detection and effective systemic adjuvant therapy. In Japan, however, the incidence is still increasing, and more than 50,000 cases were newly diagnosed in 2004, with more than 12,000 deaths reported 2009 [1].

About 50–60 % of newly diagnosed Japanese patients are estimated to have invasive cancers without regional lymph node metastases, and 10–15 % of these patients will have a local or distant recurrence within 10 years of the initial surgery [2, 3]. Therefore, it has been important to establish criteria for identifying the high-risk patient groups for whom adjuvant chemotherapy would be considered.

From 1996 to 2001, we classified patients with node-negative invasive breast cancers into high- and low-risk groups according to the histopathological criteria established for the multi-institutional phase III randomized National Surgical Adjuvant Study for Breast Cancer 01 (NSAS-BC01) clinical study [3]. These histological criteria comprised histological type, invasive size, and nuclear grade (NG) [4]. In total, 733 patients entered the study, and 5-year disease-free survival (DFS) and overall survival rates even in the high-risk group were excellent: 88 and 96 %, respectively [3].

In and around 2000, Perou et al. [5–7] introduced a novel molecular subtype classification of invasive breast cancers using a DNA microarray technique, and these subtypes were correlated with prognosis. Thereafter, a more simple intrinsic subtype classification by immunohistochemistry (IHC) has been developed for clinical use [5, 8, 9]. The surrogate intrinsic subtype classification recommended during the St. Gallen International Consensus Meeting in 2011 [10] was as follows:

Luminal A	hormone receptor (HR) [estrogen receptor (ER) and/or progesterone receptor (PR)]-positive, HER2-negative, and low (<14 %) Ki-67 labeling index (LI)
Luminal B/HER2-negative	HR-positive, HER2-negative, and high (≥14 %) Ki-67 LI
Luminal B/HER2-positive	HR-positive and HER2-positive
HER2-enriched	HR-negative and HER2-positive
Triple-negative breast cancers (TNBCs)	HR-negative and HER2-negative

Although Ki-67 LI is well established as a prognostic marker, criteria for Ki-67 LI values for distinguishing high-risk from low-risk groups in luminal breast cancers vary. Along with the 14 % cutoff value recommended at St. Gallen, there are opinions that favor other cutoff values, e.g., 10 and 20 % [11–15]. It is also mentioned that alternative assessments of tumor proliferation such as histological grade (HG) or NG may be used to distinguish luminal subtypes if the Ki-67 result is not reliable [10]. Furthermore, the prognosis for Japanese patients with node-negative invasive breast cancer has been reported to be better than that in Western patients. The 10-year recurrence rate in Japan is reported to be 10–15 % vs. up to 25–30 % after the initial surgery in the USA [2, 3, 11, 12, 14, 15].

Therefore, we examined the prognostic significance of Ki-67 LI with different cutoff values, along with that of HG and NG in patients with node-negative luminal invasive breast cancers in order to find optimal criteria to identify high-risk luminal/HER2-negative tumors in Japanese patients. We first classified 530 consecutive patients into intrinsic subtypes, and then classified the 369 patients with luminal/HER2-negative tumors into luminal A and luminal B groups according to 3 different cutoff values of Ki-67 or HG/NG. We evaluated outcomes in these groups by univariate and multivariate survival analyses. Finally, we examined concordance between Ki-67 and HG/NG and predictive value of Ki-67 with adjuvant chemotherapy in those patients with luminal breast cancers who had originally been entered in the NSAS-BC01 protocol.

Patients and methods

Patient selection and data collection

Between 1996 and 2000, 709 patients underwent surgery for primary breast cancer at the National Cancer Center Hospital in Tokyo and were pathologically diagnosed with node-negative breast cancer. We excluded 67 patients with ductal carcinoma in situ, 17 patients with bilateral breast cancers, 8 male patients with breast cancer, and 33 patients who had received neoadjuvant chemotherapy, and detailed data of 23 cases were not available. Among the 561 eligible cases remaining, formalin-fixed paraffin-embedded tissue blocks of the invasive carcinomas were available for 530. We reviewed the medical charts of these 530 patients and extracted patient data including age, regimens of adjuvant chemotherapy and endocrine therapies, and dates of recurrence, death, or the last follow-up examination.

Portions of tissues from surgically resected specimens were sampled for ER and PR assays soon after resection, and residual tissues were fixed with 10 % formalin overnight and dissected into tissue blocks. These blocks were fixed in formalin for 1 day, degreased in ethanol/chloroform for 1 day, embedded in paraffin, and subjected to routine pathology diagnosis.

For all 530 cases, we had routinely evaluated HG and NG, and the size of the invasive carcinoma component, the histological type, and lymphovascular invasion. HG was reported according to Elston and Ellis's criteria [16]. The

method for NG determination is described elsewhere [4, 16]. Histological type was assigned according to the *General Rules for Clinical and Pathological Recording of Breast Cancer in Japan* [17]. The histopathological evaluations were performed throughout the period by 2 pathologists who specialized in breast pathology, and we used those data for the present study without changes.

ER and PR status were determined by enzyme immunoassay (Abbott Molecular, Wiesbaden, Germany) using lysates of snap frozen tumor tissues. HER2 had been examined by IHC using a polyclonal antibody (Nichirei, Tokyo, Japan). These assays were not performed for all cases, and the criteria for judgment differed from those used in current practice. Therefore, we reexamined ER, PR, and HER2 using standardized IHC tests from the Department of Pathology of the National Cancer Center Hospital. The present study was approved by the institutional review board.

Tissue microarray construction, IHC, and FISH

From the formalin-fixed and paraffin-embedded tissue blocks of 530 primary breast cancers, two 2-mm cores per block were enucleated from the invasive components, including invasive fronts, and used for the construction of tissue microarrays using a manual tissue-arraying instrument (Azumaya, Tokyo, Japan). The blocks from the tissue microarrays were cut into 3- to 4-μm-thick sections for IHC and fluorescence in situ hybridization (FISH) assays.

IHC was performed using the following primary antibodies: anti-ER (1D5; Dako, Glostrup, Denmark), anti-PR (PgR636, Dako), anti-HER2 (HercepTest, Dako), and anti-Ki-67 (MIB-1, Dako). For ER, PR, HER2, and Ki-67, IHC was performed strictly according to the manufacturer's instructions using an Autostainer Link 48 (Dako). FISH was performed using a PathVysion HER2 DNA probe kit (Abbott Molecular) according to the manufacturer's instruction.

ER and PR were judged as positive when the Allred score was $\geq$3 and as negative when the score was $\leq$2 [18]. HER2 was judged as positive when IHC score was 3+ or when IHC score was 2+ and HER2/CEP17 ratio was $\geq$2.0 by FISH [19]. For Ki-67 LI, we selected 2–3 areas per tissue core including hot spots of Ki-67-positive cells, and took multiple photomicrographs of different fields [13]. The photomicrographs were printed, and the proportion of Ki-67-positive cancer cells with moderate to strong immunoreaction per 1,000 cancer cells was determined independently by 2 observers (M.O. and H.T.), with the average values adopted as Ki-67 LI. We evaluated the prognostic significance of a high Ki-67 LI based on 3 cutoff values, 10, 14, and 20 %.

Statistical analyses

Statistical analyses were performed using SPSS software (IBM SPSS Statistics, Chicago, IL, USA). Correlation analyses were performed using the chi-squared test for categorical variables. The interval from the date of initial surgery to disease recurrence was defined as disease-free survival (DFS), and the interval from the date of operation to death from breast cancer or the last follow-up examination was defined as breast cancer-specific survival (BCSS). Death from another disease was regarded as censored. DFS and BCSS curves were drawn by the Kaplan–Meier method and compared by log-rank test. Cox's univariate and multivariate regression analyses were performed to evaluate prognostic significance of each parameter in the patients with luminal-subtype breast cancers. In all analyses, differences were considered significant at $p < 0.05$.

Results

Correlation of breast cancer subtypes and grades with patient prognosis

The median duration of follow-up in a total of 530 patients was 120.1 (range, 0.6–160.3) months. Overall, 65 recurrences and 31 deaths from breast cancer occurred. Of the tumors resected from these 530 patients, 369 (70 %) were HR-positive and HER2-negative (luminal/HER2−), 40 (8 %) HR-positive and HER2-positive (luminal/HER2+), 41 (8 %) HER2-enriched, 75 (14 %) TNBC, and 5 unknown. DFS and BCSS curves differed almost significantly or significantly among these groups ($p = 0.07$ and 0.0007, respectively), and patients in the luminal/HER2− group had the best prognosis (Fig. 1a, b). In luminal/HER2−, luminal/HER2+, HER2-enriched, and TNBC groups, 10-year DFS rates were 88.3, 81.5, 81.3, and 78.2 %, respectively, and 10-year BCSS rates were 95.3, 94.9, 80.1, and 87.5 %, respectively.

In these 530 tumors, 115 (22 %), 200 (38 %), 212 (40 %), and 3 were HG1, HG2, HG3, and unknown, and 169 (32 %), 115 (22 %), 240 (45 %), and 6 were NG1, NG2, NG3, and unknown, respectively. The DFS and BCSS curves differed significantly among HG1, 2, and 3 groups ($p = 0.0012$ and <0.0001) and also among NG1, 2, and 3 groups ($p = 0.0013$ and <0.0001) (Fig. 1c–f). In the patients with HG1, 2, and 3 tumors, 10-year DFS and BCSS rates were 92.3 and 98.7 %, 89.5 and 97.4 %, and 79.2 and 86.0 %, respectively, and 10-year DFS and BCSS were 93.0 and 99.2 %, 88.1 and 96.4 %, and 79.4 and 87.1 %, in the patients with NG 1, 2, and 3 tumors, respectively.

Survival curves for patients with luminal/HER2-negative invasive breast cancer

The details of the 369 luminal/HER2− breast cancer cases are shown in Table 1. There were 187 patients (51 %) who received adjuvant hormonal therapy with tamoxifen, 153 (41 %) who received adjuvant chemotherapy, 144 (39 %) who received both adjuvant endocrine therapy and chemotherapy, and 173 (49 %) who did not receive adjuvant therapy.

