Prognostic value of Ki67 and p53 in patients with estrogen receptor-positive and human epidermal growth factor receptor 2-negative breast cancer: Validation of the cut-off value of the Ki67 labeling index as a predictive factor

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Abstract. The aim of this study was to evaluate the significance of the Ki67 labeling index and p53 status as prognostic and predictive indicators of operable estrogen receptor (ER)-positive and human epidermal growth factor receptor 2 (HER2)-negative breast cancer. Among 697 consecutive patients with primary breast cancer who underwent curative surgery between 2002 and 2013, 308 patients with ER-positive and HER2-negative breast cancer were assessed. The results of the multivariate Cox analysis demonstrated that a high Ki67 labeling index was significantly associated with a short recurrence-free interval (RFI) (P=0.004) and was marginally associated with a worse overall survival (P=0.074). A positive p53 status was not associated with worse outcomes. To validate the cut-off values of the Ki67 labeling index for identifying patients who may benefit from additional chemotherapy, prognostic factors were investigated in breast cancer patients treated postoperatively with endocrine therapy alone. Analysis of receiver operating characteristic curves demonstrated that a Ki67 labeling index cut-off of 20.0% was optimal for predicting recurrence among patients who did not receive adjuvant chemotherapy. The 5-year RFIs for patients with Ki67 <20 and ≥20% were 97.2 and 86.6%, respectively (P=0.0244). A high Ki67 labeling index (≥20%) was significantly associated with large tumors (P<0.01), lymph node metastasis (P=0.0236) and positive p53 status (P<0.001). The univariate analysis demonstrated that Ki67 labeling index ≥20%, lymph node metastasis and progesterone receptor negativity were significant worse prognostic factors for RFI (P=0.0333, 0.0116 and 0.0573, respectively). The Ki67 labeling index was found to be a useful prognostic factor in patients with ER-positive and HER2-negative breast cancer and the cut-off values of the Ki67 labeling index for making a decision regarding adjuvant treatment were validated.

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Abbreviations: AUC, area under the curve; CI, confidence interval; ER, estrogen receptor; FISH, fluorescent *in situ* hybridization; HER2, human epidermal growth factor receptor 2; HR, hazard ratio; IHC, immunohistochemistry; OS, overall survival; PgR, progesterone receptor; RFI, recurrence-free interval; ROC, receiver operating characteristic

Key words: breast cancer, Ki67, p53, prognostic factor, predictive factor

Introduction

For the majority of patients with early breast cancer, adjuvant systemic therapy is recommended following primary surgery to reduce the risk of breast cancer recurrence and to increase the likelihood of a cure. Approximately 70% of breast cancers express estrogen receptor (ER), and ER status is a powerful predictor of response to therapies that inhibit estrogen synthesis or block the action of its receptor (1). Endocrine therapies are established in the adjuvant setting (2-4). It is important to distinguish patients with ER-positive tumors at high risk for recurrence who require additional chemotherapy, from those for whom adjuvant endocrine therapy alone may suffice, as the economic burden and toxicities of chemotherapy must be minimized (5). Multi-gene assays are strong candidate tools for predicting the risk of recurrence in ER-positive patients (6). However, classification using multi-gene expression analyses is not appropriate for everyday practice. According to the St. Gallen Consensus Conference held in 2013, the intrinsic subtype affects the indication for adjuvant chemotherapy and surrogate definitions of subtype may be obtained by immunohistochemistry (IHC) of ER, progesterone receptor (PgR), Ki67 and human epidermal growth factor receptor 2 (HER2) (7).

For practical purposes, in order to reliably distinguish between 'luminal A' (more endocrine-sensitive, more indolent and better prognosis) and 'luminal B (HER2-negative)' (less endocrine-sensitive, more aggressive and worse prognosis)

breast cancer subtypes, Ki67 may be used as a proliferative index in addition to ER and PgR status. At the St. Gallen Consensus Conference, the majority of the expert panel voted that a threshold of ≥20% should be defined as 'high' Ki-67 status (7). The cut-off value of Ki67 for high proliferation remains unclear.

Breast cancers expressing high levels of Ki67, which is a nuclear marker of cell proliferation, are associated with worse outcomes (8-10). However, a standard operating procedure has not been established, and the inter-laboratory and inter-study comparabilities of Ki67 are limited (11-13). Therefore, laboratory-specific procedures and cut-off values must be examined in order to use Ki67 as an appropriate prognostic marker (7).

