Breast Cancer (2014) 21:325–333
DOI 10.1007/s12282-012-0387-5

ORIGINAL ARTICLE

Analysis of clinically relevant values of Ki-67 labeling index in Japanese breast cancer patients

Kentaro Tamaki · Takanori Ishida · Nobumitsu Tamaki · Yoshihiko Kamada · Kanou Uehara · Minoru Miyashita · Masakazu Amari · Akiko Tadano-Sato · Yayoi Takahashi · Mika Watanabe · Keely McNamara · Noriaki Ohuchi · Hironobu Sasano

Received: 16 February 2012 / Accepted: 13 June 2012 / Published online: 11 July 2012

Abstract
Background It has become important to standardize the methods of Ki-67 evaluation in breast cancer patients, especially those used in the interpretation and scoring of immunoreactivity. Therefore, in this study, we examined the Ki-67 immunoreactivity of breast cancer surgical specimens processed and stained in the same manner in one single Japanese institution by counting nuclear immunoreactivity in the same fashion.
Methods We examined 408 Japanese breast cancers with invasive ductal carcinoma and studied the correlation between Ki-67 labeling index and ER/HER2 status and histological grade of breast cancer. We also analyzed overall survival (OS) and disease-free survival (DFS) of these patients according to individual Ki-67 labeling index.
Results There were statistically significant differences of Ki-67 labeling index between ER positive/HER2 negative and ER positive/HER2 positive, ER negative/HER2 positive or ER negative/HER2 negative, and ER positive/HER2 positive and ER negative/HER2 negative groups (all $P < 0.001$). There were also statistically significant differences of Ki-67 labeling index among each histological grade ($P < 0.001$, respectively). As for multivariate analyses, Ki-67 labeling index was strongly associated with OS (HR 39.12, $P = 0.031$) and DFS (HR 10.85, $P = 0.011$) in ER positive and HER2 negative breast cancer patients. In addition, a statistically significant difference was noted between classical luminal A group and "20 % luminal A" in DFS ($P = 0.039$) but not between classical luminal A group and "25 % luminal A" ($P = 0.105$).
Conclusions A significant positive correlation was detected between Ki-67 labeling index and ER/HER2 status and histological grades of the cases examined in our study. The suggested optimal cutoff point of Ki-67 labeling index is between 20 and 25 % in ER positive and HER2 negative breast cancer patients.

Keywords Ki-67 · Breast cancer · Cutoff point · Estrogen receptor · HER2 · Histological grade

K. Tamaki (✉) · N. Tamaki · Y. Kamada · K. Uehara
Department of Breast Surgery, Nahanishi Clinic, 2-1-9 Akamine, Naha, Okinawa 901-0154, Japan
e-mail: nahanisikenta@yahoo.co.jp; k-tamaki@naha-nishi-clinic.or.jp

K. Tamaki · T. Ishida · M. Miyashita · M. Amari · A. Tadano-Sato · N. Ohuchi
Department of Surgical Oncology, Tohoku University Graduate School of Medicine, Sendai, Japan

K. Tamaki · M. Miyashita · Y. Takahashi · M. Watanabe · K. McNamara · H. Sasano
Department of Pathology, Tohoku University Hospital, Sendai, Japan

Introduction

Tumor proliferation fraction has become an established predictive marker for clinical outcome of breast cancer patients [1–3]. Uncontrolled cell proliferation has also been considered a hallmark of malignancy and can be assessed by various laboratory methods, including counting mitotic figures under light microscopy, flow or image cytometric evaluation of the fraction of the cells in S phase, and immunohistochemistry of various nuclear antigens associated with cell proliferation [3–5]. The proliferation antigen Ki-67 is localized in nuclei of the cells at all phase of the cell cycle except for those at G0 phase and, in particular, the Ki-67 labeling index (percentage of cells with Ki-67

positive nuclear immunoreactivity) is considered to represent the status of tumor proliferation [1–3, 6, 7].

The statistically significant correlation between the Ki-67 labeling index of carcinoma cells and clinical outcome has been reported in human breast cancer patients [8–10]. Trihia et al. reported that a relatively higher Ki-67 labeling index within the carcinoma was significantly associated with adverse clinical outcome regardless of the subtypes of breast cancer [9, 10]. These results indicate that the Ki-67 labeling index in breast carcinoma cells may confer a higher risk of relapse and subsequently a worse overall survival in those with early breast cancer [8–10].

While results obtained using the Ki-67 labeling index of carcinoma cells resemble those obtained by the Oncotype Dx assay in ER positive and lymph node negative breast cancer patients (largely because the results of the Oncotype Dx assay are based on the status of cell proliferation genes) [11], additional information can be gained from assessing the Ki-67 labeling index within the carcinoma cells. The information obtained from such an assessment is not limited to predictions of prognosis or clinical outcome but also includes prediction of relative responsiveness or resistance to chemotherapy or endocrine therapy in adjuvant settings and the treatment efficacy in tissue specimens obtained before, during, and after neoadjuvant therapy, particularly neoadjuvant endocrine therapy [3]. Because of this additional predictive value, results of the Ki-67 labeling index in carcinoma cells have been incorporated into surgical pathology reports of breast cancer patients in an increasing number of diagnostic pathology laboratories in many countries [3].