The Ki-67 LI was ≥10 % in 221 (60 %), ≥14 % in 163 (44 %), and ≥20 % in 87 (24 %) of the tumors. DFS curves differed significantly between the patient subgroups with low-Ki-67 and high-Ki-67 tumors under any cutoff value (10, 14, or 20 %) (Fig. 2a, c, e) ($p = 0.01$, 0.04, and 0.02, respectively). The 10-year DFS rates for patients with low-Ki-67 tumors vs. those with high-Ki-67 tumors were 93.6 vs. 84.9 %, 91.3 vs. 84.7 %, and 91.0 vs. 80.1 %, respectively, under each cutoff value. The BCSS curves also differed significantly between patients with low-Ki-67 and high-Ki-67 tumors under any of the 3 cutoff values (Fig. 2b, d, f) ($p = 0.02$, 0.01, and 0.004, respectively). The 10-year BCSS rates for patients with low-Ki-67 tumors vs. those with high-Ki-67 tumors were 99.3 vs. 92.9 %, 98.1 vs. 91.9 %, and 97.4 vs. 88.9 %, respectively, under the 3 cutoff values.

Of the 369 luminal/HER2− tumors, 104 (28 %), 175 (47 %), 88 (24 %) and 2 were HG1, HG2, HG3, and unknown, and 156 (42 %), 103 (28 %), 109 (30 %), and 1 were NG 1, NG2, NG3, and unknown, respectively. The DFS and BCSS curves differed significantly for HG1, 2, and 3 groups ($p = 0.01$ and < 0.0001) and also among the NG1, 2, and 3 groups ($p = 0.008$ and 0.0002) (Fig. 3a–d). Ten-year DFS and BCSS rates of HG1 vs. HG2 vs. HG3 were 94.3 vs. 89.3 vs. 80.0 %, and 100.0 vs. 97.8 vs. 85.7 %, respectively. Likewise, 10-year DFS and BCSS rates for NG1 vs. NG2 vs. NG3 were 95.0 vs. 87.4 vs. 80.1 %, and 100.0 vs. 96.2 vs. 88.3 %, respectively.

Hazard estimates by Cox's univariate and multivariate analyses

After Cox's univariate analyses, Ki-67 scores, regardless of the 3 cutoff values, HG, and NG, were all significant indicators of high recurrence risk in patients with luminal/HER2− breast cancers (Table 2). The hazard ratios (HaR) of recurrence in the high-Ki-67 groups were 2.74 [95 % confidence interval (95 % CI) 1.20–6.23], 1.97 (95 % CI 1.01–3.83), and 2.15 times (95 % CI 1.11–4.15) greater than those of the low-Ki-67 groups under the 10, 14, and 20 % Ki-67 LI cutoff values, respectively.

HG and NG were also powerful indicators for recurrence risk in these 369 patients. The HaR of HG3 group vs. HG1 or HG2 group was 2.40 (95 % CI 1.25–4.61), and the HaR of the NG3 group vs. NG1 or NG2 group was 2.51 (95 % CI 1.32–4.78).

By Cox's univariate analyses, Ki-67, regardless of cutoff value, HG, and NG were all significant indicators of high risk of death from breast cancer (Table 3). The HaRs in the high-Ki-67 group were 8.25 (95 % CI 1.08–63.09), 4.42 (95 % CI 1.23–15.83), and 4.21 times (95 % CI 1.46–12.14) higher than in the low-Ki-67 groups at every cutoff value.

HG and NG were also powerful indicators for risk of breast cancer death. HaR of HG3 group vs. HG1 or HG2 group was 11.28 (95 % CI 3.15–40.45), and the HaR for patients with NG3 tumors vs. NG1 or NG2 tumors was 8.50 (95 % CI 2.37–30.45).

Cox's multivariate analyses including Ki-67 LI and HG/NG for DFS and BCSS in these 369 patients produced values indicating an independent impact of Ki-67 LI, and grades fluctuated according to the Ki-67 LI cutoff values. The correlation of Ki-67 and HG with DFS was equally marginal under the 10 % cutoff value. Analyses by other combinations suggested that the impact of HG/NG on DFS was stronger than that of Ki-67 (Table 4). Under any Ki-67 cutoff value, the statistical impact of both HG and NG on BCSS was also stronger than that of Ki-67 (Table 5).

Ki-67 labeling index vs. grades

We analyzed correlations between Ki-67 LI and HG or NG in 369 luminal/HER2−, node-negative invasive breast cancers. Both HG and NG were strongly correlated with Ki-67 LI, regardless of the cutoff value of Ki-67 ($p < 0.0001$, each) (Table 6). There were a number of luminal/HER2− breast cancers that were HG1 or NG1 but had a high Ki-67 LI. From 104 HG1 tumors, 43 (19 %), 22 (21 %), and 7 (7 %) were classified as high-Ki-67 under the Ki-67 cutoff values of 10, 14, and 20 %, respectively. Likewise, in 156 NG1 tumors, 58 (37 %), 30 (19 %), and 10 (6 %) were classified as high-Ki-67 under the Ki-67 cutoff values of 10, 14, and 20 %, respectively. The percentage of HG1/high-Ki-67 tumors under the 10 % cutoff was higher in papillotubular-type invasive ductal carcinoma (IDC), IDC with a predominantly intraductal component, and mucinous carcinoma (36 of 183, 19.6 %) than in other histological types (7 of 184, 3.8 %) ($p < 0.001$). The percentage of NG1/high-Ki-67 tumors under the 10 % cutoff was also significantly higher in the former histological types (44 of 183, 24.0 %) than in other histological types (14 of 184, 7.6 %) ($p < 0.001$) (Table 7).

Ki-67 as a predictive marker for luminal subtypes

Among 409 patients with luminal node-negative invasive breast cancers, 248 (61 %) had tumors judged as high risk

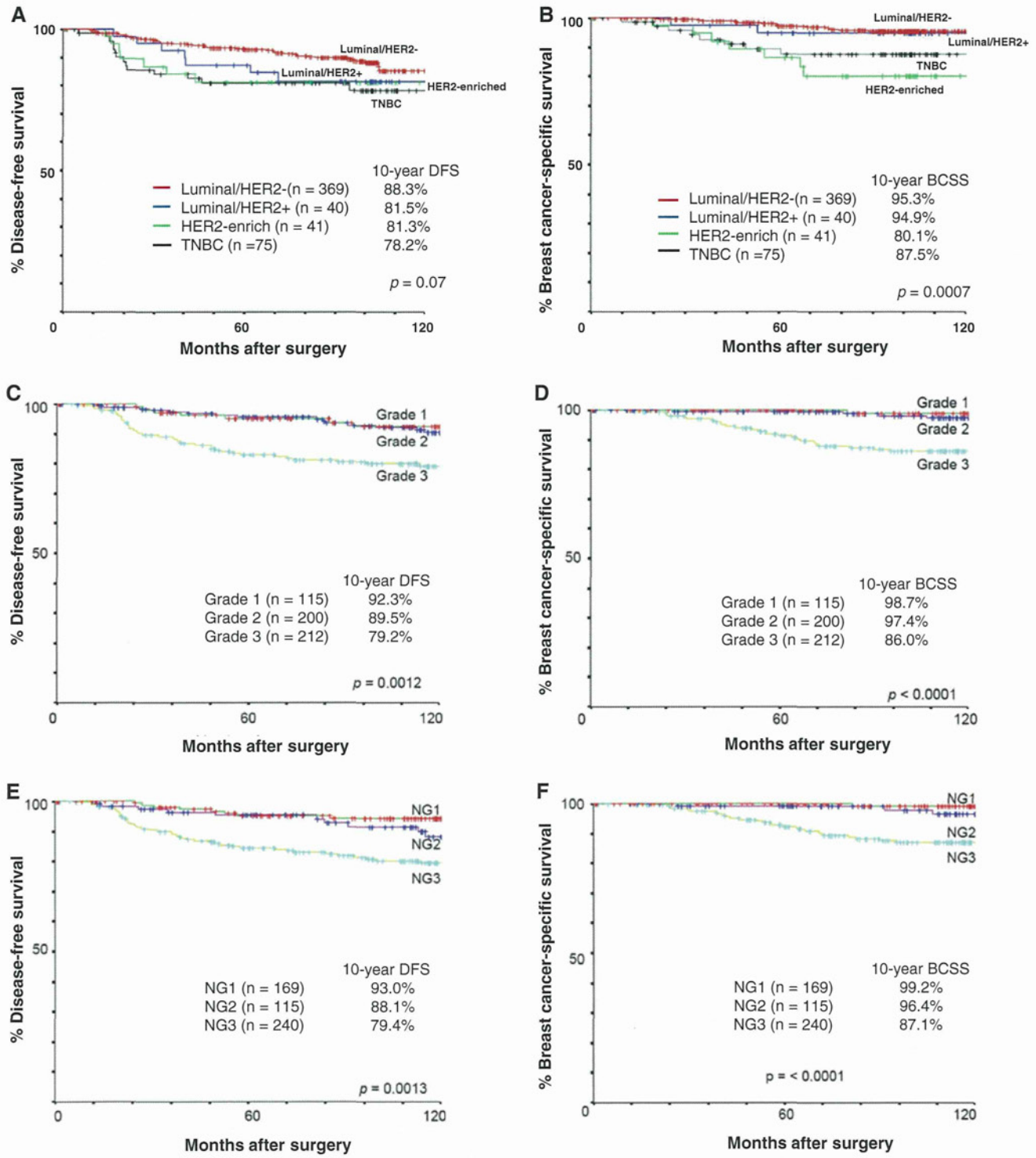

Fig. 1 Survival curves for 530 patients with node-negative invasive breast cancer. Survival curves for survival stratified by surrogate "intrinsic subtype" classification: **a** disease-free survival (DFS); **b** breast cancer-specific survival (BCSS). Curves for survival stratified by histological grade (**c**, DFS; **d**, BCSS). Curves for survival stratified by nuclear grade (**e**, DFS; **f**, BCSS)

by the NSAS-BC01 criteria. Under the Ki-67 cutoff value of 10 %, 58 were luminal A subtypes and 190 were luminal B subtypes. Both HER2−/high-Ki-67 and HER2+ cases were included in luminal B. Adjuvant chemotherapy with UFT or CMF was administered to 175 patients according to the NSAS-BC01 trial protocol. There were 73 patients who did not receive adjuvant chemotherapy; this group mostly comprised patients who refused chemotherapy, with the

remainder consisting of those who did not undergo chemotherapy because of organ dysfunction and advanced age.

DFS and BCSS curves for patient subgroups stratified by luminal A and B with/without adjuvant chemotherapy are shown in Fig. 4. There was no significant difference in DFS between patients with and without adjuvant chemotherapy in either luminal A ($p = 0.12$) or luminal B groups ($p = 0.94$). For BCSS, adjuvant chemotherapy did not affect survival in either luminal A ($p = 0.46$) or luminal B ($p = 0.69$) types.

