

## Prognostic Significance of Ki-67 in Chemotherapy-naïve Breast Cancer Patients with 10-year Follow-up

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**Abstract.** *Background/Aim:* In order to define accurate survival outcome in breast cancer, 10-year follow-up is required and such long-term survival information are few and difficult to gather. *Patients and Methods:* We recruited 253 breast cancer patients who undertook operation with no prior chemotherapy. Ten-year survival outcomes were evaluated by clinicopathological factors. *Results:* Significant univariate prognostic factors were: T factor, N factor, preoperative values of tumor markers, and biological factors. T-factor, CEA, hormone receptor, and Ki-67 were the final independent prognostic factors of recurrence-free survival through multivariate analysis. The Luminal A group except for the Ki-67-positive cases showed the best survival outcomes, while the HER2-positive or triple-negative (TN) groups showed worse prognosis than the Luminal A group, and Ki-67 was shown to be an excellent prognostic factor in each stage ( $p < 0.01$ ). *Conclusion:* Ki-67 has a great potential as a prognostic biomarker while prognostic information of this sort could be beneficial for development of novel therapeutic strategies.

In recent years, breast cancer abruptly increases in Japan and age adjustment morbidity caught-up with that of colorectal cancer, winning the first place for cancer-related mortality in females (1). It had been thought that survival outcome in breast cancer is associated with pathological stage which, during the 1990's, used to be determined based on tumor diameter and lymph node metastasis (2). The treatment plan for breast cancer was, therefore, determined based on disease tumor staging back in those days. On the other hand, we recently got accustomed to deciding therapeutic indication according to classical molecular biological factors such as either hormone receptor

(estrogen receptor, ER, and progesterone receptor, PgR) or HER2 protein status, because effective molecular-targeting against such molecules has since progressed (3). Most recently, the St. Gallen Consensus Meeting 2011 adapted novel tumor prognostic classifications according to Ki-67 status, where Ki-67 can dissociate high risk patients from hormone receptor-positive cases as a Luminal B group (4).

The Ki-67 antibody was discovered as an autoantibody in the blood of leukemia patients (5, 6). The antigen that this antibody recognizes is a Ki-67 antigen. It is the nucleoprotein with molecular weights of 395 and 345 kDa which increases in expression when a cell enters mitosis. Ki-67 is therefore regarded as an index to show proliferation ability (7, 8). Interestingly, Ki-67 antigen-specific antisense inhibited proliferation of human multiple myeloma cell lines suggesting that it has a crucial function in terms of cell proliferation (9, 10). There have been numerous reports on Ki-67 describing prognostic significance in breast cancer (11-13), however there are few to validate breast cancer patients with natural history (chemotherapy-naïve) who had been followed-up for 10 years.

In order to define accurate survival outcome of breast cancer, 10 year follow-up is required differently from other cancer types, while such long-term survival information are extremely few. We, herein, studied biological factors as well as clinicopathological characteristics in chemotherapy-naïve breast cancer patients regarding prognosis, and elucidated factors highly associated with cancer recurrence and death in order to define biological traits of breast cancer not modified by preoperative treatments. From the obtained results, we recapitulated robust relevance of pathological staining for Ki-67 in tumor tissues even among aggressive subtypes of breast cancer such as HER2-positive or triple-negative (TN) cases.

### Patients and Methods

**Patients.** A total of 253 primary breast cancer patients were registered for clinicopathological and prognostic analysis, who undertook operations with no prior chemotherapy at the Kitasato

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**Key Words:** Breast cancer, clinicopathological parameters, multivariate analysis, chemotherapy-naïve patients.

Table I. Characteristics of the 253 primary breast cancer patients.

| Variables                         | Number    | %     | Variables                              | Number | %    |
|-----------------------------------|-----------|-------|--|--------|------|
| Patient                           | 253       | 100.0 | Stage-unrelated pathological factor    |        |      |
| Preoperative factor               |           |       | Histology                              |        |      |
| Age (mean±S.D.)                   | 51.7±11.0 |       | Invasive ductal carcinoma              | 223    | 88.1 |
| Gender                            |           |       | Invasive lobular carcinoma             | 9      | 3.6  |
| Male                              | 1         | 0.4   | Others                                 | 11     | 4.4  |
| Female                            | 252       | 99.6  | Unknown                                | 10     | 3.9  |
| Preoperative CEA value            |           |       | Estrogen receptor (IHC)                |        |      |
| ≥2.5 ng/ml                        | 19        | 7.5   | Positive                               | 176    | 69.6 |
| <2.5 ng/ml                        | 228       | 90.1  | Negative                               | 77     | 30.4 |
| Unknown                           | 6         | 2.4   | Progesteron receptor (IHC)             |        |      |
| Preoperative TPA value            |           |       | Positive                               | 164    | 64.8 |
| ≥70 IU/ml                         | 24        | 9.5   | Negative                               | 89     | 35.2 |
| <70 IU/ml                         | 217       | 85.8  | HER2 (IHC)                             |        |      |
| Unknown                           | 12        | 4.7   | Positive                               | 50     | 19.8 |
| Preoperative CA15-3 value         |           |       | Negative                               | 203    | 80.2 |
| ≥28 IU/ml                         | 5         | 2.0   | Ki-67 (IHC)                            |        |      |
| <28 IU/ml                         | 242       | 95.6  | Positive                               | 36     | 14.2 |
| Unknown                           | 6         | 2.4   | Negative                               | 217    | 85.8 |
| Stage related pathological factor |           |       | Treatment factor                       |        |      |
| pT factor                         |           |       | Method                                 |        |      |
| pT1                               | 134       | 53.0  | Mastectomy                             | 172    | 68.0 |
| pT2                               | 99        | 39.1  | Lumpectomy                             | 81     | 32.0 |
| pT3                               | 15        | 5.9   | Postoperative adjuvant hormone therapy |        |      |
| pT4                               | 5         | 2.0   | Yes                                    | 95     | 37.5 |
| pN factor                         |           |       | No                                     | 158    | 62.5 |
| pN0                               | 136       | 53.7  | Postoperative adjuvant chemotherapy    |        |      |
| pN1                               | 65        | 25.7  | Yes                                    | 141    | 55.7 |
| pN2                               | 31        | 12.3  | No                                     | 112    | 44.3 |
| pN3                               | 21        | 8.3   | Prognosis                              |        |      |
| pM factor                         |           |       | Recurrence                             |        |      |
| pM0                               | 253       | 100.0 | Yes                                    | 79     | 31.2 |
| pM1                               | 0         | 0.0   | No                                     | 174    | 68.8 |
| pStage                            |           |       | Death                                  |        |      |
| I                                 | 84        | 33.2  | Yes                                    | 62     | 24.5 |
| II                                | 112       | 44.3  | No                                     | 191    | 75.5 |
| III                               | 57        | 22.5  |  |        |      |

S.D., Standard deviation; IHC, immunohistochemistry.