However, as in any study utilizing immunohistochemical staining to evaluate clinical samples, it is cardinal and pivotal to standardize the method of Ki-67 measurement, including pre-analytical, analytical, interpretation, and scoring assessment [3], because otherwise results are far from reproducible and applicable in routine clinical settings. This may be particularly true of the methodology used in the stratification of early breast cancer patients into high and low proliferation groups. This stratification is markedly important in clinical settings and many attempts have been made to define the optimal cutoff value [12–14]; however, the reported value suggested to optimally distinguish these two groups of patients has been strikingly variable, from 1 to 28.6 %, thereby markedly limiting its clinical utility [3]. The 12th St. Gallen International Breast Cancer Conference 2011 recommended that patients with ER positive and HER2 negative breast cancer with a Ki-67 labeling index of 14 % or more may be recommended to receive adjuvant chemotherapy in addition to endocrine therapy [12]. The use of this cutoff point must, however, be approached with some caution as Nishimura et al. [13] recently demonstrated that the optimal cutoff of Ki-67 was 25 % in Japanese early breast cancer patients. In addition, the International Ki-67 in Breast Cancer Working Group also proposed that the direct application of specific cutoffs for decision making must be considered unreliable unless analyses were conducted in a highly experienced laboratory with its own reference data [3].

Careful and critical review of the previously reported studies of Ki-67 in human breast cancer revealed that the great majority of Ki-67 labeling index studies have not necessarily been performed under stringent conditions as described above, especially under those recommended by the International Ki-67 in Breast Cancer Working Group. Therefore, in this study, we evaluated the Ki-67 labeling index in breast cancer surgical pathology specimens processed in the same manner in a single institute, Tohoku University Hospital, Sendai, Japan and by the same observers using the same evaluation criteria. We then evaluated the correlation between the Ki-67 labeling index and ER/HER2 status and histological grade in Japanese cases of invasive ductal carcinoma. We then attempted to determine the clinical relevant cutoff value or the percentage of Ki-67 positive invasive breast carcinoma cells that could differentiate eventual clinical outcome of ER positive breast cancer cases.

Materials and methods

Carcinomas

We examined 408 Japanese patients with invasive ductal carcinomas of the breast, all of whom had undergone surgery at Tohoku University Hospital, Sendai and Nahanishi Clinic Okinawa. The study protocol was approved by the Ethics Committee at Tohoku University Graduate School of Medicine. The median age of the patients was 56 years (range 25–89 years). Estrogen receptor (ER) and human epidermal growth factor receptor 2 (HER2) status were reevaluated and summarized as follows: ER positive and HER2 negative, ER positive and HER2 positive, ER negative and HER2 positive, and ER negative and HER2 negative. These specimens had been first cut into 5-mm slices after carefully inking the margins, fixed in 10 % formalin for 46–48 h at room temperature, and embedded in paraffin wax.

Immunohistochemistry

Immunohistochemical analyses were all performed by a single experienced histotechnician at the Department of Pathology, Tohoku University Hospital using the same protocol. All the blocks were freshly cut into 4-μm sections, placed on glue-coated glass slides (Matsunami Glass

Ind., Ltd, Osaka, Japan), and left at room temperature for 3–5 days. Sections were then deparaffinized in xylene, and hydrated with graded alcohols and distilled water at room temperature. Endogenous peroxidase activity was blocked with freshly prepared 3 % hydrogen peroxidase for 10 min at room temperature. Antigen retrieval was performed in an autoclave (Tomy SX-500 high pressure steam sterilizer, Tomy Seiko Co., Ltd., Tokyo, Japan) using citrate buffer for Ki-67 heated at 121 °C for 5 min. Sections were subsequently incubated for 30 min at room temperature in a blocking solution of 10 % rabbit serum (Nichirei Biosciences, Tokyo, Japan) for Ki-67, and then immunostained for 16 h at 4 °C with the primary antibody. The primary antibody of Ki-67 was MIB-1 mouse monoclonal antibody (code M7240; Dako, Copenhagen, Denmark) diluted at 1:300. Secondary antibody reaction for Ki-67 immunohistochemistry was performed using biotinylated rabbit anti-mouse antibody (Nichirei Bioscience) at a dilution of 1:100 for 30 min at room temperature and peroxidase-conjugated avidin (Nichirei Bioscience) was used according to the manufacture's instruction. Reacted sections were visualized using 3,3′-diaminobenzidine-tetrachloride (DAB)/30 % H_2O_2 in 0.05 mol/l Tris buffer (pH 7.6) and counterstained with hematoxylin for nuclear staining. We used the avidin–streptavidin immunoperoxidase method using the clone 6F11 antibody (Ventana, Tucson, AZ, USA) in an automated immunostainer (Benchmark System; Ventana) for immunohistochemistry of ER. A standardized immunohistochemistry kit (Hercep-Test for Immunoenzymatic Staining; Dako) was used for HER2 staining as previously reported [15, 16].

Histopathological analysis

Histopathological evaluations were based on the World Health Organization (WHO) histological classification of tumors of breast and *Rosen's Breast Pathology* [17, 18]. Histological grades were assessed according to the criteria of Elston and Ellis [17, 18]. The Ki-67 immunoreactivity was evaluated independently by two of the authors by first identifying the areas of the most densely stained areas in the whole tissue sections by scanning at low power fields and then counting 1000 carcinoma cells in these areas [3]. We used an Olympus BX50 (Olympus, Tokyo, Japan) and ×20 objectives for the analysis. Figure 1 represents characteristic immunohistochemical findings of Ki-67 positive and negative carcinoma cells (Fig. 1). The presence of ER was determined by distinctive nuclear immunoreactivity and was graded from 0 to 8 using the Allred score, with positivity of the cases defined as a score of 3 [19]. With regard to HER2 evaluation, membranous staining was graded as 0–1+, 2+, and 3+ [20]. The cases scored as 2+ were subjected to FISH to calculate the gene copy ratio of