Discussion

The surrogate intrinsic subtyping of breast cancer recommended by the St. Gallen consensus panels (2011) is now used for the selection of adjuvant systemic therapies for individual patients [10]. The importance of intrinsic subtypes is evident because they are highly expected to respond to appropriate adjuvant endocrine therapy, chemotherapy, and/or HER2-targeted therapy. However, it remains undetermined whether the criteria for luminal A and B in the luminal/HER2− group using the 14 % cutoff Ki-67 value recommended by the consensus panels are universally optimal.

As mentioned, although Ki-67 LI is an established prognostic factor for patients with HR-positive breast cancers, preferred cutoff values vary among studies [11, 12, 14, 15]. Cheang et al. [11] concluded that the best cutoff value for Ki-67 for distinction between luminal A and luminal B tumors was 13.25 %, based on a correlation with "gold standard" gene expression profiling data. Survival curves differed significantly between the high-risk and low-risk patient subgroups determined by a Ki-67 cutoff value of 14 % in patient groups who received adjuvant hormonal therapy and those who did not [11]. On the other hand, in the BIG 1-98 trial, Viale et al. [14] showed that Ki-67 LI with an 11 % of cutoff value was a prognostic factor in postmenopausal women with HR-positive breast cancer, whereas in IBCSG trials, the Ki-67 LI with a 19 % cutoff value was found to be an independent prognostic factor [15].

From the present study, Ki-67 cutoff values of 10, 14, and 20 % could all be applied to differentiate patients with luminal/HER2−, node-negative invasive breast cancers into 2 distinct groups based on clinical outcome. The highest 10-year DFS and BCSS rates in low-Ki-67 subgroups, 93.6 and 99.3 %, respectively, were found at Ki-67 cutoff values under 10 %, and the lowest 10-year DFS and BCSS rates in high-Ki-67 subgroups, 80.1 and 88.9 %, respectively, were recorded under the 20 % Ki-67 cutoff value. By Cox univariate analyses, HaRs of recurrence and breast cancer death in the high-Ki-67 patients compared to

Table 1 Clinicopathological features of 369 patients with luminal/HER2-negative, node-negative invasive breast cancers

	No. of patients (%) ($N = 369$)
Age	
≤50	146 (40)
>50	223 (60)
Menopause	
Premenopausal	152 (41)
Postmenopausal	217 (59)
Invasive tumor size (cm)	
≤2.0	276 (75)
>2.0 to ≤5.0	89 (24)
>5.0	4 (1)
Histology	
Invasive ductal carcinoma (IDC)	302 (82)
Mucinous carcinoma	26 (7)
Invasive lobular carcinoma	22 (6)
IDC with predominantly intraductal component	17 (4)
Others (medullary, tubular)	2 (1)
Ki-67 (≥10 %)	
Positive	221 (60)
Negative	148 (40)
Ki-67 (≥14 %)	
Positive	163 (44)
Negative	206 (56)
Ki-67 (≥20 %)	
Positive	87 (24)
Negative	282 (76)
Histological grade	
1	104 (28)
2	175 (47)
3	88 (24)
Unknown	2 (1)
Nuclear grade	
1	156 (42)
2	103 (28)
3	109 (30)
Unknown	1 (0)
Lymphovascular invasion	
Positive	138 (37)
Negative	230 (62)
Unknown	1 (0)
Adjuvant chemotherapy	
Yes	153 (41)
CMF	100 (65)[a]
UFT	52 (34)[a]
CEF	1 (1)
No	217 (59)
Hormone therapy (tamoxifen)	
Yes	187 (51)
No	182 (49)

[a] One patient received both CMF and UFT

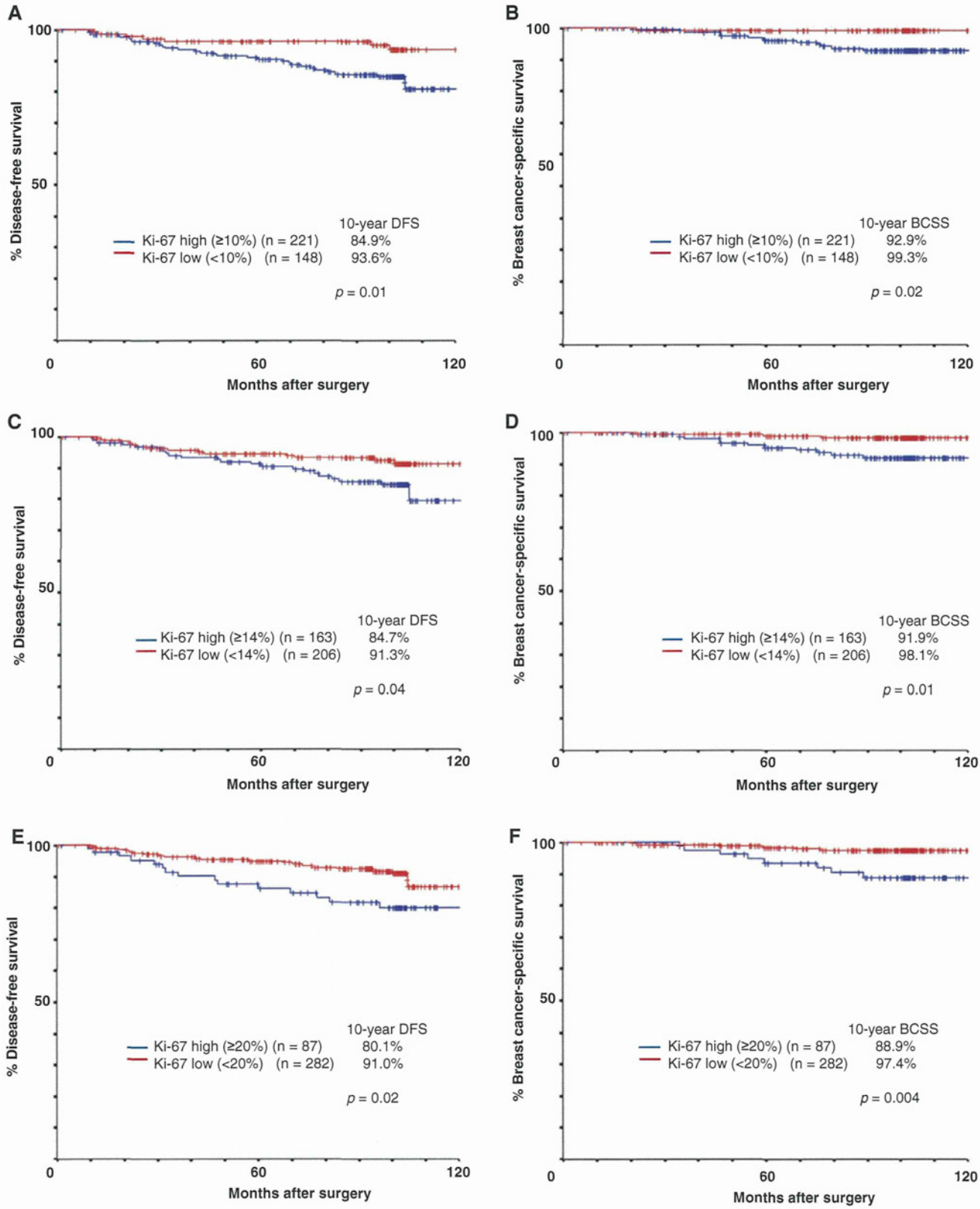

Fig. 2 Survival curves for 369 patients with luminal/HER2-negative, node-negative invasive breast cancer. Survival curves stratified by a 10 % cutoff value of Ki-67 labeling index (LI): **a** disease-free survival (DFS), **b** breast cancer specific survival (BCSS). Survival curves stratified by a 14 % cutoff value of Ki-67 LI (**c**, DFS; **d**, BCSS). Survival curves stratified by a 20 % of cutoff value of Ki-67 LI (**e**, DFS; **f**, BCSS)

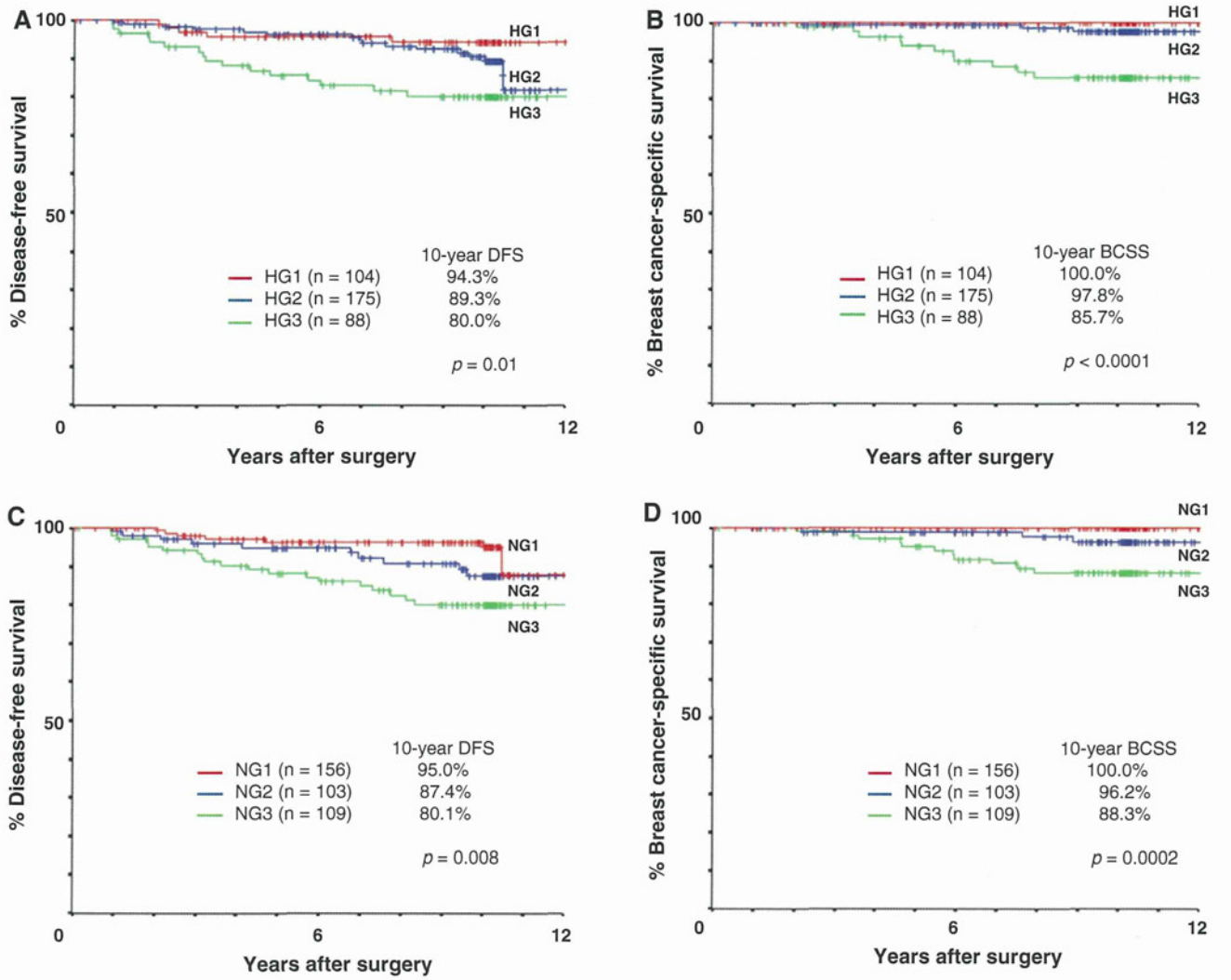

Fig. 3 Survival curves for 369 patients with luminal/HER2-negative, node-negative invasive breast cancer. Survival curves stratified by histological grade (HG): **a** disease-free survival (DFS); **b** breast cancer-specific survival (BCSS). Survival curves stratified by nuclear grade (NG) (**c**, DFS; **d**, BCSS)

the low-Ki-67 patients were largest under the 10 % Ki-67 cutoff, although these ratios were no more than relative values.