University Hospital between April, 1995 and December, 1999. We excluded cases with bilateral breast tumors and multiple primary cancers of other organs.

Patients' characteristics are shown in Table I. Two hundred and fifty three patients had information available for the 16 clinicopathological factors excluding pathological Stage (pStage) and prognosis (Table I). The 16 clinicopathological factors included 5 preoperative factors (age, sex, and preoperative serum values of CEA, CA15-3, and TPA), 3 TNM stage-determining pathological factors (pathological T, N and M factors instead of pStage itself), 5 pathological factors unrelated to TNM factors (histology, and immunohistochemistry of estrogen receptor (ER), progesterone receptor (PgR), HER2, and Ki-67), 3 treatment factors (operative methods, postoperative adjuvant chemotherapy, and postoperative adjuvant hormone therapy). Positive cut-off lines were defined in the SRL laboratories (Tokyo, Japan), as equal or beyond than 2.5

ng/ml in preoperative CEA, equal or beyond than 28 U/ml in preoperative CA 15-3, and equal or beyond than 70 U/ml in preoperative TPA. The stage-determining factors, including pStage itself, were based on the 7th Union for International Cancer Control (UICC). Prognostic analysis was performed for 10-year recurrence-free survival (RFS) and overall survival (OS).

Briefly, the average age of the patients was 51.7 (range: 22-84) years. The 253 patients were classified 84/112/57 in stage I/II/III, respectively. Sixty-two patients died, with 57 tumor-related deaths, and 191 patients survived at 10 years (5 patients died due to disease unrelated to breast cancer). A total of 141/253 patients (55.7%) who underwent surgical resection received postoperative adjuvant chemotherapy. The adjuvant regimens consisted largely of 5-fluorouracil (5-FU)-based chemotherapy as venous infusion [5-FU-only (n=6), 5-FU/methotrexate/cyclophosphamide:CMF (n=66), 5-FU/ adriamycin/cyclophosphamide: CAF (n=16), 5-FU/ therarubicin

Table II. Univariate prognostic analysis of 253 patients with breast cancer.

| Categories                             | Classification            | Number | Number proportion | 10-year RFS | <i>p</i> -Value | 10-year OS | <i>p</i> -Value |
|--|---------------------------|--------|-------------------|-------------|-----------------|------------|-----------------|
| Age                                    | ≥50 years                 | 125    | 49%               | 74.66%      | 0.002           | 84.21%     | 0.026           |
|  | <50 years                 | 128    | 51%               | 57.40%      |                 | 71.11%     |                 |
| Preoperative CEA value                 | ≥2.5 ng/ml                | 19     | 8%                | 45.22%      | <0.001          | 38.45%     | <0.001          |
|  | <2.5 ng/ml                | 228    | 92%               | 68.05%      |                 | 81.26%     |                 |
| Preoperative TPA value                 | ≥70 IU/ml                 | 24     | 10%               | 48.02%      | 0.005           | 65.22%     | 0.012           |
|  | <70 IU/ml                 | 217    | 90%               | 68.14%      |                 | 80.24%     |                 |
| Preoperative CA15-3 value              | ≥28 IU/ml                 | 5      | 2%                | 40.00%      | NS              | 30.00%     | 0.028           |
|  | <28 IU/ml                 | 242    | 98%               | 66.89%      |                 | 78.88%     |                 |
| pT factor                              | pT1                       | 134    | 53%               | 75.09%      | <0.001          | 89.16%     | <0.001          |
|  | pT2-4                     | 119    | 47%               | 56.11%      |                 | 65.55%     |                 |
| pN factor                              | pN0                       | 136    | 54%               | 78.77%      | <0.001          | 87.17%     | <0.001          |
|  | pN+                       | 117    | 46%               | 52.31%      |                 | 67.69%     |                 |
| Histology                              | Invasive ductal carcinoma | 223    | 92%               | 65.50%      | NS              | 77.37%     | NS              |
|  | Others                    | 20     | 8%                | 72.10%      |                 | 83.31%     |                 |
| Hormone receptor (IHC)                 | Positive                  | 180    | 71%               | 68.42%      | 0.003           | 83.05%     | <0.001          |
|  | Negative                  | 73     | 29%               | 60.20%      |                 | 64.88%     |                 |
| HER2 (IHC)                             | Positive                  | 50     | 20%               | 61.17%      | NS              | 63.16%     | 0.004           |
|  | Negative                  | 203    | 80%               | 67.40%      |                 | 81.26%     |                 |
| Ki-67 (IHC)                            | Positive                  | 36     | 14%               | 34.78%      | <0.001          | 39.01%     | <0.001          |
|  | Negative                  | 217    | 86%               | 71.19%      |                 | 84.50%     |                 |
| Method                                 | Total                     | 172    | 68%               | 61.27%      | 0.007           | 73.22%     | 0.007           |
|  | Partial                   | 81     | 32%               | 76.51%      |                 | 87.95%     |                 |
| Postoperative adjuvant hormone therapy | Yes                       | 95     | 38%               | 79.00%      | 0.002           | 89.31%     | 0.002           |
|  | No                        | 158    | 62%               | 58.25%      |                 | 71.06%     |                 |
| Postoperative adjuvant chemotherapy    | Yes                       | 141    | 56%               | 56.34%      | <0.001          | 68.07%     | <0.001          |
|  | No                        | 112    | 44%               | 79.23%      |                 | 91.47%     |                 |

RFS, Recurrence-free survival; OS, overall survival; NS, not significant; IHC, immunohistochemistry.

(n=1)], tegafur/uracil (UFT)-based chemotherapy (n=25) as oral therapy and doxifluridine based chemotherapy (n=27) as oral therapy. A total of 95/253 patients (37.5%) who underwent surgical resection received postoperative adjuvant hormone therapy. The adjuvant hormone therapy regimens consisted largely of tamoxifen citrate (n=60) as oral therapy, toremifene citrate (n=22) as oral therapy, fadrozole (n=5) as oral therapy and goserelin acetate (n=8) as hypodermic injection.

**Immunohistochemistry.** Immunohistochemistry was performed for hormone receptors such as ER (ER antibody; 1D5 DAKO, DENMARK, dilution 1:100) and PgR (PgR antibody; PgR636 DAKO, dilution 1:100), human epidermal growth factor receptor-2 (HER2; Hercep Test DAKO, dilution 1:200), and Ki-67 (Ki-67 antibody; MIB-1 DAKO, dilution 1:200): ER and PgR were judged for positive cases which stained nuclei more than 1% according to the previous report (14).

