=179) Placebo (n=100) Tamoxifen (n=79) | 1 0.78 (0.58 – 1.05) | 0.100 |
| ER positives (n=139) Placebo (n=85) Tamoxifen (n=54) | 1 0.63 (0.45 – 0.88) | 0.007 |
| ER negative (events: n=40) Placebo (n=15) Tamoxifen (n=25) | 1 1.65 (0.87 – 3.12) | 0.127 |

**Table 2**

Major Demographics of the IHC sample set

|  | **Tamoxifen** | **Placebo** |
|---|---|---|
| **Number of patients** | 79 | 100 |
|  |  |  |
| Age: median (range) | 48 yrs (37 – 67) | 49 yrs (30 – 67) |
| HRT (n,%) | 17 (21) | 21 (21) |
| Size: median (range) | 15mm (4 – 50) | 15mm (2 – 60) |
| Grade (n,%): |  |  |
| I | 13 (16) | 16 (16) |
| II | 24 (30) | 35 (35) |
| III | 23 (29) | 30 (30) |
| Others* | 19 (25) | 19 (19) |
| Nodal Status (n,%): |  |  |
| Positive | 22 (28) | 22 (22) |
| Negative/ Unknown | 77 (72) | 78 (78) |
|  |  |  |
| **Follow-up time** |  |  |
| 0-8 years | 40 | 44 |
| > 8 years | 39 | 56 |
|  |  |  |
| **ER +ve cases** |  |  |
| ALL | 54 | 85 |
| 0-8 years | 29 | 37 |
| >8 years | 25 | 48 |
|  |  |  |
| **ER -ve cases** |  |  |
| ALL | 25 | 15 |
| 0-8 years | 11 | 7 |
| >8 years | 14 | 8 |

* Others – unknown, MOD, poor, well, not assessable

**Table 3:**
Comparison of biomarker expression between placebo and tamoxifen groups in overall follow-up period

| Parameter | | Tamoxifen (n=79) | Placebo (n=100) | p-value |
|---|---|---|---|---|
| **ER** | Median H-score (IQR) | 86.4 (0 – 163.9) | 150.8 (73.6 – 184.6) | 0.001 |
| **ER+** | Median H-score (IQR) | 135.8 (84.0 – 182.2) | 164 (110.6 – 191.4 | 0.053 |
| | Status    +ve | 54 (68%) | 85 (85%) | |
| | -ve | 25 (32%) | 15 (15%) | 0.008 |
| | | | | |
| **PgR** | Median H-score (IQR) | 52.9 (0 – 153.7) | 87.2 (1.3 – 168.4) | 0.180 |
| | Median H-score (IQR) | 110.7 (58.8 – 184.5) | 115.9 (66.9 – 180.6) | 0.643 |
| | Status    +ve | 50 (63%) | 76 (76%) | |
| | -ve | 29 (37%) | 24 (24%) | 0.064 |
| | | | | |
| **ER/PgR** | ER + PgR + | 49 (62%) | 76 (76%) | 0.800* |
| **status** | ER + PgR - | 5 (6%) | 9 (9%) | |
| | ER - PgR - | 24 (30%) | 15 (15%) | |
| | ER - PgR + | 1 (1%) | 0 | |
| | | | | |
| **Ki67** | Median % +ve (IQR) | 10.2 (5.1 – 34.5) | 10.2 (4.2 – 18.3) | 0.280 |
| | | | | |
| **HER2** | Status    +ve | 12 (15%) | 10 (10%) | |
| | -ve | 67 (85%) | 90 (90%) | 0.294 |
| | | | | |
| **EGFR** | Status    +ve | 15 (19%) | 12 (12%) | |
| | -ve | 64 (81%) | 88 (88%) | 0.205 |

* P-value relates to the difference between PgR+ and PgR- among the ER+ by chi-square test.