The ratio of patients with high-Ki-67 and low-Ki-67 luminal/HER2− breast cancers substantially fluctuated according to the Ki-67 cutoff values. The ratios of high-risk (high-Ki-67) subgroups in luminal/HER2− cases under the Ki-67 cutoff values of 10, 14, and 20 % were approximately 2/3, 1/2, and 1/4, respectively. In the Oncotype DX assay [20], the ratios of low, intermediate, and high-risk score cases were 52, 22, and 27 %, and 10-year distant recurrence rates were 6.8, 14.3, and 30.5 %, respectively [20]. In Japanese cases, the proportion and 10-year distant recurrence rates of high-risk score cases by Oncotype DX were similar [21]. From these comparisons, it might be reasonable that a 14 or 20 % Ki-67 cutoff value might be more compatible than the 10 % cutoff value in terms of attempting to predict both recurrence rate and patient distribution in Japanese populations.

Cheang et al. [11] reported that 10-year DFS and BCSS rates in node-negative luminal A (Ki-67 LI <14 %) breast cancers treated with adjuvant tamoxifen only were 85 % and 91 %, respectively. These rates were very similar to those determined for the luminal B (Ki-67 LI ≥14 % or ≥10 %)/HER2− subtype in this study (84.7–84.9 % DFS and 91.9–92.9 % BCSS).

HG and NG were also correlated with DFS and BCSS in the patients with luminal/HER2−, node-negative invasive breast cancers, and appeared to be alternative powerful and simple methods to evaluate recurrence and the risk of recurrence and death in those patients. Patients with HG1/NG1 tumors had an excellent DFS rate (94.2–96.3 %), and those with HG1/NG1 and HG2/NG2 tumors also showed excellent BCSS rates (100 % and 96.2–97.8 %,

Table 2 Relative recurrence risk according to clinicopathological parameters in 369 patients with luminal/HER2-negative, node-negative invasive breast cancers calculated by Cox's univariate model analyses

Parameter	Hazard ratio	95 % CI	*p* value
Age (≤50 vs. >50 years)	0.94	0.49–1.82	0.86
Menopause (pre vs. post)	1.07	0.56–2.05	0.83
Invasive tumor size (>2.0 vs. ≤2.0 cm)	1.55	0.79–3.05	0.20
Histology (IDC vs. others)	1.51	0.59–3.87	0.40
Histological grade (3 vs. 1, 2)	2.40	1.25–4.61	0.008
Nuclear grade (3 vs. 1, 2)	2.51	1.32–4.78	0.005
Lymphovascular invasion (+ vs. −)	0.71	0.35–1.44	0.34
Ki-67 ≥10 vs. <10 %	2.74	1.20–6.23	0.02
Ki-67 ≥14 vs. <14 %	1.97	1.01–3.83	0.05
Ki-67 ≥20 vs. <20 %	2.15	1.11–4.15	0.02

CI confidence interval, *IDC* invasive ductal carcinoma

Table 3 Relative risk of breast cancer death according to clinicopathological parameters in 369 patients with luminal/HER2-negative, node-negative invasive breast cancers calculated by Cox's univariate model analyses

Parameter	Hazard ratio	95 % CI	*p* value
Age (≤50 vs. >50 years)	1.02	0.35–2.93	0.98
Menopause (pre vs. post)	0.81	0.29–2.32	0.81
Invasive tumor size (>2.0 vs. ≤2.0 cm)	2.08	0.72–6.00	0.17
Histology (IDC vs. others)	3.06	0.40–23.38	0.28
Histological grade (3 vs. 1, 2)	11.28	3.15–40.45	<0.001
Nuclear grade (3 vs. 1, 2)	8.50	2.37–30.45	0.001
Lymphovascular invasion (+ vs. −)	0.91	0.31–2.72	0.87
Ki-67 ≥10 vs. <10 %	8.25	1.08–63.09	0.04
Ki-67 ≥14 vs. <14 %	4.42	1.23–15.83	0.02
Ki-67 ≥20 vs. <20 %	4.21	1.46–12.14	0.008

CI confidence interval, *IDC* invasive ductal carcinoma

respectively). Furthermore, high Ki-67 LI with any cutoff value was strongly correlated with HG and NG, and when these parameters were included in the multivariate analyses, the impact of HG/NG was stronger than Ki-67 LIs. Another advantage of determining HG or NG to assist with guiding treatment and predicting risk consists in the simplicity of method, i.e., examination of hematoxylin–eosin stained slides only, without Ki-67 measurement, although inter-rater disagreement may present a challenge [22–24].

It is reasonable to consider that adjuvant chemotherapy could be omitted for Japanese patients with luminal

Table 4 Independent relative recurrence risk according to the parameters that were significant by univariate analyses in 369 patients with luminal/HER2-negative, node-negative invasive breast cancers calculated by Cox's multivariate model analysis

Multivariate analysis	Hazard ratio	95 % CI	*p* value
Ki-67 (≥10 vs. <10 %)	2.31	0.99–5.41	0.05
Histological grade (3 vs. 1, 2)	1.95	0.99–3.82	0.05
Ki-67 (≥14 vs. <14 %)	1.54	0.75–3.16	0.24
Histological grade (3 vs. 1, 2)	2.03	1.00–4.10	0.05
Ki-67 (≥20 vs. <20 %)	1.61	0.77–3.39	0.21
Histological grade (3 vs. 1, 2)	1.95	0.94–4.06	0.08
Ki-67 (≥10 vs. <10 %)	2.18	0.92–5.15	0.08
Nuclear grade (3 vs. 1, 2)	2.02	1.03–3.95	0.04
Ki-67 (≥14 vs. <14 %)	1.48	0.72–3.06	0.29
Nuclear grade (3 vs. 1, 2)	2.14	1.06–4.33	0.03
Ki-67 (≥20 vs. <20 %)	1.57	0.75–3.26	0.23
Nuclear grade (3 vs. 1, 2)	2.09	1.02–4.28	0.05

Table 5 Independent relative risk of breast cancer-specific survival according to the parameters that were significant by univariate analyses in 369 patients with luminal/HER2-negative, node-negative invasive breast cancers calculated by Cox's multivariate model analysis

Multivariate analysis	Hazard ratio	95 % CI	*p* value
Ki-67 (≥10 vs. <10 %)	4.29	0.54–34.12	0.17
Histological grade (3 vs. 1, 2)	8.36	2.28–30.72	0.001
Ki-67 (≥14 vs. <14 %)	1.95	0.50–7.60	0.33
Histological grade (3 vs. 1, 2)	8.77	2.26–34.11	0.002
Ki-67 (≥20 vs. <20 %)	1.69	0.54–5.29	0.37
Histological grade (3 vs. 1, 2)	8.99	2.27–35.68	0.002
Ki-67 (≥10 vs. <10 %)	4.32	0.54–34.82	0.17
Nuclear grade (3 vs. 1, 2)	6.02	1.62–22.32	0.007
Ki-67 (≥14 vs. <14 %)	2.18	0.56–8.48	0.26
Nuclear grade (3 vs. 1, 2)	6.31	1.62–24.56	0.008
Ki-67 (≥20 vs. <20 %)	2.00	0.64–6.26	0.23
Nuclear grade (3 vs. 1, 2)	6.37	1.61–25.14	0.008

B/HER2− tumors, just as adjuvant chemotherapy can be omitted from postsurgical management of Western breast cancer patients with node-negative invasive luminal A tumors. It is also reasonable to consider the withdrawal of adjuvant chemotherapy for Japanese patients with node-negative invasive HG1/NG1 or HG2/NG2 tumors. Further

Table 6 Correlation of Ki-67 labeling index with histological/nuclear grades in 369 luminal/HER2-negative, node-negative invasive breast cancers

Ki-67 labeling index	Number of tumors (%)								
	Total	Histological grade			*p* value	Nuclear grade			*p* value
		1	2	3		1	2	3	
Low (<10 %)	148	61 (41)	73 (49)	14 (10)	<0.0001	98 (66)	33 (22)	17 (12)	<0.0001
High (≥10 %)	221[a]	43 (19)	102 (47)	74 (33)		58 (26)	70 (32)	92 (42)	
Low (<14 %)	206[b]	82 (40)	102 (50)	21 (10)	<0.0001	126 (61)	52 (25)	28 (14)	<0.0001
High (≥14 %)	163[c]	22 (13)	73 (45)	67 (41)		30 (18)	51 (31)	81 (50)	
Low (<20 %)	282[b]	97 (34)	144 (51)	40 (14)	<0.001	146 (52)	81 (29)	55 (19)	<0.0001
High (≥20 %)	87[c]	7 (8)	31 (36)	48 (55)		10 (11)	22 (25)	54 (62)	
Total	369[a]	104	175	88		156	103	109	

[a] The number includes 2 cases with unknown histological grade and 1 case with unknown nuclear grade

[b] The number includes 1 cases with unknown histological grade

[c] The number includes 1 cases with unknown histological grade and 1 case with unknown nuclear grade

Table 7 Discordance between high Ki-67 labeling index (10 % cutoff) and histological grade 1/nuclear grade 1 in various histological types of luminal/HER2-negative breast cancers

Histological type	Total	No. of tumors with Ki-67 ≥10 % (%)	
		Histological grade 1 ($N = 43$)	Nuclear grade 1 ($N = 58$)
Invasive ductal carcinoma (IDC)			
Papillotubular	140	20 (14)	22 (16)*
Solid-tubular	70	3 (4)	4 (6)
Scirrhous	91	2 (2)	6 (7)
Unknown	1	0 (0)	0 (0)
Mucinous carcinoma	26	9 (35)	12 (46)*
IDC with a predominantly intraductal component	17	7 (41)	10 (59)*
Invasive lobular carcinoma	22	2 (9)	4 (18)
Total	367	43 (12)	58 (16)

* $p < 0.001$ between the groups, including papillotubular IDC, mucinous carcinoma, IDC with a predominantly intraductal component, and others

studies will be needed to determine the benefit of adjuvant chemotherapy in luminal B/HER2− (or HG1/NG1 or HG2/NG2), node-negative invasive breast cancers in Far Eastern countries.