Ki-67 was evaluated in positive cases which stained more than 20% according to a previous report (15). HER2-positive cases were evaluated according to Hercep Test definition (16).

**Statistical analysis.** The Kaplan-Meier method was used to evaluate 10-year recurrence-free survival (RFS) and overall survival (OS), and differences in survival rates were assessed by the log-rank test. RFS was measured from the date of operation to the date of cancer recurrence. OS was defined from the date of operation to the date of

death, including death unrelated to cancer after 10 years of follow-up (7-120, median 87.4). Variables that had prognostic potential in the univariate analysis ( $p<0.05$ ) were applied to the multivariate proportional hazard model. A value of  $p<0.05$  was considered significant. All statistical analyses were carried out with the SAS software package JMP version 9.0 (SAS Institute, Cary, NC, USA).

## Results

### *Univariate analysis for 10-year RFS and 10-year OS in 253 chemo-naïve breast cancers patients who underwent operation.*

All significant prognostic factors ( $p<0.05$ ) selected in a univariate manner are presented in Table II. The prognostic factors for 10-year RFS with statistically significant difference were age ( $p=0.0021$ ), preoperative CEA value ( $p=0.0002$ ), preoperative TPA value ( $p=0.0053$ ), tumor diameter (T factor,  $p=0.0003$ ), lymph node metastasis (N factor,  $p<0.0001$ ), hormone receptor status ( $p=0.0029$ ), and Ki-67 status ( $p<0.0001$ ). Regarding treatment factors, postoperative adjuvant hormone therapy ( $p=0.0018$ ) and postoperative adjuvant chemotherapy ( $p<0.0001$ ) were also significant.

On the other hand, poor prognostic factors for 10-year OS with statistically significant difference, following univariate

Table III. Multivariate prognostic analysis for 10-year RFS in breast cancer.

| Prognostic factor                                     | HR   | 95% CI    | p-Value |
|---|------|-----------|---------|
| Ki-67 (IHC)   | 1.80 | 1.36-2.34 | <0.001  |
| Preoperative value of CEA 2.5 ng/ml or over           | 1.65 | 1.11-2.31 | 0.015   |
| Below 50 years of age against 50 years of age or over | 1.43 | 1.12-1.83 | 0.004   |
| Preoperative value of TPA 70 IU/ml or over            | 1.31 | 0.93-1.78 | NS      |
| Adjuvant hormone therapy                              | 1.26 | 0.95-1.71 | NS      |
| pN+ against pN0                                       | 1.20 | 0.85-1.73 | NS      |
| Hormone receptor (IHC)                                | 1.19 | 0.92-1.53 | NS      |
| pT2/3/4 against pT1                                   | 1.14 | 0.89-1.48 | NS      |
| Postoperative adjuvant chemotherapy                   | 0.95 | 0.65-1.41 | NS      |
| Method  | 0.92 | 0.67-1.25 | NS      |

HR; Hazard Ratio. CI; Confidence interval. IHC; Immunohistochemistry. NS; not significant.

Table IV. Multivariate prognostic analysis for 10-year OS in breast cancer.

| Prognostic factor                                     | HR   | 95% CI    | p-Value |
|---|------|-----------|---------|
| Ki-67 (IHC)   | 2.31 | 1.67-3.19 | <0.001  |
| Preoperative value of CEA 2.5 ng/ml or over           | 2.02 | 1.32-2.96 | 0.002   |
| Hormone receptor (IHC)                                | 1.67 | 1.19-2.33 | 0.003   |
| Below 50 years of age against 50 years of age or over | 1.49 | 1.08-2.08 | 0.015   |
| pT2/3/4 against pT1                                   | 1.49 | 1.06-2.17 | 0.022   |
| Postoperative adjuvant hormone therapy                | 1.21 | 0.81-1.88 | NS      |
| HER2 (IHC)  | 1.19 | 0.84-1.65 | NS      |
| Preoperative value of CA15-3 28 IU/ml or over         | 1.17 | 0.55-2.13 | NS      |
| Preoperative value of TPA 70 IU/ml or over            | 1.15 | 0.73-1.71 | NS      |
| Method  | 0.97 | 0.61-1.46 | NS      |
| pN+ against pN0                                       | 0.76 | 0.49-1.23 | NS      |
| Postoperative adjuvant chemotherapy                   | 0.58 | 0.34-0.99 | 0.045   |

HR; Hazard Ratio. CI; Confidence interval. IHC; Immunohistochemistry. NS; Not significant.

analysis, were age ( $p=0.026$ ), preoperative CEA value ( $p<0.0001$ ), preoperative TPA value ( $p=0.012$ ), preoperative CA15-3 ( $p=0.028$ ), T factor ( $p<0.0001$ ), N factor ( $p=0.0003$ ), hormone receptor status ( $p<0.0001$ ), HER2 status ( $p=0.004$ ), and Ki-67 status ( $p<0.0001$ ). Postoperative adjuvant hormone therapy ( $p=0.0021$ ) and postoperative adjuvant chemotherapy ( $p<0.0001$ ) were also significant prognostic factors.

*Multivariate proportional hazard model for 10-year RFS and 10-year OS in chemotherapy-naïve breast cancer patients who underwent operation.* Among the 253 patients, the multivariate proportional hazard model was applied to 237 patients and to 235 patients for whom all information were available for the 10 and 12 clinicopathological factors, respectively for 10-year RFS and 10-year OS. In the multivariate prognostic analysis for 10-year RFS, Ki-67 [Hazard ratio (HR)=1.80;  $p=0.0001$ ], preoperative CEA value (HR=1.65;  $p=0.015$ ), and age (HR=1.43;  $p=0.038$ ) were independent prognostic factors (Table III).

On the other hand, in the multivariate prognostic analysis for 10-year OS, Ki-67 (HR=2.31;  $p=0.0001$ ), preoperative CEA value (HR=2.02;  $p=0.0019$ ), HR status (HR=1.67;  $p=0.0030$ ), age (HR=1.49;  $p=0.015$ ), and T factor (HR=1.49;  $p=0.022$ ) were independent prognostic factors (Table IV).

Kaplan-Meier curve of the independent prognostic factors (T-factor, age, Ki-67, and preoperative CEA value) are shown for 10-year RFS and 10-year OS in Figure 1. Among the robust prognostic factors, Ki-67 was the strongest one and could potentially predict for breast cancer patient prognosis, so we focused on Ki-67 for further analysis in the present study.