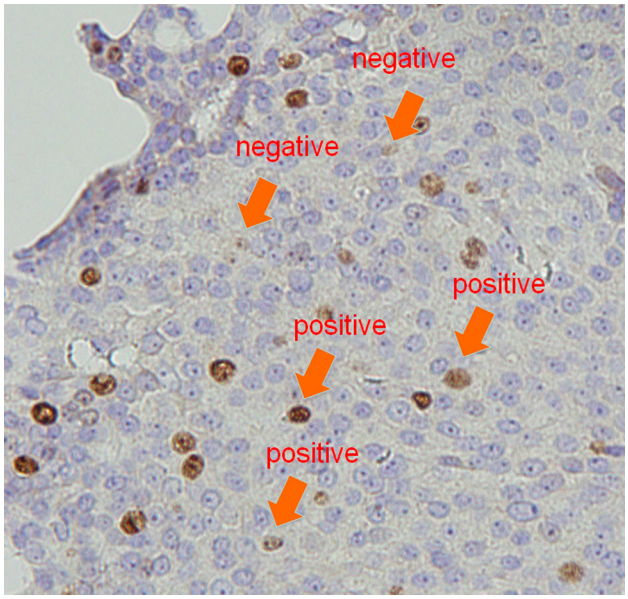

Fig. 1 Representative immunohistochemical findings of Ki-67 positive and negative carcinomas. The specimens were fixed in neutral buffered 10 % formalin and sections stained for Ki-67 with MIB1 antibody (*brown stain*) and counterstained with Mayer's hematoxylin (*blue stain*) (color figure online)

HER2 to CEP17 (PathVysion HER2 DNA Probe kit; Abbott, Chicago, IL, USA), as previously reported [15, 21]. HER2 positive cases were defined as a HER2/CEP17 signal ratio (FISH score) greater than 2.2 [20].

On the basis of the values obtained in the manner above, we examined the correlation between the Ki-67 labeling index and ER/HER2 status and histological grade. We also analyzed overall survival (OS) and disease-free survival (DFS) stratified according to the Ki-67 labeling index, in order to examine the utility of various cutoff points of Ki-67 in predicting clinical outcome within various ER+ breast cancer subgroups (luminal A, luminal B). In order to do this we tentatively assigned luminal A cases as follows: "classical luminal A" as the ER positive and HER2 negative group [22]; "14 % luminal A", based upon the proposal made at the St. Gallen 2011 consensus meeting [12], with a Ki-67 labeling index of less than 14 %; "20 % cutoff luminal A" with a Ki-67 labeling index of less than 20 %; "25 % cutoff luminal A" with a Ki-67 labeling index of less than 25 %; and "30 % cutoff luminal A" with a Ki-67 labeling index of less than 30 % [14, 23]. As for luminal B, we defined "classical luminal B" as ER positive and HER2 positive [24]; "14 % luminal B", proposed at St. Gallen 2011 [12], with a Ki-67 labeling index of more than 14 %; "20 % cutoff luminal B" with a Ki-67 labeling index of more than 20 %; "25 % cutoff luminal B" with a Ki-67 labeling index of more than 25 %; and "30 % cutoff luminal B" with a Ki-67 labeling index of more than 30 % [14, 22].

Statistical analyses

Statistically analyses were performed using StatMate IV for Windows (ATMS, Tokyo, Japan). The Mann–Whitney test was used to assess the correlation between the Ki-67 labeling index and ER/HER2 status and histological grade. The Cox proportional hazards regression model was used for multivariate analyses to evaluate each factor including the Ki-67 labeling index, TNM stages, ER expression, HER2 status, and adjuvant therapy of the patients. The analyses of OS or DFS curves were performed using the Kaplan–Meier method. The results were considered significant at $P < 0.05$.

Results

Correlation between Ki-67 labeling index and ER and HER2 status

Figure 2 summarizes the Ki-67 labeling index results according to ER and HER2 status of the cases examined. The Ki-67 labeling index in carcinoma cells was 11 % (median) and 17.9 % (average) in ER positive/HER2 negative, 40 % (median) and 36.4 % (average) in ER positive/HER2 positive, 40 % (median) and 46.8 % (average) in ER negative/HER2 positive, and 60 % (median) and 56.3 % (average) in ER negative/HER2 negative groups. There were statistically significant differences of the Ki-67 labeling index between ER positive/HER2 negative and ER positive/HER2 positive, ER negative/HER2 positive or ER negative/HER2 negative, and ER positive/HER2 positive and ER negative/HER2 negative groups (all $P < 0.001$).

Correlation between Ki-67 labeling index and histological grades

Figure 3 summarizes the Ki-67 labeling results index in each histological grade of the cases examined. The Ki-67 labeling index was 6 % (median) and 8.5 % (average) in grade 1, 19 % (median) and 24.0 % (average) in grade 2, and 60 % (median) and 55.8 % (average) in grade 3. The Ki-67 labeling index was significantly different between histological grades ($P < 0.001$, respectively).

OS of luminal A and B groups according to Ki-67 labeling index

Table 1 shows the distribution of patients according to the subtypes classical luminal, 14 % luminal, 20 % luminal, 25 % luminal, and 30 % luminal. The 5-year OS rates of patients in luminal A groups were 0.949 in classical luminal A, 1.000 in "14 % luminal A", 1.000 in "20 % luminal A", 1.000 in "25 % luminal A", and 1.000 in "30 % luminal A". There were no statistically significant differences of OS rates among these groups. The 5-year OS rates of luminal B were 1.000 in classical luminal B, 0.875 in "14 % luminal B", 0.853 in "20 % luminal B", 0.822 in "25 % luminal B", and 0.812 in "30 % luminal B". No statistically significant differences were detected among these groups.