While Ki-67 LI was strongly correlated with HG or NG, under the Ki-67 cutoff values of 10 or 14 %, a relatively large number of HG1 or NG1 tumors were classified as high Ki-67 LI. In particular, this discordance was observed relatively frequently in 3 histological types, i.e., papillotubular-type IDC, IDC with a predominantly intraductal component, and mucinous carcinoma. Papillotubular-type IDC, a classification used only in Japan [17], is composed, in a substantial part, of cases of "non-classic" tubular carcinoma and invasive cribriform carcinoma described by the World Health Organization classification [25]. In the guidelines of the National Comprehensive Cancer Network and in St. Gallen 2011, mucinous carcinoma is included in a low-risk group of tumors for which endocrine therapy only is recommended as the adjuvant therapy [10, 26]. IDC with a predominantly intraductal component is an early invasive carcinoma group which is overwhelmingly (≥80 %) intraductal and contains foci of stromal invasion [27]. Patients with this tumor type are known to have excellent prognoses, especially when invasive tumor size is <0.5 cm [28].

Paik et al. [29] have shown that the Oncotype DX recurrence risk score was able to identify patients with high-risk luminal breast cancers for whom adjuvant chemotherapy can be beneficial. Viale et al. [15] did not find support for such benefit when using Ki-67 LI values. We could not obtain positive data for the predictive role of Ki-67 from the present study, particularly when focused on the group of patients with luminal A/HER2− tumors. In this group, the patient subgroup that did not receive chemotherapy tended to show a higher rate of DFS than the subgroup that chose adjuvant chemotherapy, although the

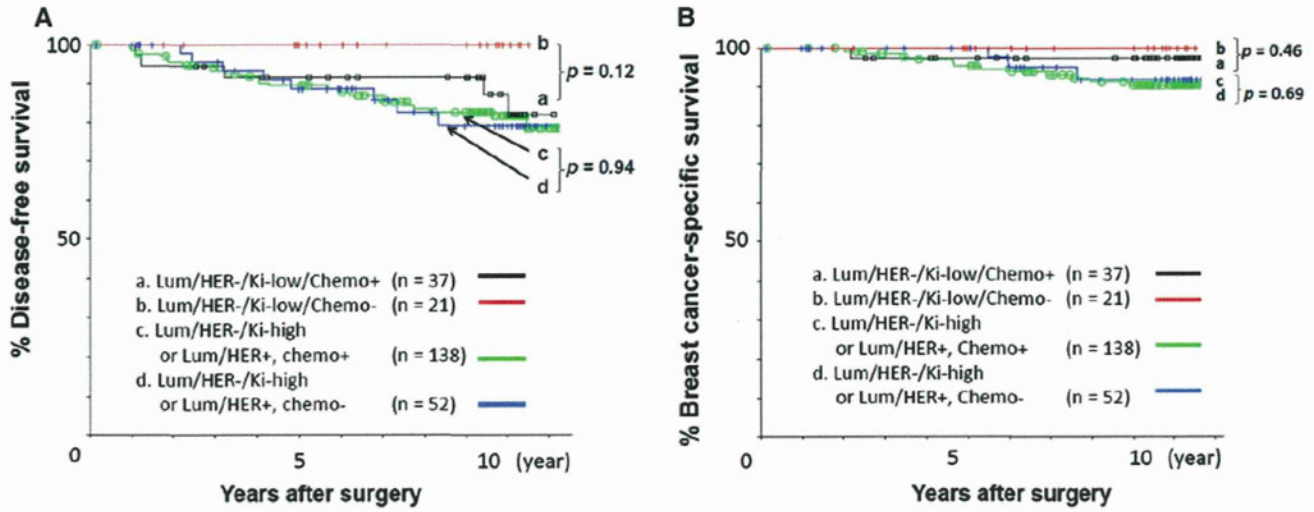

Fig. 4 Survival curves for 409 patients with luminal node-negative invasive breast cancer. **a** Disease-free survival curves, and **b** breast cancer-specific survival curves stratified by luminal subtype and with and without adjuvant chemotherapy. Curves for patient groups with luminal/HER2−/low-Ki-67 (<10 %) tumors with (**a**) and without (**b**) chemotherapy. Curves for patient groups with luminal/HER2−/high-Ki-67 (≥10 %) or luminal/HER2 + tumors with (**c**) and without (**d**) chemotherapy. There were no significant differences between patient groups with and without adjuvant chemotherapy in either luminal A or B groups

difference was not significant. There might have been bias in patient selection for adjuvant therapies, especially among the group of low-risk patients who were not candidates for the NSAS-BC01 protocol.

In the present study, we introduced tissue microarrays, and this point might be flaw when intratumor heterogeneity of Ki-67 immunoreaction is taken into account. Nonetheless, we enucleated two 2-mm cores, including the invasive front, from each tumor tissue in order to reproduce the condition of routine diagnosis as much as possible. The evaluation of Ki-67 LI by two observers would have contributed to improve the reliability of the data.

In conclusion, we confirmed the prognostic utility of 3 different cutoff values Ki-67 LI, HG, and NG for patients with luminal/HER2−, node-negative invasive breast cancers. Any of the Ki-67 LI values, regardless of cutoff value, could be applicable for the classification of high-risk and low-risk luminal/HER2−, node-negative invasive breast cancers. The 10 % cutoff value for Ki-67 LI revealed the highest RR, but 14 or 20 % appeared better in terms of determining the proportion of high-risk patients. Luminal A/B subtyping according to Ki-67 LI or HG/NG values, in combination with histological type, appeared to be able to create an optimum risk estimation system for patients with luminal/HER2−, node-negative invasive breast cancers in Japan. We could not determine the predictive value of luminal A/B subtyping in the current study. For this, another study examining a larger cohort will be necessary to make further conclusions.

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Conflict of interest The authors declare that they have no conflict of interest.

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ORIGINAL ARTICLE

Marked lymphovascular invasion, progesterone receptor negativity, and high Ki67 labeling index predict poor outcome in breast cancer patients treated with endocrine therapy alone

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Abstract
Purpose Whether postoperative chemotherapy should be added to endocrine therapy or not is an important issue in patients with hormone receptor-positive and human epidermal growth factor receptor (HER)2-negative breast cancer. To identify patients who should be treated with additional chemotherapy, prognostic factors were investigated in breast cancer patients postoperatively treated with endocrine therapy alone.
Patients and methods Tumor samples and clinicopathological data were collected from patients who underwent curative surgery and were postoperatively treated with endocrine therapy alone between 1999 and 2003 in three different institutes. Expression levels of estrogen receptor (ER), progesterone receptor (PgR), and HER2 in primary tumors were centrally retested. Patients with ER-negative and/or HER2-positive tumors and/or with unknown nodal status were excluded from the study subjects. Immunohistochemical analysis of Ki67, HER1, insulin-like growth factor-1 receptor, and aldehyde dehydrogenase-1 was also performed. Prognostic factors were investigated by univariate and multivariate analyses.
Results A total of 261 patients were the subjects of this study. The median age was 59 years old, the mean tumor size was 1.9 cm, the node-positive rate was 20 %, and 65 % received tamoxifen alone. Distant metastases were observed in 11 patients at a median follow-up of 98 months, and four patients had died of breast cancer at a median follow-up of 99 months. Univariate analysis showed that marked lymphovascular invasion (LVI), PgR negativity, high Ki67 labeling index (LI), and high nuclear grade were significantly worse prognostic factors for distant metastasis. Multivariate analysis revealed that marked LVI [hazard ratio (HR) 21.8] and PgR negativity (HR 10.3) were independently worse prognostic factors for distant metastasis, respectively. Multivariate analysis also revealed that marked LVI (HR 287.3), PgR negativity (HR 25.1), and high Ki67 LI (HR 19.6) were independently worse prognostic factors for breast cancer-specific death, respectively.
Conclusions The results of this multi-institute cohort study indicated that endocrine therapy alone could not prevent distant metastasis in breast cancer patients with PgR-negative tumors and/or with tumors showing marked LVI or high cell proliferation. These patients may need postoperative adjuvant chemotherapy in addition to endocrine therapy.

Keywords Endocrine therapy · Distant metastasis · Lymphovascular invasion · Progesterone receptor · Ki67 labeling index

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Introduction

According to the surrogate definition of intrinsic subtypes advocated at the St. Gallen Consensus Conference held in 2011, breast cancer is a heterogeneous disease including at least five distinct subtypes, luminal A subtype [hormone receptor (HR)-positive/human epidermal growth factor receptor (HER)2 negative/low Ki67 labeling index (LI)], luminal B/HER2-negative subtype (HR positive/HER2 negative/high Ki67 LI), luminal B/HER2-positive subtype (HR positive/HER2 positive), HER2-positive/nonluminal subtype (HR negative/HER2 positive), and triple-negative subtype (HR negative/HER2 negative). Whether postoperative adjuvant chemotherapy should be added to endocrine therapy or not is a major clinical issue in patients with breast tumors of luminal A or luminal B subtype [1]. To answer this important question, not only conventional clinicopathological factors but also newly developed molecular biomarkers have been investigated in the past decade; however, a definitive answer to this question has not yet been provided [2].

According to the meeting highlight paper of the St. Gallen Consensus Conference 2009, ER/progesterone receptor (PgR) status, nodal status, histological grade, tumor size, extent of peritumoral vessel invasion, and cell proliferation rate such as Ki67 LI were indicated to be important clinicopathological factors for selection of postoperative chemotherapy additional to endocrine therapy in patients with ER-positive, HER2-negative breast cancer [3]. To confirm this and also to explore new additional prognostic biomarkers, we conducted a retrospective multi-institute cohort study to investigate prognostic factors in early breast cancer patients who were postoperatively treated with endocrine therapy alone.