Recent studies have demonstrated that abnormalities of the p53 gene and accumulation of the p53 protein in the nuclei are prognostic indicators in ER-positive and HER2-negative breast cancer patients (14-17). Whole-genome analysis identified the presence of a p53 gene mutation in 12% of luminal A and 32% of luminal B breast cancers (18). Therefore, the expression of p53 has been suggested to be useful in distinguishing between luminal A and B subtypes. However, p53 assessment is not recommended for routine clinical use in breast cancer.

The present study aimed to determine the clinical value of Ki67 and p53 as prognostic markers in patients with ER-positive and HER2-negative breast cancer, in order to help stratify patients into prognostic subgroups with a better predictive response to adjuvant treatment.

Patients and methods

Patients. Between February, 2002 and March, 2013, 697 consecutive patients with primary breast cancer underwent curative surgery at our institution. Among 615 patients with invasive breast cancer, 85 who received preoperative chemotherapy and 161 with ER-negative, HER2-negative, or unknown subtypes were excluded from the analysis. A total of 6 patients with unknown Ki67 labeling indices and 55 who did not undergo endocrine therapy were also excluded. Data from 308 patients with a median age of 59.0 years (range, 26-91 years) and ER-positive/HER2-negative breast cancer, who were treated with endocrine therapy, were finally analyzed (Fig. 1). Patients who were considered to be at high risk according to prevalent breast cancer guidelines received chemotherapy (19-23). The follow-up period (median, 57.9 months) was terminated on 31 December, 2013. Demographic and medical data including age, type of breast surgery and a history of treatment for breast cancer and endocrine therapy were collected from medical charts. The Institutional Review Board approved this study (approval no. H25-98) and waived the requirement for informed consent from individual patients.

Clinicopathological factors. Clinicopathological factors such as age, tumor size, lymph node metastasis, nuclear grade, lymphovascular invasion positivity and PgR positivity, which are established prognostic factors for breast cancer, were compared between patients assigned to groups based on Ki67 labeling index cut-offs. Nuclear grade was determined according to the General Rules for Clinical and Pathological Recording of Breast Cancer, 16th edition (24). ER and PgR positivity was assessed by IHC and scored according to the Allred system (25). HER2

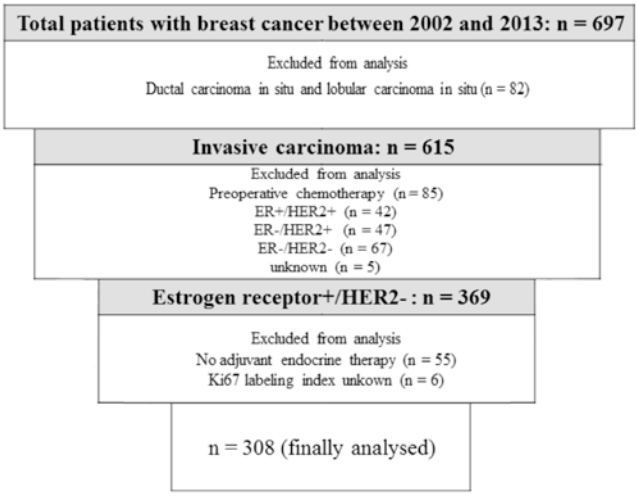

Figure 1. Patient selection process. ER, estrogen receptor. HER2, human epidermal growth factor receptor 2.

positivity was defined as 3^+ by IHC or 2^+ by gene amplification using fluorescent *in situ* hybridization (FISH) >2.0.

The surgical specimens were stained using mouse monoclonal anti-Ki67 antibody (MIB-1; M7240; dilution 1:80; Dako, Glostrup, Denmark) and mouse DO-7 anti-p53 antibody (DO-7; dilution 1:800; cat. no. NCL-L-p53-DO7; Leica-Novocastra Laboratories Ltd., Newcastle upon Tyne, UK). Areas of dense staining were selected under a microscope, and >500 cancer cells were assessed to determine the levels of Ki67 expression. Ki67 immunoreactivity was recorded as a continuous variable based on the proportion of positive tumor cells (0-100%), regardless of staining intensity. Cells with nuclear p53 immunostaining were defined as positive. When <10 or ≥10% of tumor cells expressed p53, the specimens were defined as negative or positive, respectively, for p53 expression (26).

Follow-up. All the patients were followed up from the day of surgery onwards. Follow-up care plans included regular physical examinations and annual mammograms. Recurrence was defined as any unequivocal occurrence of new cancer foci in a hitherto disease-free patient. The site of the first cancer recurrence and the interval between surgery and recurrence were determined. The recurrence-free interval (RFI) was calculated as the elapsed time between the date of surgery and that of the first confirmation of cancer recurrence or the last clinical contact attesting to recurrence-free status. Overall survival (OS) was defined as the interval from the day of surgery until death from any cause.