*Association of Ki-67 status and other clinicopathological factors.* We investigated on the prognostic relevance of Ki-67 according to pStage or molecular classification. Firstly, we compared Ki-67 staining frequency according to various prognostic factors. Ki-67 positivity was more frequently

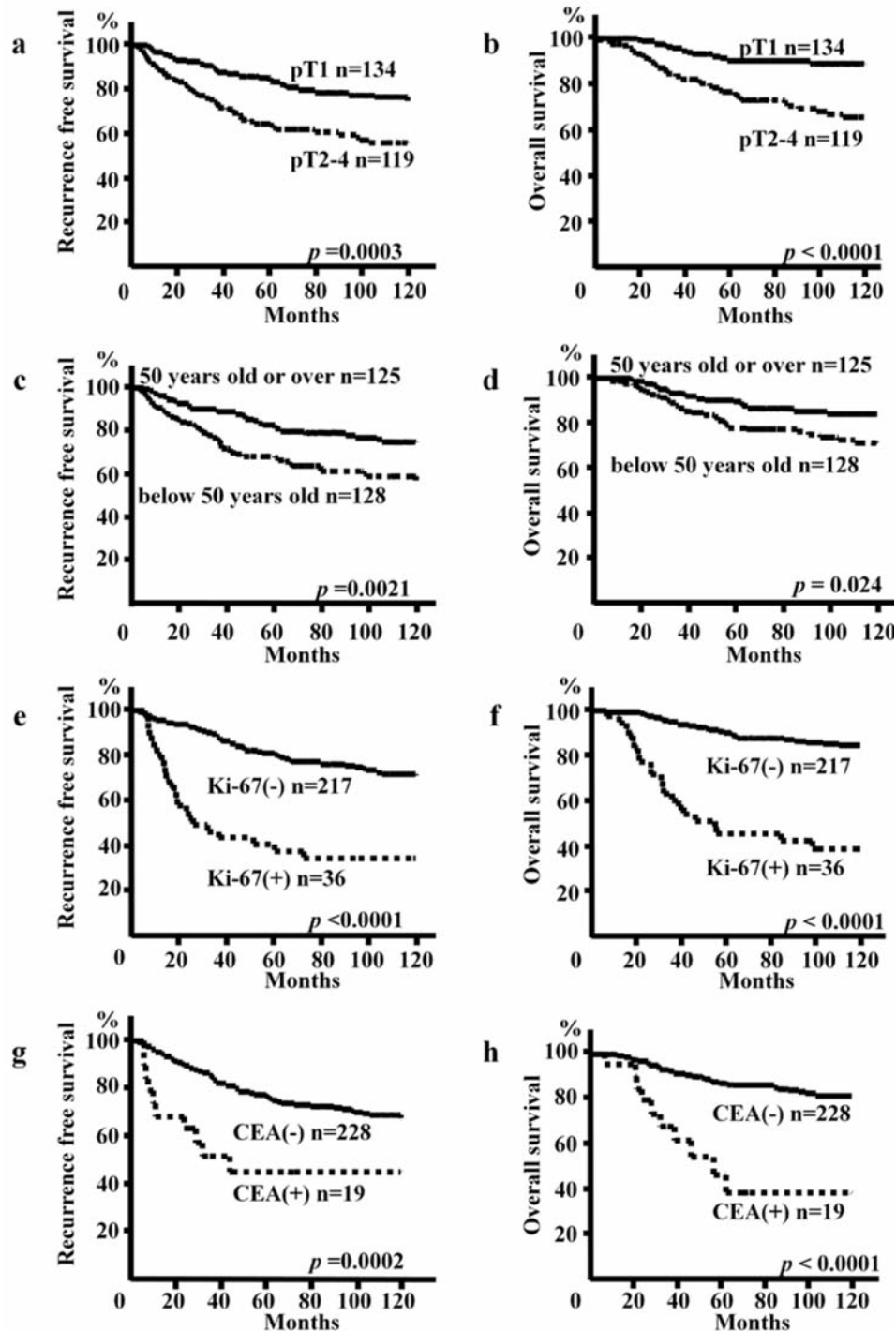


Figure 1. Kaplan-Meier curves of 10-year recurrence-free survival (RFS) and 10-year overall survival (OS) by independent prognostic factors from multivariate analysis. a. Breast cancer patients with high pT factor showed significantly poorer recurrence free survival than those with low pT factor ( $p=0.0003$ ). b. Breast cancer patients with high pT factor showed significantly poorer overall survival than patients with low pT factor ( $p<0.0001$ ). c. Breast cancer patients with age below 50 years showed significantly poorer recurrence free survival than those with age equal or over 50 years ( $p=0.0021$ ). d. Breast cancer patients with age below 50 years showed significantly poorer overall survival than those with age equal to or over 50 years ( $p=0.024$ ). e. Breast cancer patients with positive staining of Ki-67 showed significantly poorer recurrence-free survival than those with negative staining ( $p<0.0001$ ). f. Breast cancer patients with positive staining of Ki-67 showed significantly poorer overall survival than those with negative staining ( $p<0.0001$ ). g. Breast cancer patients with high preoperative CEA value showed significantly poorer recurrence-free survival than those with low value ( $p=0.0002$ ). h. Breast cancer patients with high preoperative CEA value showed significantly poorer overall survival than those with low value ( $p<0.0001$ ).