Patients and methods

Patients

At first, 292 breast cancer patients were selected as the study subjects. All patients with invasive breast cancer had undergone curative surgery and received postoperative adjuvant endocrine therapy alone at three different hospitals (Kawasaki Medical School Hospital, National Hospital Organization Shikoku Cancer Center, and National Cancer Center Hospital) between January 1999 and December 2003. Secondly, 31 patients with ER-negative and/or HER2-positive tumors and/or with unknown nodal status were excluded to obtain more accurate prognostic factors for patients with endocrine-responsive tumors. Clinicopathological data and ten unstained 5-μm sections of each formalin-fixed paraffin-embedded tumor sample were collected for immunohistochemical (IHC) analysis. The study protocol was approved by the Institutional Review Boards of Kawasaki Medical School, National Hospital Organization Shikoku National Cancer Center, and the National Cancer Center, respectively.

Histopathological parameters

ER and PgR status were retested by IHC at the Department of Pathology, Kawasaki Medical School using anti-ER monoclonal antibody 1D5 (1:50 dilution; Dako, Glostrup, Denmark) and anti-PgR monoclonal antibody PgR636 (1:800 dilution; Dako), respectively. The cutoff value for both ER and PgR positivity was 1 % [4]. HER2 expression was also retested at the same department by IHC using HercepTest (Dako). The results were evaluated according to the criteria of the HercepTest. These biomarkers were evaluated by a pathologist (N.K.) in blinded manner.

Histologic grading was evaluated according to the modified Bloom and Richardson method by Elston and Ellis (Nottingham's grading system) [5]. Lymphovascular invasion (LVI) was assessed at the periphery of the tumor by standard pathological evaluation using hematoxylin and

Table 1 Clinicopathological characteristics of the study subjects

Variable	Category	Number	%
Age (years)	>50	184	70.5
	≤50	77	29.5
Surgery	Breast-conserving	155	59.4
	Mastectomy	106	40.6
Nodal status	Negative	210	80.5
	Positive	51	19.5
Tumor size	≤2 cm	189	72.4
	>2 cm	71	27.2
	Unknown	1	0.4
ER	Positive	261	100
	Negative	0	0
PR	Positive	192	73.6
	Negative	69	26.4
Grade	I	90	34.5
	II	130	49.8
	III	41	15.7
HER2	0	163	62.5
	1+	98	37.5
Endocrine therapy	Tamoxifen	170	65.1
	Aromatase inhibitor	41	15.7
	LH–RH agonist + tamoxifen	33	12.6
	Tamoxifen → aromatase inhibitor	12	4.6
	LH–RH agonist	5	1.9

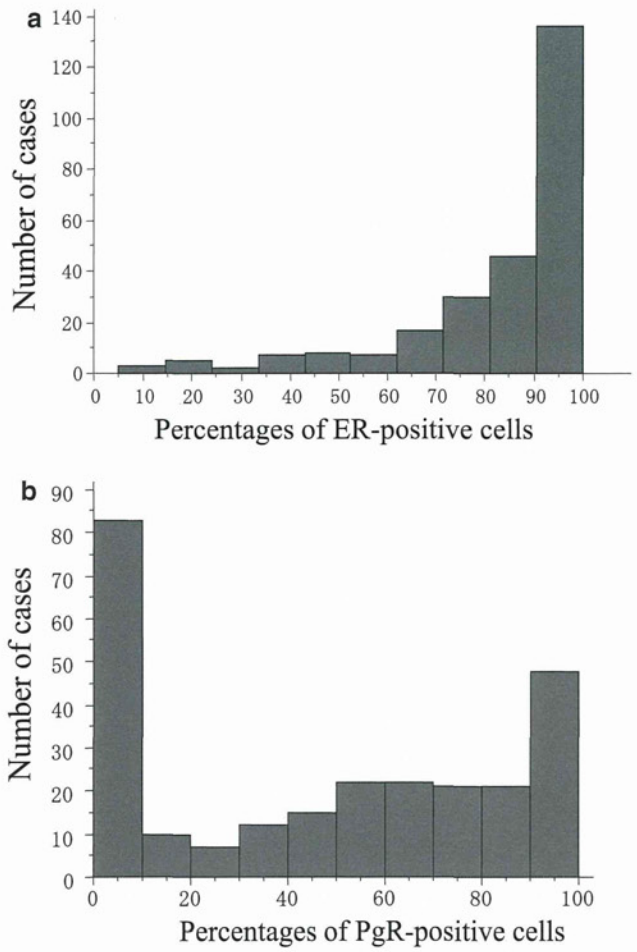

Fig. 1 Histogram of percentages of positively stained tumor cells for ER (**a**) and PgR (**b**)

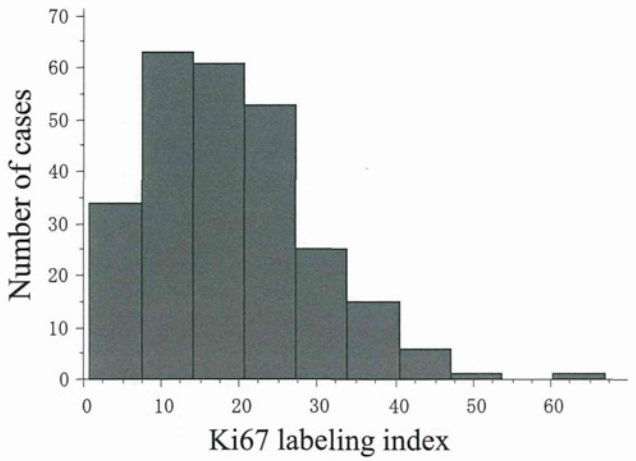

Fig. 2 Histogram of Ki67 LI

Table 2 Immunohistochemical analyses of exploratory biomarkers

Variable	Category	Number	%
Ki67 labeling index	<30 %	219	83.9
	≥30 %	40	15.3
	Unknown	2	0.8
HER1	Negative	239	91.6
	Positive	22	8.4
IGFR	Negative	64	24.5
	Weak	171	65.5
	Strong	26	10.0
ALDH1	<10 %	111	42.5
	10–50 %	115	44.1
	>50 %	34	13.0
	Unknown	1	0.4

eosin staining. Special immunostaining markers were not used to identify endothelial cells as part of the routine pathological evaluation. The degree of LVI was semi-quantitatively scored as mild (one positive lymph vessel within the specimen), moderate (two or three positive lymph vessels), and marked (four or more positive lymph vessels) in the section of maximum tumor diameter [6].

To explore additional prognostic markers, the Ki67 LI and the expression levels of insulin-like growth factor-1 receptor (IGF-1R), epidermal growth factor receptor (EGFR, HER1), and a putative cancer stem cell marker, aldehyde dehydrogenase (ALDH) 1, were measured by IHC. Representative paraffin blocks were extracted, and thin sections of 5 μm were cut and placed on Matsunami adhesive slide (MAS)-coated glass slides (Matsunami, Osaka, Japan). After deparaffinization and hydration, they were place in a hot bath of target retrieval solution, pH 9.0 (Dako) at 95 °C for 40 min. Endogenous peroxidase activity was quenched by incubation in 3 % hydrogen peroxide for 5 min. The sections were then incubated at 4 °C overnight with primary antibodies for Ki67 and IGF-1R, or for 30 min at room temperature with the antibody for ALDH1. Primary antibodies used were mouse monoclonal anti-Ki67 antibody (clone MIB-1, 1:50 dilution; Dako), anti-IGF-1R antibody (clone 24–31, 1:50 dilution; Millipore, Temecula, CA, USA), and anti-ALDH1 antibody (clone 44/ALDH, 1:50 dilution; BD Biosciences, Franklin Lakes, NJ, USA). The samples were then washed with Tris-buffered saline, and the color was developed using the EnVision+ system (Dako), according to the manufacturer's instructions. The chromogen used was diaminobenzidine. Sections were counterstained with hematoxylin and mounted. EGFR pharmDx kit (Dako) was used for evaluation of HER1 expression. Samples in which the primary antibody was omitted served as negative controls.

MIB-1 staining for Ki67 was examined with 4× and 10× object lenses to identify the area of most intense staining ("hot spot"). Scoring was performed by counting at least 500 malignant invasive tumor cells in high-power fields with a 40× object lens. All brown-stained nuclei, regardless of staining intensity, were counted as positive [7].