DFS of luminal A and B groups according to the Ki-67 labeling index

Figure 4 summarizes the DFS rates of the patients according to each subgroup determined by the Ki-67 labeling index of individual cases. The 5-year DFS rates of patients in luminal A groups were 0.956 in classical luminal A, 1.000 in "14 % luminal A", 0.993 in "20 % luminal A", 0.989 in "25 % luminal A", and 0.983 in "30 % luminal A". There were statistically significant differences between classical luminal A and "14 % luminal A" or "20 % luminal A" ($P = 0.010$ and $P = 0.039$, respectively). A similar tendency was also noted between classical luminal A and "25 % luminal A" or "30 % luminal A" ($P = 0.105$ and 0.159, respectively) but the difference did not reach statistical significance. The 5-year DFS rates of patients in luminal B groups were 0.885 in classical luminal B, 0.880 in "14 % luminal B", 0.871 in "20 % luminal B", 0.840 in "25 % luminal B" and 0.835 in "30 % luminal B". There were no statistically significant differences among these groups above.

Multivariate analyses of OS and DFS according to Ki-67 labeling index

Among the factors examined, including the Ki-67 labeling index, tumor size, nodal status, stage, and adjuvant chemotherapy status, the Ki-67 labeling index was markedly associated with OS (HR 39.12, $P = 0.031$) and DFS (HR 10.85, $P = 0.011$) in ER positive and HER2 negative breast cancer patients. However, the Ki-67 labeling index was not statistically associated with OS (HR 9.28, $P = 0.198$) and DFS (HR 5.76, $P = 0.420$) in all cases including ER positive/HER2 positive, ER negative/HER2 negative, and ER negative/HER2 positive breast cancer patients.

Determination of Ki-67 labeling index cutoff values of carcinoma cells according to the clinical outcome of ER positive breast cancer cases

We evaluated the statistical significance of cutoff values of the Ki-67 labeling index in carcinoma cells segregated by 5 %. There were no statistically significant differences in OS of the patients. A statistically significant difference was

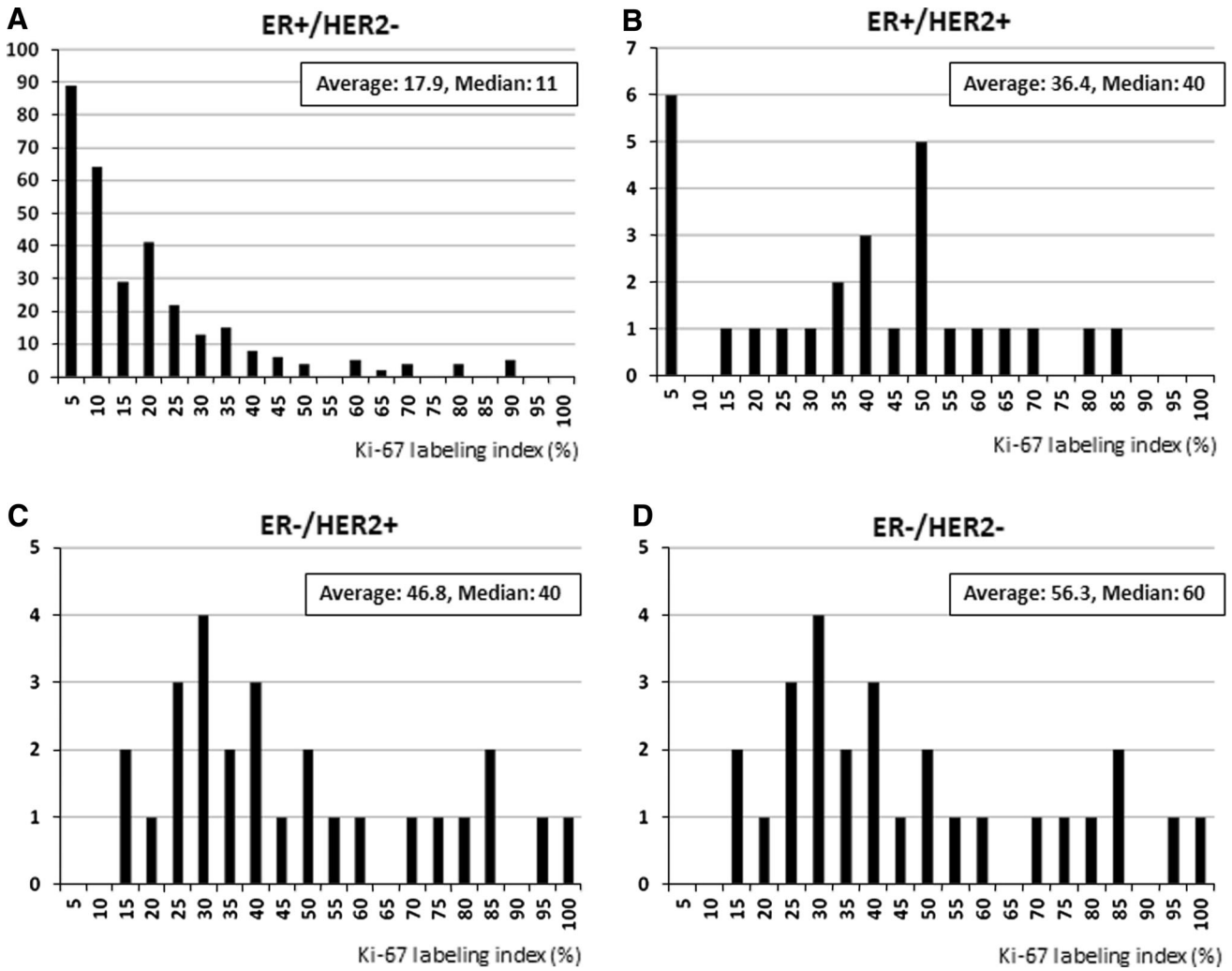

Fig. 2 Correlation between Ki-67 labeling index and ER or HER2 status. The distribution of Ki-67 labeling index in **a** ER positive and HER2 negative cases, **b** ER positive and HER2 positive cases, **c** ER negative and HER2 positive cases, **d** ER negative and HER2 negative cases