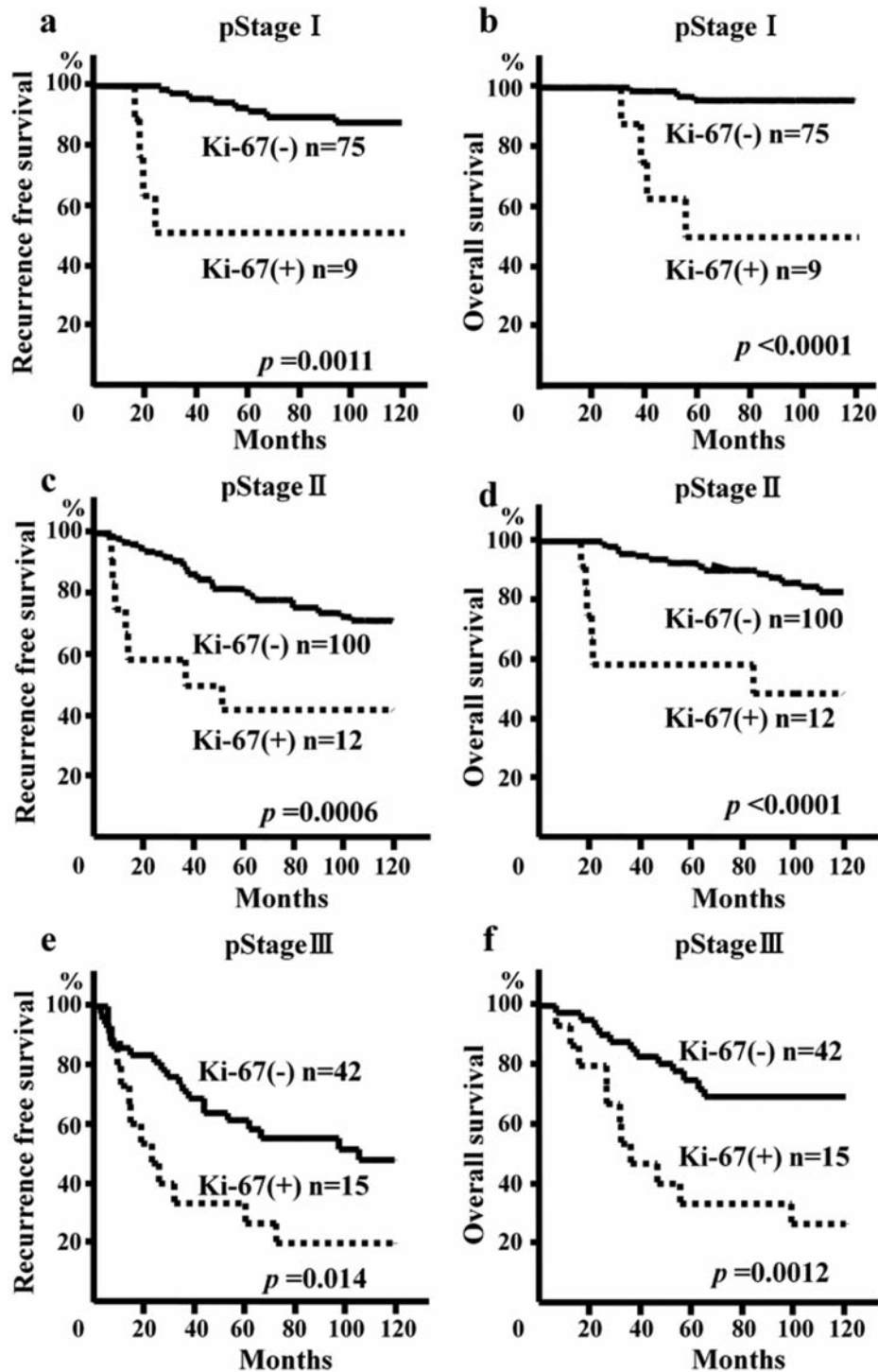


Figure 2. Kaplan-Meier curves of 10-year RFS and 10-year OS according to pathological Stage (pStage) and Ki-67. a. In pStage I breast cancer, patients with positive staining of Ki-67 showed significantly poorer prognosis for 10-year RFS than those with its negative staining for Ki-67 ( $p=0.0011$ ). b. In pStage I breast cancer, patients with positive staining for Ki-67 showed significantly poorer prognosis for 10-year OS than those with negative staining ( $p<0.0001$ ). c. In pStage II breast cancer, patients with positive Ki-67 staining showed significantly poorer prognosis for 10-year RFS than those with negative staining ( $p=0.0006$ ). d. In pStage II breast cancer, patients with positive Ki-67 staining showed significantly poorer prognosis for 10-year OS than those with negative staining of Ki-67 ( $p<0.0001$ ). e. In pStage III breast cancer, patients with positive Ki-67 staining of showed significantly poorer prognosis for 10-year RFS than those with negative staining ( $p=0.014$ ). f. In pStage III breast cancer, patients with positive staining of Ki-67 showed significantly poorer prognosis for 10-year OS than those with negative staining ( $p=0.0012$ ).