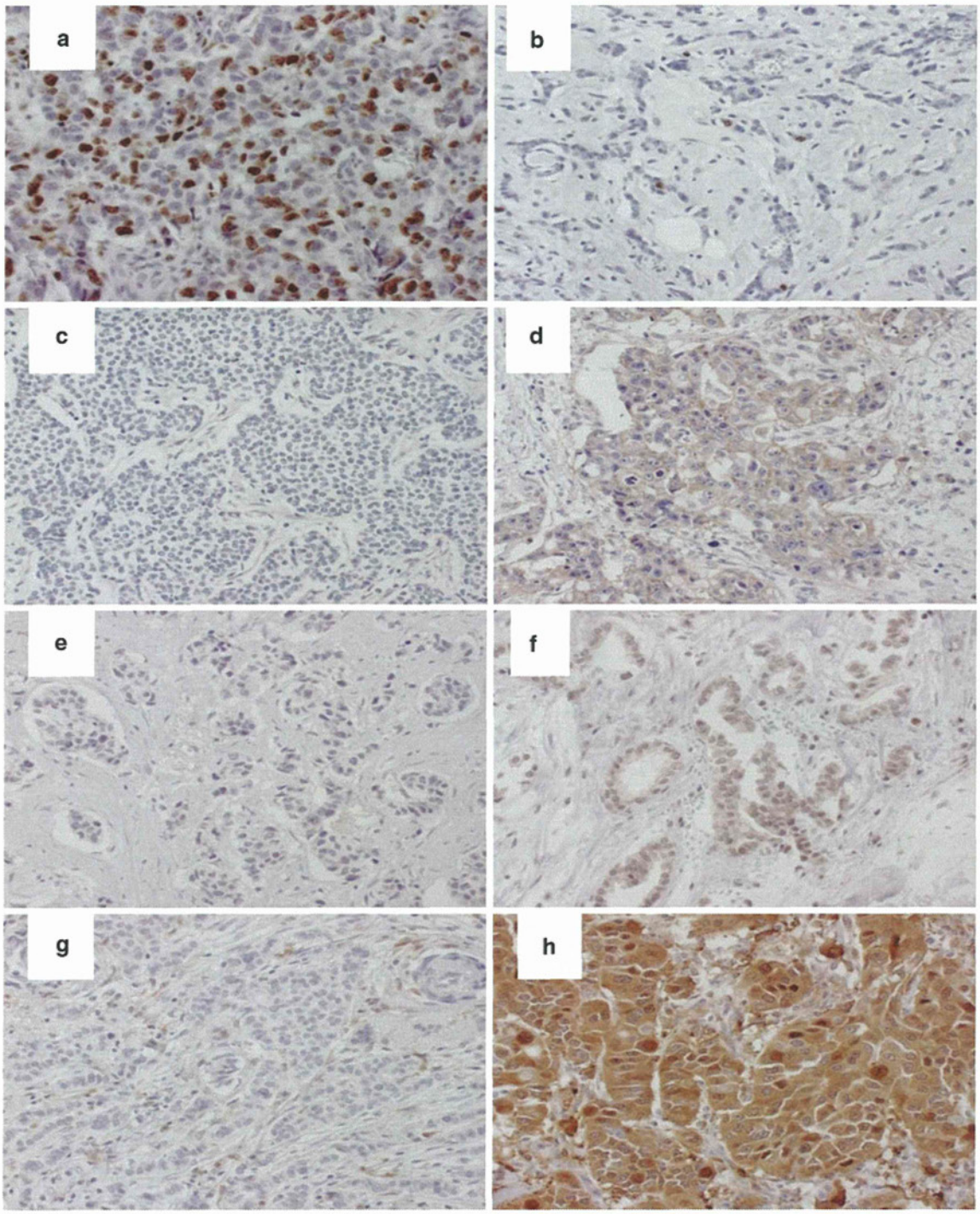

Fig. 3 Representative IHC staining of tumor samples for Ki67 (**a** high, **b** low), HER1 (**c** negative, **d** positive), IGF-1R (**e** negative, **f** strong), and ALDH1 (**g** negative, **h** strong)

Statistical analysis

The χ^2 test, Fisher's exact test, and analysis of variance (ANOVA) were used to analyze whether significant differences existed in the clinicopathological factors among the patients. Distant relapse-free survival (DRFS) and breast cancer-specific survival (BCSS) curves were drawn using the Kaplan–Meier method, and the differences were assessed by the log-rank test [8]. We also analyzed prognostic factors for distant recurrence and cancer-related death using multivariate analyses with the Cox proportional hazard regression model. $P < 0.05$ was considered significant. All analyses were performed using StatView version 5.0.

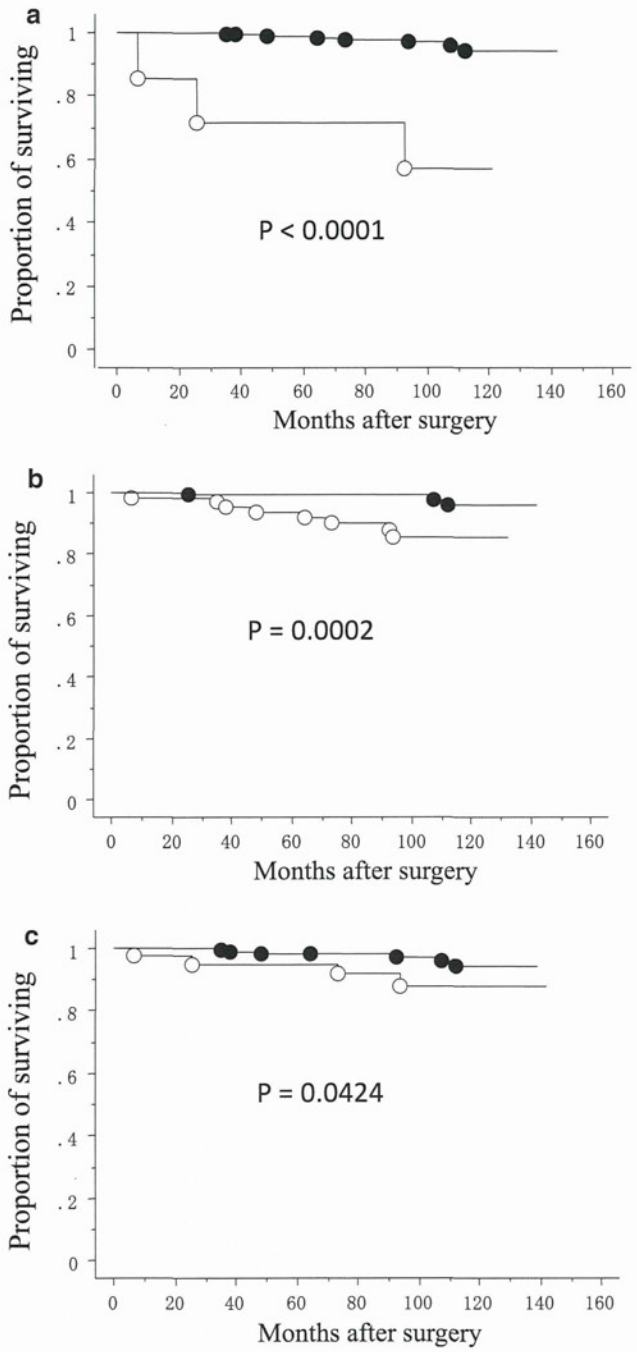

Fig. 4 DRFS according to LVI (**a**), PgR status (**b**), and Ki67 LI (**c**). **a** *Open circles*, marked LVI, *filled circles* without marked LVI. **b** *Open circles* PgR-negative tumors, *filled circles* PgR-positive tumors. **c** *Open circles* tumors showing high Ki67 LI, *filled circles* tumors not showing high Ki67 LI

Results

Clinicopathological characteristics of the study subjects

The median patient age was 59 years; 59 % received breast-conserving surgery; the mean tumor size was 1.9 cm; and 80 % were node negative. All patients had ER-positive breast tumors. Tamoxifen alone was given to 170 (65 %) of the patients (Table 1). At the median follow-up period of 98 months, 11 patients had distant recurrence (six in bone, two in bone and lung, two in liver, and one in lung). At the median follow-up period of 99 months, four patients had died of breast cancer.

The distribution of the percentages of hormone receptor-positive cells in each tumor sample is shown in Fig. 1. The percentages of ER-positive cells were over 90 % in most cases (Fig. 1a), but those of PgR-positive cells were widely distributed from 0 to 100 % (Fig. 1b). These findings indicate that the majority of tumors were strongly positive for ER.

The distribution of Ki67 LI is shown in Fig. 2. The median Ki67 LI was 17.6 % (range 0.8–67.0 %). The Ki67 LI correlated well with the histological grade. The average Ki67 LIs were 14.7 % for grade I, 19.1 % for grade II, and 27.0 % for grade III. When the cutoff values of Ki67 LI were changed, the value of 30 % discriminated patients with poor or good prognosis; therefore, the cutoff value of Ki67 LI was defined as 30 % in this study (Table 2).

For HER1, any completely membranous staining was considered positive, regardless of its intensity or proportion [9]. HER1 expression was observed in only 8.4 % of cases (22 out of 261, Table 2). The mean Ki67 LI was significantly higher in HER1-positive tumors than in HER1-negative tumors (25.7 versus 18.2 %, $P = 0.0012$).

For IGF-1R, cytoplasmic staining was scored by the Histoscore system, multiplying the products of the percentage of cells stained at a given staining intensity (0–100) by the staining intensity score (0, none; 1, weak; 2, moderate; 3, intense): 0–10 points were considered as negative, 11–100 points as weak, 101–200 points as moderate, and 201–300 points as strongly positive [10]. The IGF-1R expression was categorized as negative in 64 cases (24.5 %), weak in 171 cases (65.5 %), and strong in 26 cases (10.0 %) (Table 2). No significant correlation among IGF-1R expression and other clinicopathological factors was observed. IGF-1R expression in tumor cells was not a significant prognostic factor for either DRFS or BCSS.

For ALDH1, tumor cells with cytoplasmic staining were considered to be ALDH1-positive cells [11]. The proportions of ALDH1-positive cells and the number of cases were as follows: <10 % in 111 cases (42.5 %), 10–50 % in 115 cases (44.1 %), and >50 % in 34 cases (13.0 %, Table 2). No significant correlation between ALDH1 expression and other clinicopathological factors was observed. ALDH1 expression in tumor cells was not a significant prognostic factor for either DRFS or BCSS.

Representative findings of IHC for Ki67, HER1, IGF-1R, and ALDH1 are shown in Fig. 3.

Table 3 Analyses of distant relapse-free survival

Variable	Category	Number	Univariate analysis	Multivariate analysis		
			P value	Hazard ratio	95 % CI	*P* value
LVI	Marked	7	<0.0001	21.8	5.5–86.2	<0.0001
	Not	250				
PgR	Negative	69	0.0002	10.3	2.7–39.8	0.0007
	Positive	192				
Ki67 LI	≥30 %	40	0.0424	NA	NA	NA
	<30 %	219				
Grade	III	41	0.0071	NA	NA	NA
	I/II	220				

NA not applicable

Univariate analyses of DRFS and BCSS

Marked LVI (Fig. 4a, $P < 0.0001$), PgR negativity (Fig. 4b, $P = 0.0002$), high Ki67 LI (Fig. 4c, $P = 0.0424$), and histological grade III ($P = 0.0071$) were significant prognostic factors for DRFS by the log-rank test (Table 3). In contrast, other factors, such as tumor size, age, nodal status, HER2 status, HER1 status, ALDH1 status, and IGF-1R status, were not significant.

Marked LVI (Fig. 5a, $P < 0.0001$), high Ki67 LI (Fig. 5b, $P = 0.0009$), PgR negativity (Fig. 5c, $P = 0.0205$), and histological grade III ($P = 0.0021$) were significant prognostic factors for BCSS by the log-rank test (Table 4). In contrast, other factors such as age, tumor size, nodal status, HER2 status, HER1 status, ALDH1 status, and IGF-1R status were not significant.

Multivariate analyses of distant metastasis and breast cancer-specific death

Cox proportional hazards model revealed that marked LVI [hazard ratio (HR) 21.8, 95 % confidence interval (CI) 5.5–86.2, $P < 0.0001$] and PgR negativity (HR 10.3, 95 % CI 2.7–39.8, $P = 0.0007$) were independently worse prognostic factors for distant metastasis (Table 3). The Cox proportional hazards model revealed that marked LVI (HR 287.3, 95 % CI 5.6–14817.5, $P = 0.0049$), high Ki67 LI (HR 19.6, 95 % CI 1.4–250.0, $P = 0.0292$), and PgR negativity (HR 25.1, 95 % CI 1.2–536.5, $P = 0.0389$) were independently worse prognostic factors for breast cancer-specific death (Table 4).