noted between classical luminal A group and "20 % luminal A" in DFS ($P = 0.039$) but not between classical luminal A group and "25 % luminal A" ($P = 0.105$). Therefore, the optimal cutoff point of the Ki-67 labeling index was suggested to be between 20 and 25 %.

Discussion

Ki-67 has been established as a well-known biomarker of cell proliferation in many human malignancies including breast cancer. The Ki-67 labeling index has been utilized to obtain both prognosis and prediction of the sensitivity to systemic therapy of breast cancer patients [2, 10, 21]. Some examples of this are the statistically significant correlation between a high Ki-67 labeling index of carcinoma cells and increased risk of cancer relapse and death in breast cancer patients [10] and the utility of mid-course evaluation of Ki-67 labeling index, even after 2 weeks of endocrine therapy, in predicting the subsequent response to endocrine therapy in ER positive breast cancer patients [23]. In addition the group of breast cancer patients associated with a high Ki-67 labeling index studied in the Breast International Group trial (BIG) 1-98 was associated with a potential clinical benefit in selecting letrozole over tamoxifen in post-menopausal patients [2]. Despite these important aspects of Ki-67 immunohistochemistry, the necessary standardized guidelines have not been developed [12, 25].

The International Ki-67 in Breast Cancer Working Group recently recommended the fixation of the specimens with neutral buffered formalin for 4–48 h or more and the counting of at least 500 invasive carcinoma cells using MIB-1 mouse monoclonal antibody [3]. In our present study, all the specimens examined had been processed in the same manner and according to the guidelines above and the Ki-67 labeling index was also evaluated accordingly.