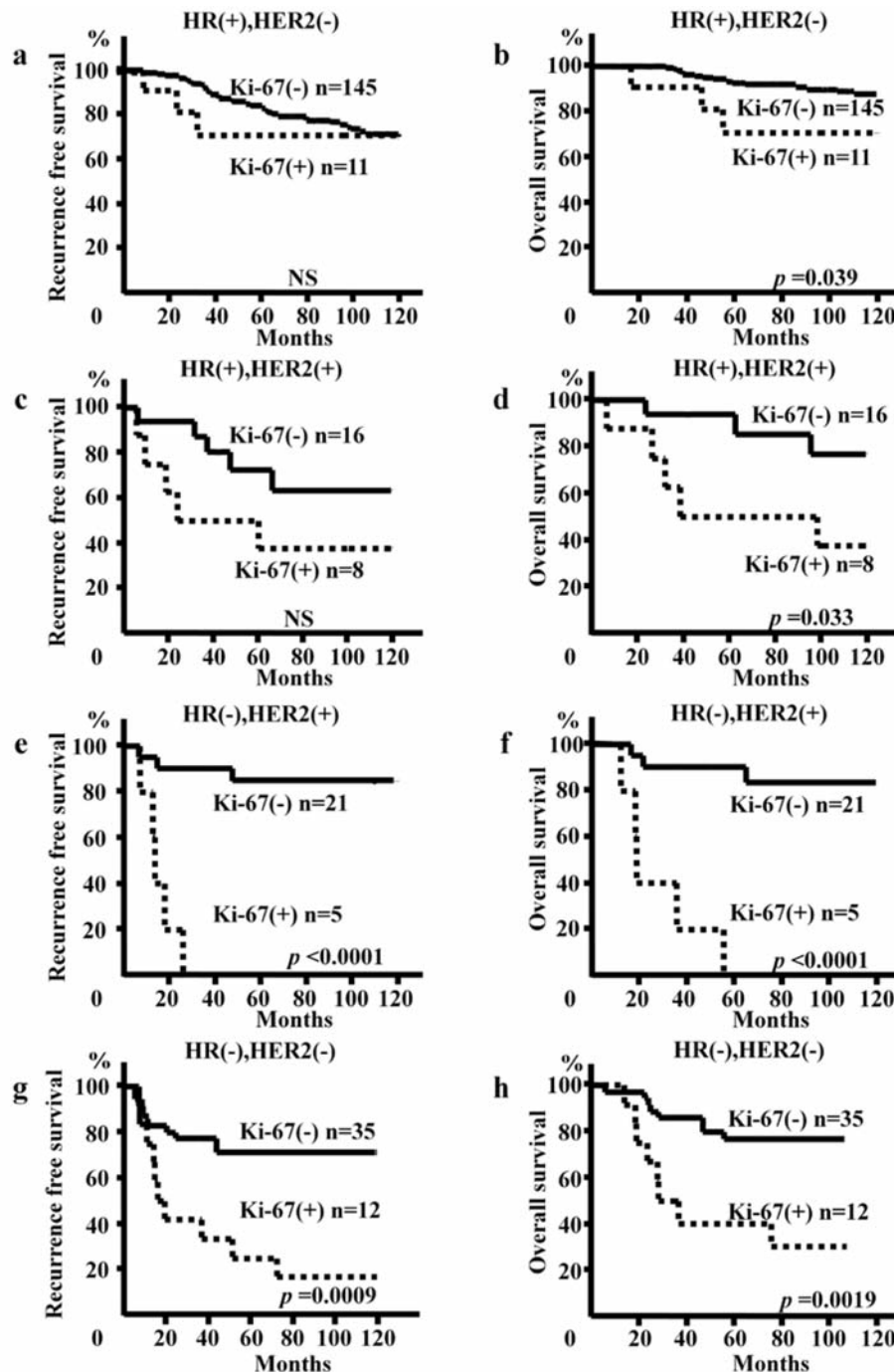


Figure 3. Kaplan-Meier curve of 10-year RFS and 10-year OS according to breast cancer molecular subtype and Ki-67. a. In hormone receptor-positive, HER2-negative breast cancer, patients with positive Ki-67 staining did not show poorer prognosis for 10-year RFS than those with its negative staining ( $p=0.46$ ). b. In hormone receptor-positive, HER2-negative breast cancer, patients with positive staining for Ki-67 showed significantly poorer prognosis for 10-year OS than those with negative staining ( $p=0.039$ ). c. In hormone receptor-positive, HER2-positive breast cancer, patients with positive staining for Ki-67 did not show poorer prognosis for 10-year RFS than those with negative staining ( $p=0.079$ ). d. In hormone receptor-positive, HER2-positive breast cancer, patients with positive staining of Ki-67 showed significantly poorer prognosis for 10-year OS than those with its negative staining ( $p=0.033$ ). e. In hormone receptor-negative, HER2-positive breast cancer, patients with positive staining of Ki-67 showed significantly poorer prognosis for 10-year RFS than those with its negative staining ( $p<0.0001$ ). f. In hormone receptor-negative, HER2-positive breast cancer, patients with positive staining of Ki-67 showed significantly poorer prognosis for 10-year OS than those with its negative staining ( $p<0.0001$ ). g. In triple-negative (TN) breast cancer, patients with positive staining of Ki-67 showed significantly poorer prognosis than those with its negative staining ( $p=0.0009$ ). h. In TN breast cancer, patients with positive staining of Ki-67 showed significantly poorer prognosis for 10-year OS than those with negative staining ( $p=0.0019$ ).

found breast cancer patients with high preoperative CEA values ( $p=0.019$ ) or TPA ( $p=0.032$ ), lymph node metastasis ( $p<0.0001$ ), ER-negative ( $p=0.018$ ), PgR-negative ( $p<0.0001$ ), HER2-positive ( $p=0.008$ ), and triple-negative TN ( $p=0.014$ ), suggesting that Ki-67 is involved in aggressive breast cancer phenotypes.

On the other hand, patients with positive Ki-67 showed significantly poorer prognosis than those with negative Ki-67 in pStage I (Figure 2a and 2b), pStage II (Figure 2c and 2d), and pStage III (Figure 2e and 2f). This may show that Ki-67 was the most robust independent prognostic factor in multivariate prognostic analysis.

We then investigated the prognostic stratification by Ki-67 for the 4 definite breast cancer phenotypes that are designated as Luminal A (hormone receptor-positive, HER2-negative-, Luminal B (hormone receptor-positive, HER2-positive-, HER2 (hormone receptor-negative, HER2-positive-, and TN (hormone receptor-negative, HER2-negative (-type)). The Luminal A group showed the best survival outcomes among chemo-naïve breast cancer patients (Figure 3a). Interestingly, the Luminal A group did not show significant differences according to Ki-67 status for 10-year RFS, while Ki-67 clarified prognostic stratification with significant difference for 10-year OS in the Luminal A group (Figure 3b). This similar tendency was also found in the Luminal B group (hormone receptor-positive, and HER2-positive). Among Luminal-B group patients, Ki-67 showed marginal significance for 10-year RFS (Figure 3c), while it showed significant difference for 10-year OS (Figure 3d). These findings may suggest that Ki-67 is the indicator reflecting resistant phenotypes against post-recurrence phenotype in Luminal A/B breast cancer.

Most intriguingly, Ki-67 showed potent prognostic relevance even in aggressive subgroups such as the HER2-positive and the TN group. HER2-positive or TN groups showed worse prognosis than the Luminal A, and Ki-67 showed potent prognostic relevance ( $p<0.01$ ) in HER2-positive (Figure 3e and, f) and TN group (Figure 3g and h) for both 10-year RFS and OS.

The multivariate sub-analysis again revealed that Ki-67 could be an independent prognostic factor in aggressive breast cancer. In the multivariate prognostic analysis for 10-year RFS in the TN group ( $n=47$ ), Ki-67 ( $HR=2.30$ ;  $p<0.0001$ ) and preoperative CEA value ( $HR=2.91$ ;  $p=0.004$ ) were independent prognostic factors among the significant univariate prognostic factors. In terms of 10-year OS in TN group, Ki-67 ( $HR=2.39$ ;  $p=0.0002$ ) and preoperative CEA value ( $HR=2.95$ ;  $p=0.004$ ) were independent prognostic factors among the significant univariate prognostic factors. On the other hand, in the multivariate prognostic analysis for 10-year RFS in the HER2-positive group ( $n=50$ ), Ki-67 ( $HR=2.58$ ;  $p=0.0001$ ) was only remnant among the significant univariate prognostic factors such as preoperative CEA value, preoperative TPA value, and Ki67. In terms of

10-year OS in the HER2-positive group, Ki-67 ( $HR=2.43$ ;  $p=0.0002$ ) was also an independent prognostic factor among the significant prognostic factors such as preoperative TPA value, pN factor, and Ki67.

## Discussion

Both adjuvant and neo-adjuvant chemotherapy, as well as hormonal treatment, have contributed majorly in improving recurrence-free survival (RFS) and overall survival (OS) in breast cancer (17-20). However, they may include the possibility of high toxicity and, therefore, a potential limit to the patient for therapeutic continuation. Physicians should, thus, consider both advantage and disadvantage to the patients. In order to guide therapeutic decisions, physicians use clinical and histopathological variables as well as biomarkers as prognostic or predictive tools, these latter being the most effective, if linked with well-evidenced targeted therapies, such as ER and HER2 (21-23). In our current study, we recapitulated that Ki-67 has a great potential as a prognostic biomarker in aggressive breast cancer as well as in the Luminal groups among patients with no prior chemotherapy. Current breast cancer treatment diversifies, and preoperative therapy is indicative for many cases. So far, there have been few reports describing a prognostic value of Ki-67 for chemo-naïve patients, including 10-year follow-up information as in our current study.