Discussion

Whether chemotherapy should be postoperatively administered in addition to endocrine therapy to patients with ER-positive and/or PgR-positive, HER2-negative breast cancer remains unanswered. In daily practice, physicians always take into account two important issues: the risk of recurrence, and the biological characteristics responsible for response to certain therapies. The former includes clinicopathological factors such as nodal status, tumor size, histological grade, LVI, and others. The latter includes biomarkers such as ER, PgR, HER2, Ki67 LI, and others; however, it is difficult for physicians to distinguish which factors are more important than others [1–3]. To clarify the significance of these factors and also to explore new prognostic factors, we conducted this multi-institute cohort study.

To exclude the effect of adjuvant chemotherapy on patients' prognosis, breast cancer patients postoperatively treated with endocrine therapy alone were recruited to this study. As expected, the majority of patients had ER-positive, HER2-negative breast cancer at earlier stages. They received standard adjuvant endocrine therapies, tamoxifen alone, aromatase inhibitor alone, or tamoxifen followed by aromatase inhibitor in cases of postmenopausal status or tamoxifen alone, or luteinizing hormone-releasing hormone (LH–RH) agonist plus tamoxifen or LH–RH agonist alone in cases of premenopausal status (Table 1). Although they were recruited from three different hospitals, the clinicopathological characteristics and adjuvant endocrine therapy used were not significantly different (data not shown). This is probably because most physicians working for the respective hospitals basically followed the recommendations for the selection of postoperative adjuvant treatment for early breast cancer provided by the St. Gallen Consensus Conference. Additionally, there was no significant difference in DRFS or BCSS among the types of endocrine therapy (data not shown).

To focus on the occurrence of fatal recurrence and exclude the influence of local therapies on overall recurrence rates, DRFS and BCSS rather than disease-free survival (DFS) and overall survival were investigated in this study. It was found that, because there were some

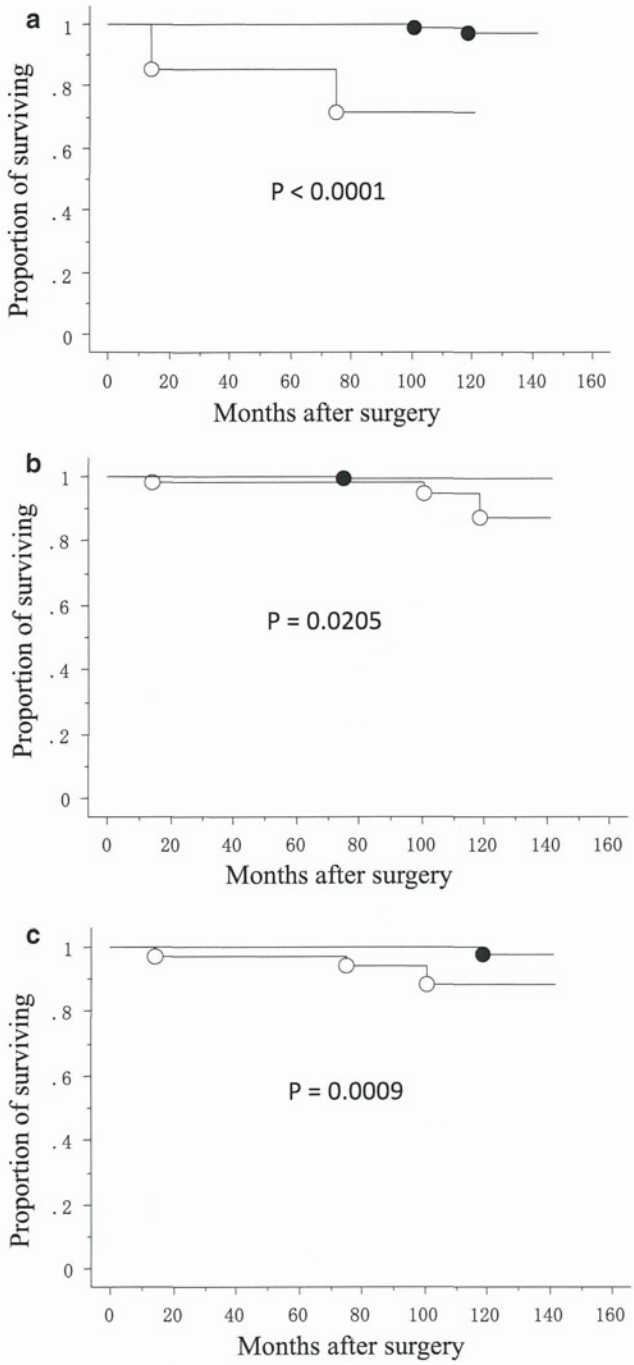

Fig. 5 BCSS according to LVI (**a**), PgR status (**b**), and Ki67 LI (**c**). **a** *Open circles* marked LVI, *filled circles* without marked LVI. **b** *Open circles* PgR-negative tumors, *filled circles* PgR-positive tumors. **c** *Open circles* tumors showing high Ki67 LI, *filled circles* tumors not showing high Ki67 LI

differences in the indication for breast-conserving surgery among the three hospitals, local recurrence rates significantly differed among them (data not shown).

Multivariate analyses of both distant metastasis and breast cancer-specific death showed that three pathological biomarkers, marked LVI, PgR negativity, and high Ki67 LI, were independently worse prognostic factors in this study population (Tables 3, 4). It is likely that, because of the small proportion of cases with large and/or node-positive tumors, the nodal status and tumor size became nonindependent prognostic factors in this study (Table 1). It should be noted that histological grade was not selected as an independent prognostic factor. Histological grade correlated well with Ki67 LI in this study population. When removing Ki67 LI from prognostic variables, histological grade became an independent prognostic factor (data not shown). These findings indicate that histological grade and Ki67 LI influenced each other in terms of prognostic variables and Ki67 LI may have removed histological grade from significant prognostic factors in this study.

Lymphovascular invasion has been associated with poor outcome in patients with breast cancer, in particular, node-negative and/or small tumors [12]. Very recently, a large cohort study reported that LVI provided a strong predictor of outcome in patients with invasive breast cancer and should be incorporated into breast cancer staging systems [13]. The results of this study totally support these findings; however, some points should be clarified before the routine use of LVI as a prognostic factor. First, how to objectively evaluate LVI in breast tumors remains to be resolved. The use of IHC for detecting an endothelial-specific marker such as D2-40 and quantitative imaging techniques may contribute to the prognostic significance of LVI. In addition, the significance of LVI grading is still unclear. In this study, study subjects were divided into two groups according to the grade of LVI, marked LVI or not [6]. This categorization successfully predicted the risk of distant recurrence and breast cancer-specific death. In contrast, when using other categorizations such as absence and mild versus moderate and marked, LVI became a nonindependent prognostic factor.

It has been indicated that PgR status is an independent predictive factor of benefit from adjuvant endocrine therapy and that PgR status should be taken into account when discussing risk reductions expected from endocrine therapy with individual breast cancer patients [14]. In addition, it was hypothesized that loss of PgR in ER-positive breast cancer is a surrogate marker for increased activity of growth factor receptor tyrosine kinases, such as HER1 and HER2, that cause lower PgR expression and tamoxifen resistance in some patients [15].

The significance of HER1 expression in tumor cells remains controversial in terms of prognostic power in breast cancer patients [16]. Recent studies have suggested that HER1 overexpression correlated with worse outcome in patients with breast cancer of the triple-negative phenotype [17–20]. HER1 expression was not an independent prognostic factor in this study population, which consisted

Table 4 Analyses of breast cancer-specific survival

Variable	Category	Number	Univariate analysis	Multivariate analysis		
			P value	Hazard ratio	95 % CI	*P* value
LVI	Marked	7	<0.0001	287.3	5.6–14817.5	0.0049
	Not	250				
Ki67 LI	≥30 %	40	0.0009	19.6	1.4–250.0	0.0292
	<30 %	219				
PgR	Negative	69	0.0205	25.1	1.2–536.5	0.0389
	Positive	192				
Grade	III	41	0.0021	NA	NA	NA
	I/II	220				

NA not applicable

of patients with ER-positive, HER2-negative breast cancer. Otherwise, the proportion of HER1-expressing tumors was very small but these tumors showed higher Ki67 LI in this study as described in the "Results." Further studies are clearly needed to develop a quantitative assay procedure for HER1 expression and to elucidate an appropriate cutoff point of HER1 expression as a prognostic marker in breast cancer patients.

It has been indicated that the IGF1-R signaling pathway plays important roles in cell survival and resistance to endocrine therapy and anti-HER2 therapy through the PI3K/AKT/mTOR cascade [21–23]; however, the clinical significance of IGF1-R expression in tumor cells remains controversial. It is well known that the expression level of IGF1-R is not the sole mediator to regulate activity of the PI3K/AKT/mTOR signaling pathway. In addition, there are confounding factors influencing IGF signaling, such as the activation mechanism of IGF1-R and various IGF binding proteins [24].

The cancer stem cell theory in solid tumors including breast cancer has been widely accepted as a key paradigm to develop new anticancer strategies [4]. Because breast cancer stem cells may play an important role in developing resistance to endocrine therapy, expression levels of a putative cancer stem cell marker, ALDH1 [25], in tumor cells were investigated in this study. Unexpectedly, the proportion of ALDH1-positive tumor cells was not a prognostic factor in this study. Recent studies have suggested that ALDH1 positivity may have prognostic value in patients with triple-negative breast cancer [26]. It has been speculated that suitable cancer stem cell markers are different among breast cancer subtypes [27, 28]. It seems that ALDH1-positive tumor cells were not cancer stem cells in the ER-positive, HER2-negative breast tumors investigated in this study. Other cancer stem cell markers such as cell surface markers CD44 and CD24 might be more useful in studying the significance of the proportion of cancer stem cells as a prognostic variable in ER-positive, HER2-negative breast cancer.

In conclusion, this retrospective multi-institute cohort study indicated that three pathological factors, marked LVI, PgR negativity, and high Ki67 LI, were independently worse prognostic factors in early breast cancer patients treated with endocrine therapy alone. These factors were representative biomarkers for tumor invasiveness, endocrine responsiveness, and tumor cell proliferation. Although it is uncertain whether additional chemotherapy is beneficial or not for patients with these worse prognostic factors, because these factors are relatively easy to identify in daily practice, physicians should take into account these prognostic factors for the choice of chemotherapy additional to endocrine therapy in patients with ER-positive, HER2-negative breast cancer.

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