**Table 4:**
Comparison of biomarker expression between placebo and tamoxifen groups in on-treatment and post-treatment follow-up periods

| Parameter | | | Years 0-8, on treatment | | | Years >8, post-treatment | | |
|---|---|---|---|---|---|---|---|---|
| | | | Tamoxifen | Placebo | P-value | Tamoxifen | Placebo | P-value |
| | | N | 40 | 44 | | 39 | 56 | |
| | | | | | | | | |
| ER | All | Median H-score (IQR) | 83.0 (0 – 151.9) | 118.9 (30.7 – 181.2) | 0.043 | 107.5 (0 – 182.3) | 162.1 (103.0 – 195.0) | 0.018 |
| | ER+ | Median H-score (IQR) | 104 (71.2 – 165.0) | 148.5 (81.1 – 182.7) | 0.148 | 162.8 (110.5 – 191.4) | 169.5 (134.2 – 196.3) | 0.390 |
| | All | Status +ve | 29 (73%) | 37 (84%) | | 25 (64%) | 48 (86%) | |
| | | -ve | 11 (27%) | 7 (16%) | 0.196 | 14 (36%) | 8 (14%) | 0.014 |
| | | | | | | | | |
| PgR | All | Median H-score (IQR) | 55.2 (0 – 111.3) | 73.4 (0.8 – 167.6) | 0.582 | 52.9 (0 – 167.7) | 101.3 (3.5 – 168.4) | 0.272 |
| | PgR+ | Median H-score (IQR) | 92.3 (52.3 – 172.8) | 96.5 (67.0 – 180.8) | 0.988 | 146 (90.6 – 187.7) | 121.4 (59.6 – 180.0) | 0.291 |
| | All | Status +ve | 27 (68%) | 32 (73%) | | 23 (59%) | 44 (79%) | |
| | | -ve | 13 (32%) | 12 (27%) | 0.601 | 16 (41%) | 12 (21%) | 0.039 |
| | | | | | | | | |
| ER/PgR | | ER + PgR + | 26 | 32 | | 23 | 44 | |
| | | ER + PgR - | 3 | 5 | | 2 | 4 | |
| | | ER - PgR + | 1 | 0 | | nil | nil | |
| | | ER - PgR - | 10 | 7 | | 14 | 8 | |
| | | | | | | | | |
| Ki67 | All | Median % +ve (IQR) | 9.9 (4.7 – 20.4) | 9.1 (3.3 – 18.3) | 0.660 | 10.9 (5.4 – 47.0) | 10.9 (4.6 – 18.2) | 0.180 |
| | | | | | | | | |
| HER2 | All | Status +ve | 5 (12%) | 6 (14%) | | 7 (18%) | 4 (7%) | |
| | | -ve | 35 (88%) | 38 (86%) | 0.877 | 32 (82%) | 52 (93%) | 0.105 |
| | | | | | | | | |
| EGFR | All | Status +ve | 6 (15%) | 6 (14%) | | 9 (23%) | 6 (11%) | |
| | | -ve | 34 (85%) | 37 (86%) | 0.892 | 30 (77%) | 50 (89%) | 0.104 |

**Supplementary Table 1** Immunohistochemical analysis of biomarkers

| Biomarker | Antibody | Antigen Retrieval and Scoring Methods |
|---|---|---|
| ER | Clone 6F11 Vector VP E-614 dilution 1/40 | 10 minutes microwaving Full power in pre-heated citrate buffer pH 6 <br><br> H Score: % staining positive & intensity. Cut-off $\geq 1$ |
| PgR | 321 clone Vector Labs VP-P976 dilution 1/100 | 10 minutes microwaving full power in pre-heated citrate buffer pH 6 <br> H Score: % staining positive & intensity. Cut-off $\geq 1$ |
| HER2 | HercepTest+ K5207 Dako | 40 minutes 10 mM Epitope retrieval buffer in water bath at 95-99$^0$C <br> 0, 1+,2+,3+ positive membrane staining <br> Cut-off 3+ is positive <br> 2+ scores are subjected to FISH analyses |
| FISH | Fluorescence *in situ* hybridization of HER2 using PathVysion Abbott HER-2 neu Kit when IHC 2+ cases | According to Kit instructions. <br> HER2 red coloured and C17 centromere green ratio (1.8-2.2 borderline) |
| EGFR | Clone31G7Invitrogen clone 31G7 Zymed dilution 1/50 | 15 minutes in pre-warmed 5% pronase E (Sigma P6911) buffer <br> % positive membrane staining. Cut-off >0 is +ve |
| Ki67 | MIB-1 clone Dako (M7240) Dilution 1/40 | 10 minutes microwaving full power in pre-heated citrate buffer pH 6 <br> % positive nuclei score. No cutoff |