Ki-67 was discovered in the beginning of 1980's. And it has been used as growth-related factor of a malignant tumor represented by malignant lymphoma, and has been repeatedly reported as an aggressive prognostic marker in breast cancer (15, 24-26). Importantly, the St. Gallen consensus meeting held in 2011, additionally clarified the new Luminal B entity to be separated from Luminal A by Ki-67 (4). Moreover, the TN breast cancer showed aggressive clinical course, and recent reports assumed that Ki-67 is a prognostic factor even among the TN group (27, 28). These results are quite consistent with our current results. In the present study, it was shown for the first time that Ki-67 can be a potent prognostic factor in HER2-positive breast cancer, which has also an aggressive clinical potential in chemo-naïve conditions. Although our analysis included a small number of cases as a sub-analysis, Ki-67 was shown to be an independent prognostic factor by multivariate analysis in the aggressive breast cancer such as TN and HER2-positive cases. Our results emphasize on the clinical importance of Ki-67 as a prognostic indicator in breast cancer with no prior chemotherapy. Seeing prognosis of Luminal A/B type stratified by Ki-67, more robust significance was found in OS rather than RFS, suggesting that Ki-67 indicates likelihood to show resistant phenotype for hormone therapy. Resistance of Ki-67-positive breast cancer against hormone therapy (29, 30) or chemotherapy (31, 32) was also actually supported by our clinical experience.

We used a 20% cut-off value in this current study, where positive/negative judgment was relatively obvious. Namely, the positive cases of Ki-67 staining largely showed strong positivity in much more than 20%, while we experienced few cases with judgment of ~20% positive. We thus feel that Ki-67 is a relatively objective and excellent indicator to predict prognosis in breast cancer.

In conclusion, Ki-67 has a great potential as a prognostic biomarker in aggressive breast cancer as well as in the Luminal groups, and such prognostic information could be beneficial for the development of therapeutic strategy. Moreover, cell surface molecules like tyrosine kinases that could be therapeutically targeted and which are associated in expression with and actually involved in indication of Ki-67 may have a great potential for development of the new active drug for the breast cancer harboring augmented expression of Ki-67 and exhibiting aggressive traits in the near future.

## Acknowledgements

The Authors thank Ms. Inoue for her technical guidance in this manuscript.

## References

- 1 Ferlay J, Shin HR, Bray F, Forman D, Mathers C and Parkin DM: Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 127: 2893-2917, 2008.
- 2 Bear HD, Anderson S, Smith RE, Geyer CE Jr., Mamounas EP, Fisher B, Brown AM, Robidoux A, Margolese R, Kahlenberg MS, Paik S, Soran A, Wickerham DL and Wolmark N: Sequential preoperative or postoperative docetaxel added to preoperative doxorubicin plus cyclophosphamide for operable breast cancer: National Surgical Adjuvant Breast and Bowel Project Protocol B-27. *J Clin Oncol* 24: 2019-2027, 2006.
- 3 Goldhirsch A, Wood WC, Gelber RD, Coates AS, Thurlimann B and Senn HJ: Progress and promise: highlights of the international expert consensus on the primary therapy of early breast cancer 2007. *Ann Oncol* 18: 1133-1144, 2007.
- 4 Goldhirsch A, Wood WC, Coates AS, Gelber RD, Thurlimann B and Senn HJ: Strategies for subtypes – dealing with the diversity of breast cancer: highlights of the St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2011. *Ann Oncol* 22: 1736-1747, 2011.
- 5 Lopez F, Belloc F, Lacombe F, Dumain P, Reiffers J, Bernard P and Boisseau MR: Modalities of synthesis of Ki67 antigen during the stimulation of lymphocytes. *Cytometry* 12: 42-49, 1991.
- 6 Birner P, Ritz M, Musahl C, Knippers R, Gerdes J, Voigtlander T, Budka H, Hainfellner JA: Immunohistochemical detection of cell growth fraction in formalin-fixed and paraffin-embedded murine tissue. *Am J Pathol* 158: 1991-1996, 2001.
- 7 Pinder SE, Wencyk P, Sibbering DM, Bell JA, Elston CW, Nicholson R, Robertson JF, Blamey RW and Ellis IO: Assessment of the new proliferation marker MIB1 in breast carcinoma using image analysis: associations with other prognostic factors and survival. *Br J Cancer* 71: 146-149, 1995.
- 8 van Dierendonck JH, Keijzer R, van de Velde CJ and Cornelisse CJ: Nuclear distribution of the Ki-67 antigen during the cell cycle: comparison with growth fraction in human breast cancer cells. *Cancer Res* 49: 2999-3006, 1989.
- 9 Vandewalle B, Hornez L, Lassalle B, Revillion F, Bertout M and Lefebvre J: Intracellular calcium and breast-cancer cell-growth and differentiation. *Int J Oncol* 2: 613-620, 1993.
- 10 Ermiah E, Buhmeida A, Abdalla F, Khaled BR, Salem N, Pyrhonen S and Collan Y: Prognostic value of proliferation markers: immunohistochemical ki-67 expression and cytometric s-phase fraction of women with breast cancer in libya. *J Cancer* 3: 421-431, 2012.
- 11 Wang Y, Yin Q, Yu Q, Zhang J, Liu Z, Wang S, Lv S and Niu Y: A retrospective study of breast cancer subtypes: the risk of relapse and the relations with treatments. *Breast Cancer Res Treat* 130: 489-498, 2011.
- 12 Millar EK, Graham PH, McNeil CM, Browne L, O'Toole SA, Boulghourjian A, Kearsley JH, Papadatos G, Delaney G, Fox C, Nasser E, Capp A and Sutherland RL: Prediction of outcome of early ER+ breast cancer is improved using a biomarker panel, which includes Ki-67 and p53. *Br J Cancer* 105: 272-280, 2011.
- 13 Jung SY, Han W, Lee JW, Ko E, Kim E, Yu JH, Moon HG, Park IA, Oh DY, Im SA, Kim TY, Hwang KT, Kim SW and Noh DY: Ki-67 expression gives additional prognostic information on St. Gallen 2007 and Adjuvant! Online risk categories in early breast cancer. *Ann Surg Oncol* 16: 1112-1121, 2009.
- 14 Viale G, Regan MM, Maiorano E, Mastropasqua MG, Dell'Orto P, Rasmussen BB, Raffoul J, Neven P, Orosz Z, Braye S, Ohlschlegel C, Thurlimann B, Gelber RD, Castiglione-Gertsch M, Price KN, Goldhirsch A, Gusterson BA and Coates AS: Prognostic and predictive value of centrally reviewed expression of estrogen and progesterone receptors in a randomized trial comparing letrozole and tamoxifen adjuvant therapy for postmenopausal early breast cancer: BIG 1-98. *J Clin Oncol* 25: 3846-3852, 2007.
- 15 Luporsi E, Andre F, Spyrtos F, Martin PM, Jacquemier J, Penault-Llorca F, Tubiana-Mathieu N, Sigal-Zafrani B, Arnould L, Gompel A, Egele C, Poulet B, Clough KB, Crouet H, Fourquet A, Lefranc JP, Mathelin C, Rouyer N, Serin D, Spielmann M, Haugh M, Chenard MP, Brain E, de Cremoux P and Bellocq JP: Ki-67: level of evidence and methodological considerations for its role in the clinical management of breast cancer: analytical and critical review. *Breast Cancer Res Treat* 132: 895-915, 2012.
- 16 Good E, Hammond M, Martin C, Burns C and Groos A: An audit of local government planning tools for their potential use in addressing community food and nutrition issues. *Health Promot J Austr* 21: 5-11, 2010.
- 17 Group EBCTC: Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet* 365: 1687-1717, 2005.
- 18 Harbeck N, Salem M, Nitz U, Gluz O and Liedtke C: Personalized treatment of early-stage breast cancer: present concepts and future directions. *Cancer Treat Rev* 36: 584-594, 2010.
- 19 Penault-Llorca F, Andre F, Sagan C, Lacroix-Triki M, Denoux Y, Verriele V, Jacquemier J, Baranzelli MC, Bibeau F, Antoine M, Lagarde N, Martin AL, Asselain B and Roche H: Ki67 expression and docetaxel efficacy in patients with estrogen receptor-positive breast cancer. *J Clin Oncol* 27: 2809-2815, 2009.