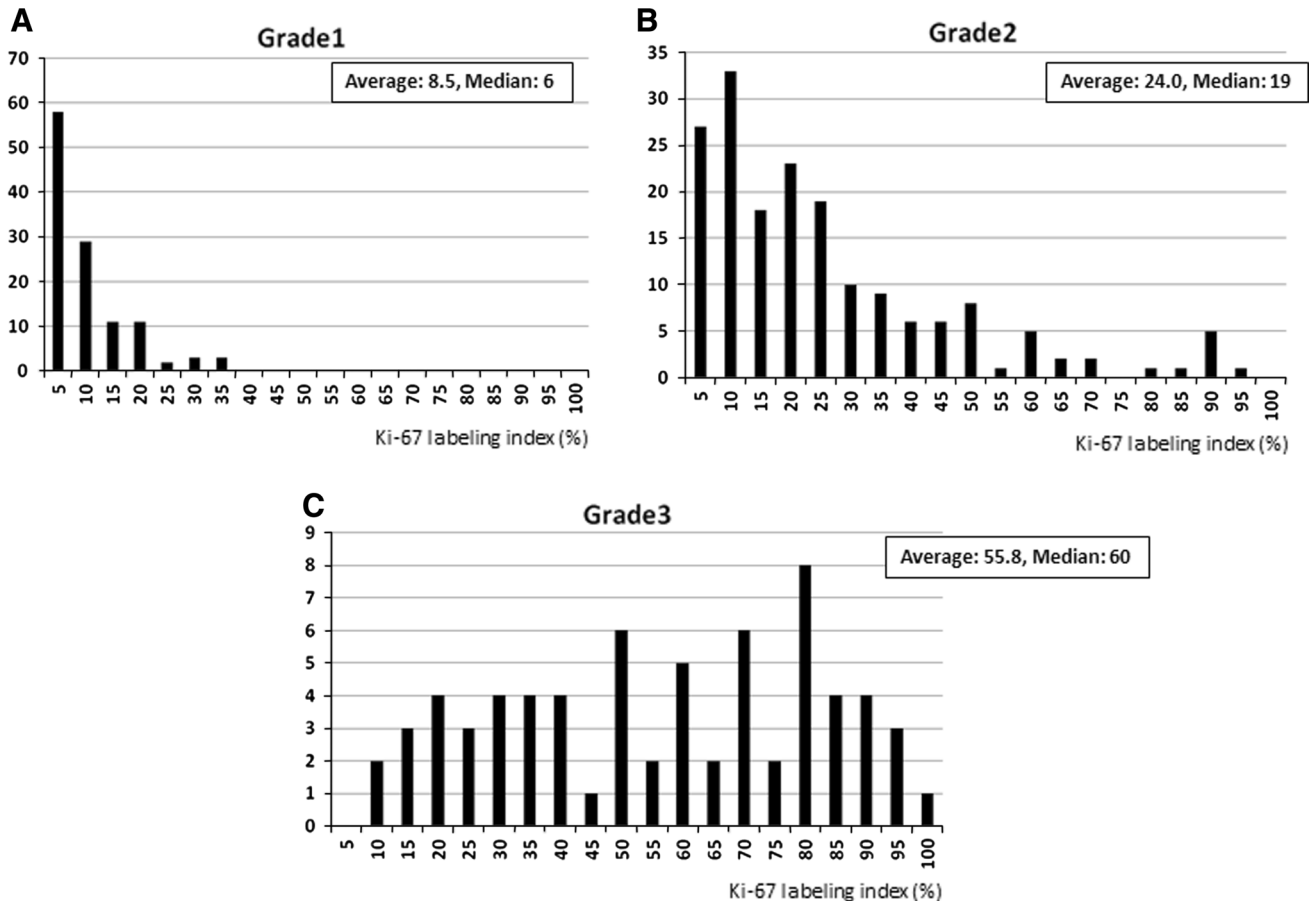

Fig. 3 Correlation between Ki-67 labeling index and histological grade of the patients. The distribution of Ki-67 labeling index in **a** grade 1, **b** grade 2, **c** grade 3 groups

Table 1 Distribution of patients according to the subtypes classical luminal, 14 % luminal, 20 % luminal, 25 % luminal, and 30 % luminal

	n	Ki-67 (median %)	Ki-67 (average %)
Classical lum A	289	11	17.9
14 % lum A	160	5	6.0
20 % lum A	186	6	7.5
25 % lum A	215	8	9.2
30 % lum A	225	9	10.1
Classical lum B	23	40	36.4
14 % lum B	152	27	33.2
20 % lum B	126	31	36.7
25 % lum B	97	35	41.1
30 % lum B	87	40	42.9

Previous studies conducted by Nishimura et al. [26–28] on Japanese breast cancer patients demonstrated that the Ki-67 value as significantly higher in triple negative cases. However, the Ki-67 labeling index was also statistically lower in ER positive/HER2 negative cases [26–28]. We therefore examined the correlation between the Ki-67 labeling index and hormone receptor, HER2 status, or histological grade using surgical pathology specimens processed in the same manner and immunostained in the same fashion by one single experienced histotechnician in one single institution.

The results of our present study demonstrated that the ER positive and HER2 negative group was associated with a significantly lower Ki-67 labeling index of carcinoma cells than in other subtypes examined. The cases with a high Ki-67 labeling index in the ER positive and HER2 negative group have been considered as potential candidates for receiving chemotherapy in addition to endocrine therapy as in the patients with a high histological grade [12–14]. In our present study, there was also a statistically significant correlation between the Ki-67 labeling index and histological grades of individual cases. Collectively our findings suggest that it may be better to review the slides when there is a significant discrepancy between the results of Ki-67 labeling index and histological grade in invasive ductal carcinoma cases. The results of our present study also demonstrated that subtyping of the tumors using immunohistochemical surrogate markers such as ER,

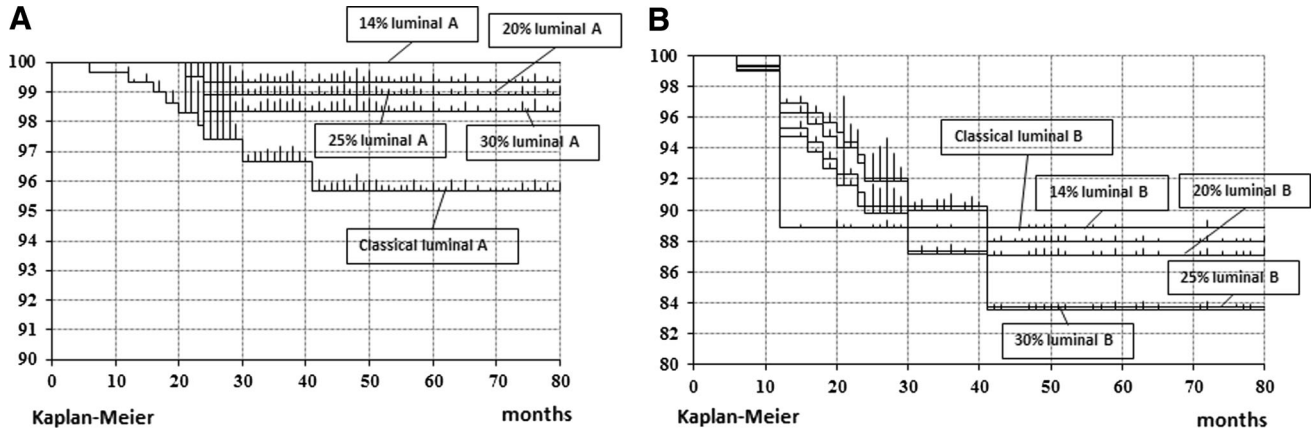

Fig. 4 DFS according to Ki-67 labeling index of the patients. **a** Luminal A: *classical luminal A* ER positive and HER2 negative; *14 % luminal A* Ki-67 labeling index less than 14 %; *20 % luminal A* Ki-67 labeling index less than 20 %; *25 % luminal A* Ki-67 labeling index less than 25 %; *30 % luminal A* Ki-67 labeling index less than 30 %. **b** Luminal B: *classical luminal B* ER positive and HER2 positive; *14 % luminal B* Ki-67 labeling index more than 14 %; *20 % luminal B* Ki-67 labeling index more than 20 %; *25 % luminal B* Ki-67 labeling index more than 25 %; *30 % luminal B* Ki-67 labeling index more than 30 %