Statistical analysis. Data are presented as numbers (%) or as means unless otherwise stated. Frequencies were compared using the χ^2 test for categorical variables, and small samples were assessed using the Fisher's exact test. The patient population was subdivided according to the Ki67 labeling index cut-offs, and the duration of RFI was determined using Kaplan-Meier analyses. Differences in RFI were assessed using the log-rank test. The potential independent effects of the Ki67 labeling index on RFI and OS were determined by multivariate analyses using a Cox proportional hazards model that included variables with $P<0.05$ in the univariate analyses

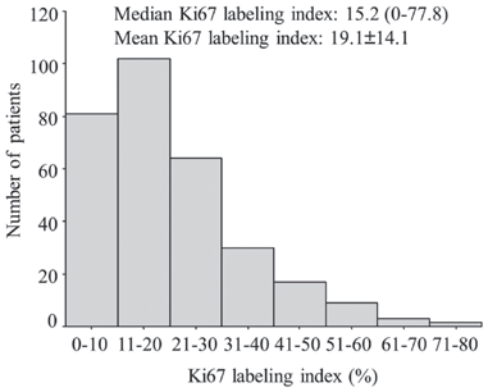

Figure 2. Distribution of patients according to the Ki67 labeling index.

and P<0.05 was considered to indicate a statistically significant difference. Receiver operating characteristic (ROC) curves of the Ki67 labeling index for the prediction of recurrence were generated to determine the cut-off that yielded optimal sensitivity and specificity according to the Youden index. Data were statistically analyzed using EZR (27), which is a graphical user interface for R (version 2.13.0; The R Foundation for Statistical Computing, Vienna, Austria). More precisely, it is a modified version of R commander (version 1.6-3) that was designed to add statistical functions frequently used in biostatistics.

Results

Characteristics of patients and tumors. The clinicopathological characteristics of the 308 patients included in this study are summarized in Table I. The tumors were mainly ductal (91.6%) or lobular (1.6%) invasive carcinomas. The majority of the patients had pathological T1-stage tumors, 20 (6.5%) had lymph node involvement, 248 (80.5%) of the tumors were positive for lymphovascular invasion, and 257 (83.5%) were PgR-positive on IHC. Adjuvant chemotherapy had been administered to 86 (27.9%) patients. A total of 24 patients developed recurrence and 8 succumbed to the disease during the follow-up period. The distribution of the patients according to the Ki67 labeling index, ranging from 0 to 77.8% (median, 15.2%), is shown in Fig. 2.

Univariate and multivariate analyses of RFI and OS. The variables included in the univariate analysis of RFI of the 308 patients were age, tumor size, nodal status, nuclear grade, lymphovascular invasion status, PgR status, Ki67 labeling index, p53 status and adjuvant chemotherapy. Positive nodal status and a high Ki67 labeling index were significantly associated with a short RFI. Moreover, a multivariate analysis that included nodal status and the Ki67 labeling index, identified positive nodal status [hazard ratio (HR)=8.92, 95% confidence interval (CI): 3.79-21.0, P<0.001] and a high Ki67 labeling index (HR=1.03, 95% CI: 1.01-1.06, P=0.004) as independent prognostic factors for RFI (Table II). Larger tumors, positive nodal status and a high Ki67 labeling index were significantly associated with a short OS. A multivariate analysis that included tumor size, nodal status and Ki67 labeling index identified positive nodal status (HR=29.5, 95% CI: 4.91-177.0, P<0.001) as an independent prognostic factor for OS. A high

Table I. Patient characteristics (n=308).

Characteristics	No. (%)
Age, years [median (range)]	59.0 (26-91)
Histology	
IDC	282 (91.6)
ILC	5 (1.6)
Others	21 (6.8)
pT stage	
T1	226 (73.4)
T2	67 (21.8)
T3	1 (0.3)
T4	14 (4.5)
Nodal status	
Negative	288 (93.5)
Positive	20 (6.5)
Nuclear grade	
I	38 (12.3)
II	214 (69.5)
III	32 (10.4)
Unknown	24 (7.8)
Lymphovascular invasion	
Negative	57 (18.5)
Positive	248 (80.5)
Unknown	3 (1.0)
Progesterone receptor status	
Negative	50 (16.2)
Positive	257 (83.5)
Unknown	1 (0.3)
Ki67 labeling index, % [median (range)]	15.2 (0.0-77.8)
p53 status	
Positive	83 (26.9)
Negative	225 (73.1)
Type of surgery	
Breast-conserving surgery	221 (71.8)
Modified radical mastectomy	87 (28.2)
Endocrine therapy	
Tamoxifen	88 (28.5)
Tamoxifen + Gn-RH agonist	40 (13.0)
Gn-RH agonist	3 (1.0)
Anastrozole	97 (31.5)
Letrozole	76 (24.7)
Exemestane	4 (1.3)
Chemotherapy	86 (27.9)
Oral 5-FU	4 (1.3)
Anthracycline	29 (9.4)
Anthracycline + taxane	35 (11.4)
CMF	2 (0.6)
TC	16 (5.2)
Recurrence	
Local	11 (3.6)
Distant	13 (4.2)
No event	284 (92.2)
Death	8 (2.5)

IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; Gn-RH, gonadotrophin-releasing hormone; 5-FU, 5-fluorouracil; CMF, cyclophosphamide, methotrexate and 5-fluorouracil; TC, docetaxel and cyclophosphamide.

Table II. Univariate and multivariate analyses of recurrence-free interval and overall survival.

	Univariate analysis		Multivariate analysis	
Variables	HR (95% CI)	P-value	HR (95% CI)	P-value
Recurrence-free interval				
Age (years)				
High	1.02 (0.984-1.05)	0.327		
pT stage				
pT2,3,4	2.04 (0.910-4.57)	0.084		
Nodal status				
Positive	9.10 (3.88-21.3)	0.007	8.92 (3.79-21.0)	<0.001
Nuclear grade				
III	1.91 (0.640-5.67)	0.247		
Lymphovascular invasion				
Positive	5.17 (0.697-38.4)	0.108		
Progesterone receptor status				
Negative	0.827 (0.367-1.86)	0.646		
Ki67 labeling index				
High	1.03 (1.02-1.05)	0.003	1.03 (1.01-1.06)	0.004
p53 status				
Positive	1.32 (0.576-3.02)	0.512		
Chemotherapy				
Yes	1.51 (0.647-3.54)	0.339		
Overall survival				
Age (years)				
High	1.05 (0.985-1.11)	0.140		
pT stage				
pT2,3,4	6.45 (1.29-32.3)	0.023	1.52 (0.257-9.03)	0.643
Nodal status				
Positive	40.0 (7.94-201.0)	<0.001	29.5 (4.91-177.0)	<0.001
Nuclear grade				
III	4.05 (0.740-22.2)	0.107		
Lymphovascular invasion				
Positive	27.2 (0.009-84.3)	0.420		
Progesterone receptor status				
Negative	0.697 (0.170-2.86)	0.616		
Ki67 labeling index				
High	1.04 (1.01-1.08)	0.021	1.04 (0.996-1.09)	0.074
p53 status				
Positive	2.02 (0.503-8.11)	0.321		
Chemotherapy				
Yes	3.14 (0.784-12.6)	0.106		

HR, hazard ratio; CI, confidence interval.

Ki67 labeling index was marginally associated with OS (HR=1.04, 95% CI: 0.996-1.09, P=0.074) (Table II).

The predictive value of the Ki67 labeling index should be determined when considering additional chemotherapy decisions. Therefore, whether Ki67 may be used to determine the likelihood of a poor prognosis among patients who did not receive chemotherapy, and who had undergone breast surgery before the Ki67 labeling index was adopted as a breast cancer guideline (18) for implementing decisions regarding adjuvant therapy. The ROC curve revealed an optimal Ki67 labeling index cut-off value of 20.0% for predicting recurrence in 138 patients who did not receive adjuvant chemotherapy (area under the curve = 0.650; sensitivity, 66.7%; specificity, 63.4%) (Fig. 3A).

Comparison of clinicopathological parameters between Ki67 labeling index <20 and ≥20%. At a cut-off value of 20.0%, a