- 20 Taylor WC and Muss HB: Recent advances: adjuvant therapy for older women with breast cancer. *Cancer J* 16: 289-293, 2010.
- 21 Dowsett M, Procter M, McCaskill-Stevens W, de Azambuja E, Dafni U, Rueschoff J, Jordan B, Dolci S, Abramovitz M, Stoss O, Viale G, Gelber RD, Piccart-Gebhart M and Leyland-Jones B: Disease-free survival according to degree of HER2 amplification for patients treated with adjuvant chemotherapy with or without 1 year of trastuzumab: the HERA Trial. *J Clin Oncol* 27: 2962-2969, 2009.
- 22 Piccart-Gebhart MJ, Procter M, Leyland-Jones B, Goldhirsch A, Untch M, Smith I, Gianni L, Baselga J, Bell R, Jackisch C, Cameron D, Dowsett M, Barrios CH, Steger G, Huang CS, Andersson M, Inbar M, Lichinitser M, Lang I, Nitz U, Iwata H, Thomssen C, Lohrisch C, Suter TM, Rueschoff J, Suto T, Gatreux V, Ward C, Strahle C, McFadden E, Dolci MS and Gelber RD: Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. *N Engl J Med* 353: 1659-1672, 2005.
- 23 Untch M, Gelber RD, Jackisch C, Procter M, Baselga J, Bell R, Cameron D, Bari M, Smith I, Leyland-Jones B, de Azambuja E, Wermuth P, Khasanov R, Feng-Yi F, Constantin C, Mayordomo JI, Su CH, Yu SY, Lluch A, Senkus-Konefka E, Price C, Haslbauer F, Suarez Sahui T, Srimuninnimit V, Colleoni M, Coates AS, Piccart-Gebhart MJ and Goldhirsch A: Estimating the magnitude of trastuzumab effects within patient subgroups in the HERA trial. *Ann Oncol* 19: 1090-1096, 2008.
- 24 Loi S: Molecular analysis of hormone receptor positive (luminal) breast cancers: what have we learnt? *Eur J Cancer* 44:2813-2818,2008.
- 25 Nishimura R, Osako T, Okumura Y, Hayashi M, Toyozumi Y and Arima N: Ki-67 as a prognostic marker according to breast cancer subtype and a predictor of recurrence time in primary breast cancer. *Exp Ther Med* 1: 747-754, 2010.
- 26 Raica M, Jung I, Cimpean AM, Suciu C and Muresan AM: From conventional pathologic diagnosis to the molecular classification of breast carcinoma: are we ready for the change? *Rom J Morphol Embryol* 50: 5-13, 2009.
- 27 Jiyoung Rhee S-WH and Do-Youn Oh: The clinicopathologic characteristics and prognostic significance of triple-negativity in node-negative breast cancer. *BMC Cancer* 8: 307, 2008.
- 28 Munzone E, Botteri E, Sciandivasci A, Curigliano G, Nole F, Mastropasqua M, Rotmensz N, Colleoni M, Esposito A, Adamoli L, Luini A, Goldhirsch A and Viale G: Prognostic value of Ki-67 labeling index in patients with node-negative, triple-negative breast cancer. *Breast Cancer Res Treat* 134: 277-282, 2012.
- 29 Dowsett M, Smith IE, Ebbs SR, Dixon JM, Skene A, A'Hern R, Salter J, Detre S, Hills M and Walsh G: Prognostic value of Ki67 expression after short-term presurgical endocrine therapy for primary breast cancer. *J Natl Cancer Inst* 99: 167-170, 2007.
- 30 Viale G, Giobbie-Hurder A, Regan MM, Coates AS, Mastropasqua MG, Dell'Orto P, Maiorano E, MacGrogan G, Braye SG, Ohlschlegel C, Neven P, Orosz Z, Olszewski WP, Knox F, Thurlimann B, Price KN, Castiglione-Gertsch M, Gelber RD, Gusterson BA and Goldhirsch A: Prognostic and predictive value of centrally reviewed Ki-67 labeling index in postmenopausal women with endocrine-responsive breast cancer: results from Breast International Group Trial 1-98 comparing adjuvant tamoxifen with letrozole. *J Clin Oncol* 26: 5569-5575,2008.
- 31 Choy ET, Chiu A, Butow P, Young J and Spillane A: A pilot study to evaluate the impact of involving breast cancer patients in the multidisciplinary discussion of their disease and treatment plan. *Breast* 16: 178-189, 2007.
- 32 Carey LA, Dees EC, Sawyer L, Gatti L, Moore DT, Collichio F, Ollila DW, Sartor CI, Graham ML and Perou CM: The triple negative paradox: primary tumor chemosensitivity of breast cancer subtypes. *Clin Cancer Res* 13: 2329-2334, 2007.

*Received September 25, 2013*

*Revised November 19, 2013*

*Accepted November 21, 2013*