HER2, and Ki-67, if using appropriately processed surgical pathology specimens and well-controlled immunohistochemical procedures, could at least contribute to identifying high-risk Japanese breast cancer patients within the hormone receptor positive subgroup of breast cancers. Nishimura et al. [26] also indicated that ER/PgR, HER2, and Ki-67 are all important biological markers for predicting prognosis and making effective treatment decisions in Japanese breast cancer patients by using only these biomarkers. The combination of these markers has been proposed at least in defining luminal A and B types of breast cancer without necessarily performing gene profiling studies with some exceptions [12, 29]. Luminal B type breast cancer represents a clinically important subgroup generally associated with adverse clinical outcome regardless of systemic adjuvant therapy [19]. It was recently recommended at the St. Gallens consensus meeting that chemotherapy was indicated for the majority of these patient defined as ER positive and with a Ki-67 labeling index of more than 14 % [12]. However, it is also true that the optimal cutoff points of the Ki-67 labeling index in these cases have been reported as 10–25 % [3, 12]. For instance, no pathological responders were reported in the cases with more than 25 % Ki-67 in neoadjuvant chemotherapy of Japanese breast cancer patients [13]. These discrepancies or variations of proposed values of Ki-67 labeling may be all due to differences of methodologies involved in obtaining the Ki-67 labeling index including pre-analytical factors such as fixation of the specimens and/or ethnical or racial backgrounds of the patients and further investigations are required for clarification.

The direct application of a specific cutoff for clinical decision making may be considered unreliable unless analyses are conducted in a highly experienced laboratory with its own reference data [3]. The International Ki-67 in Breast Cancer Working Group demonstrated that no consensus has been reached regarding the ideal cutoff point of the Ki-67 labeling index. The results of our present study demonstrated that there were statistically significant differences of DFS between classical luminal A and luminal with a 14 or 20 % cutoff of Ki-67. In addition, we examined the cutoff values of the Ki-67 labeling index segregated by 5 %. A statistically significant difference was noted between classical luminal A group and "20 % luminal A" in DFS but not between classical luminal A group and "25 % luminal A". Therefore, we propose an optimal cutoff point of the Ki-67 labeling index of between 20 and 25 %. These results were similar to that of a previous study from Japan mentioned above [13]. Therefore, ER positive and HER2 negative Japanese breast cancer patients with a Ki-67 labeling index of 20–25 % are associated with more aggressive biological course than those not and additional chemotherapy may be of further help or benefit to these patients.

It was recently proposed that the prognostic information provided by ER, PgR, HER2, and Ki-67 immunostaining performed in a rigorously controlled fashion was considered at least equivalent to that provided by 21 gene signature analysis and highlights the relevance of these readily available routine histopathological parameters in the clinical management of early ER positive breast cancer [30]. In addition, we demonstrated using multivariate analysis that the Ki-67 labeling index was one of the most important prognostic factors for the ER positive and HER2 negative group in this study. Therefore, it has become important to standardize the type of fixation, time to fixation, appropriate primary antibody, and methods of immunostaining and interpretation, especially in countries like Japan where

the expensive gene signature tests are and will be out of reach for the great majority of breast cancer patients. We also noted the statistically significant correlation between the Ki-67 labeling index and ER/HER2 status and histological grade of individual patients performed in a single institution. It is true that our present study was retrospective, the number of the patients is relatively small, and the patients were all Japanese but the results still provided sufficient evidence to support the value of the Ki-67 labeling index in the clinical management of breast cancer patients. Further investigations employing larger numbers of patients with longer periods of clinical follow-up may be required for determining the most clinically relevant cutoff points of the Ki-67 labeling index in breast cancer patients, especially those in the early stage in order to confer the maximal clinical benefits upon individual breast cancer patients.

Acknowledgments The authors appreciate the continuous excellent technical assistance of the staff in the Department of Pathology, Tohoku University Hospital, Sendai, Japan, especially their uninterrupted laboratory service toward the care of breast cancer patients despite enormous and unprecedented damages inflicted upon glass slides and instruments such as tissue processors, cryostat instruments, and automatic stainers, and harsh working conditions such as continuous aftershocks, total blackout, and interruption of running water in our laboratory as a result of the 3/11 earthquake. This work was supported in part by a Grant-in Aid from the "Kurokawa Cancer Research Foundation".

Conflict of interest The authors have no conflict of interest.

References

1. Clahsen PC, van de Velde CJ, Duval C, Pallud C, Mandard AM, Delobelle-Deroide A, et al. The unit of mitotic index, oestrogen receptor and Ki-67 measurements in the creation of novel prognostic indices for node-negative breast cancer. Eur J Surg Oncol. 1999;25:356–63.
2. Viale G, Giobbie-Hurder A, Regan MM, Coates AS, Mastropasqua MG, Dell'Orto P, et al. 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. 2008;26:5569–75.
3. Dowsett M, Nielsen TO, A'Hern R, Bartlett J, Coombes RC, Cuzick J, et al. Assessment of Ki67 in breast cancer: recommendations from the International Ki67 in Breast Cancer Working Group. J Natl Cancer Inst. 2011;103:1–9.
4. Dressler LG, Seamer L, Owens MA, Clark GM, McGuire WL. Evaluation of a modeling system for S-phase estimation in breast cancer by flow cytometry. Cancer Res. 1987;47:5294–302.
5. Tovey SM, Witton CJ, Bartlett JM, Stanton PD, Reeves JR, Cooke TG. Outcome and human epidermal growth factor receptor (HER) 1–4 status in invasive breast carcinomas with proliferation indices evaluated by bromodoxyuridine labeling. Breast Cancer Res. 2004;6:246–51.
6. Gerdes J, Schwab U, Lemke H, Stein H. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. Int J Cancer. 1983;3:13–20.
7. Lehr HA, Hansen DA, Kussick S, Li M, Hwang H, Krummenauer F, et al. Assessment of proliferative activity in breast cancer: MIB-1 immunohistochemistry versus mitotic figure count. Hum Pathol. 1999;30:1314–20.
8. Tamaki K, Moriya T, Sato Y, Ishida T, Maruo Y, Yoshinaga K, et al. Vasohibn-1 in human breast carcinoma: a potential negative feedback regulator of angiogenesis. Cancer Sci. 2009;100:88–94.
9. Trihia H, Murray S, Price K, Gelber RD, Golouh R, Goldhirsch A, et al. Ki-67 expression in breast carcinoma: its association with grading system, clinical parameters, and other prognostic factors-a surrogate marker? Cancer. 2003;97:1321–31.
10. de Azambuja E, Cardoso F, de Castro G, Colozza M Jr, Mano MS, Durbecq V, et al. Ki-67 as prognostic marker in early breast cancer: a meta-analysis of published studies involving 12,155 patients. Br J Cancer. 2007;96:1504–13.
11. Paik S, Shak S, Tang G, Kim C, Baker J, Cronin M, et al. A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. N Engl J Med. 2004;351:2817–26.
12. Goldhirsch A, Wood WC, Coates AS, Gelber RD, Thurlimann B. Panel members. 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. 2011;22:1736–47.
13. Nishimura R, Osako T, Okumura Y, Hayashi M, Arima N. Clinical significance of Ki-67 in neoadjuvant chemotherapy for primary breast cancer as a predictor for chemosensitivity and for prognosis. Breast Cancer. 2010;17:269–75.
14. Goldhirsch A, Ingle JN, Gelber RD, Coates AS, Thürlimann B. Panel members. Thresholds for therapies: highlights of the St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2009. Ann Oncol. 2009;20:1319–29.
15. Tamaki K, Ishida T, Miyashita M, Amari M, Mori N, Ohuchi N, et al. Multidetector row helical computed tomography for invasive ductal carcinoma of breast: the correlation between radiological findings and the corresponding biological characteristic of the patients. Cancer Sci. 2012;103:67–72.
16. Tamaki K, Sasano H, Ishida T, Ishida K, Miyashita M, Takeda M, et al. Comparison of core needle biopsy (CNB) and surgical specimens for accurate preoperative evaluation of ER, PgR and HER2 status of breast cancer patients. Cancer Sci. 2010;101:2074–9.
17. Tavassoli FA, Devilee P. World Health Organization classification of tumors. Tumor of the breast and female gential organs. Lyon: IARC; 2003.
18. Rosen PP. Rosen's breast pathology. 3rd ed. Philadelphia: Lippincott Williams & Wilkins; 2009.
19. Allred DC, Harvey JM, Berardo M, Clark GM. Prognostic and predictive factors in breast cancer by immunohistochemical analysis. Mod Pathol. 1998;11:155–68.
20. Wolff AC, Hammond ME, Schwartz JN, Hagerty KL, Allred DC, Cote RJ, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. J Clin Oncol. 2007;25:118–45.
21. Miyashita M, Ishida T, Ishida K, Tamaki K, Amari M, Watanabe M, et al. Histopathological subclassification of triple negative breast cancer using prognostic scoring system: five variables as candidates. Virchows Arch. 2011;458:65–72.
22. Jalava P, Kuopio T, Juntti-Patinen L, Kotkansalo T, Kronqvist P, Collan Y. Ki67 immunohistochemistry: a valuable marker in prognostication but with a risk of misclassification: proliferation subgroups formed based on Ki67 immunoreactivity and standardized mitotic index. Histopathology. 2006;48:674–82.
23. Dowsett M, Smith IE, Ebbs SR, Dixon JM, Skene A, A'Hern R, Salter J. Prognostic value of Ki67 expression after short term presurgical endocrine therapy for primary breast cancer. J Natl Cancer Inst. 2007;99:167–70.

24. Spitale A, Mazzola P, Soldini D, Mazzucchelli L, Bordoni A. Breast cancer classification according to immunohistochemical markers: clinicopathologic features and short-term survival analysis in a population-based study from the South of Switzerland. Ann Oncol. 2009;20:628–35.
25. Cheang MCU, Chia SK, Voduc D, Gao D, Leung S, Snider J. Ki67 index, Her2 status, and prognosis of patients with luminal B breast cancer. J Natl Cancer Inst. 2009;101:736–50.
26. Nishimura R, Osako T, Okumura Y, Tashima R, Toyozumi Y, Arima N. Changes in the ER, PgR, HER2, p53 and Ki-67 biological markers between primary and recurrent breast cancer: discordance rates and prognosis. World J Surg Oncol. 2011;9:131.
27. Nishimura R, Okumura Y, Arima N. Trastuzumab monotherapy versus combination therapy for treating recurrence breast cancer: time to progression and survival. Breast Cancer. 2008;15:57–64.
28. Nishimura R, Arima N. Is triple negative a prognostic factor in breast cancer? Breast Cancer. 2008;15:303–8.
29. Bhargava R, Striebel J, Beriwal S, Flickinger JC, Onisko A, Ahrendt G. Prevalence, morphologic features and proliferation indices of breast carcinoma molecular classes using immunohistochemical surrogate markers. Int J Clin Exp Pathol. 2009;2:444–55.
30. Cuzick J, Dowsett M, Wale C. Prognostic value of a combined ER, PgR, Ki67, HER2 immunohistochemical (IHC4) score and comparison with the GHI recurrence score-results from TransATAC. Cancer Res. 2009;69:503s.