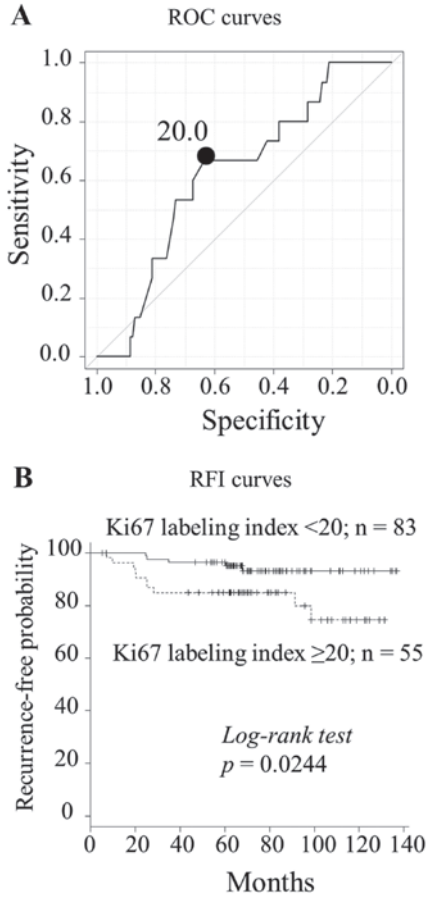

Figure 3. Recurrence and recurrence-free interval (RFI) determined from receiver operating characteristic (ROC) curves according to Ki67 labeling index cut-offs in patients prior to 2009 who did not receive adjuvant chemotherapy. (A) The optimal cut-off of the Ki67 labeling index is 20.0% (n=138, area under the curve = 0.650; sensitivity, 66.7%; specificity, 63.4%). (B) The 5-year RFI for patients with Ki67 <20 and ≥20% was 97.2 and 86.6%, respectively (P=0.0244).

high Ki67 labeling index was significantly associated with large tumors, lymph node positivity and p53 positivity (Table III). The 5-year RFI for patients with Ki67 labeling indices <20 and ≥20% was 97.2 and 86.6%, respectively (P=0.0244; Fig. 3B). The univariate analysis demonstrated that a high Ki67 labeling index (≥20%), lymph node metastasis and PgR negativity were significantly worse prognostic factors for RFI (P=0.0333, 0.0116 and 0.0573, respectively) (Table IV).

Discussion

The clinical value of Ki67 and p53 as prognostic markers in patients with ER-positive and HER2-negative breast cancer was investigated. The present findings confirmed that Ki67 expression is a prognostic factor for both RFI and OS in patients with ER-positive and HER2-negative breast cancer. The multivariate model demonstrated that Ki67 expression remained significant for RFI, a trend was evident for OS, and these results were consistent with the majority of published data (10,28).

The predictive value of Ki67 IHC for adjuvant treatment of ER-positive and HER2-negative breast cancer has not been investigated in a prospective, randomized study. In the present study, a cut-off Ki67 labeling index of 20.0% identified patients with a poor prognosis among those who did not receive adjuvant chemotherapy. Although this retrospective study included significant selection bias for adjuvant chemotherapy, the results indicated that patients with a Ki67 labeling index ≥20.0% should not be treated with adjuvant endocrine therapy alone. Likewise, Criscitiello *et al* (29) retrospectively analyzed the ability of Ki67 to predict adjuvant chemotherapy in patients with ER-positive/HER2-negative, node-positive breast cancer, using propensity scores to minimize bias related to the non-random assignment of treatment. Their analysis of Subpopulation Treatment Effect Pattern Plots found that Ki67 was dichotomous at the 32% level. Certainly, not only the Ki67 labeling index but also nodal status, PgR status and other prognostic factors, should be considered in the decision regarding adjuvant treatment (7). Further prospective validation studies are required to evaluate the appropriateness of the Ki67 labeling index cut-off value as a predictive factor.

There is a lack of consensus regarding the use of the Ki67 labeling index, which results in inconsistencies in inter-laboratory methodology (11-13). In particular, the method of assessing Ki67 has been argued. It may be done by several scoring approaches: Hot-spot scoring, inclusion of hot spots in general across the section scoring, and by overall average score across the whole section only. A working party of the International Ki67 in Breast Cancer Working Group has been established to assess which method is more robust (30). Although it remains under evaluation, Honma *et al* (31) reported that Ki67 estimation at the 'hottest spot' was found to be superior to that determined by the average score across the whole section as a predictor of outcome in patients with ER-positive and HER2-negative breast cancer treated with tamoxifen. In the present study, hot-spot scoring was used, and it demonstrated the prognostic significance of the Ki67 labeling index. However, establishment of an international standard methodology for using Ki67 is required.

The results of the present study did not reveal a prognostic significance for p53. The prognostic significance of p53 protein expression in ER-positive and HER2-negative breast cancer has been reported in a limited number of studies (14-17). However, several items of information are required for the interpretation of p53 protein expression. The correlation between p53 accumulation measured using IHC and p53 mutations detected using sequencing has been estimated to be <75% in breast cancer (32). Not all mutations yield a stable protein, and certain mutations lead to a truncated protein that cannot be detected using IHC. Done *et al* (33) demonstrated strong p53 nuclear staining in all tumors known to harbour missense mutations, but in no tumors with truncation mutations. Wild-type p53 may also accumulate in certain tumors as a result of a response to DNA damage or by binding to other cellular proteins (34). Moreover, at the single-tumor level, p53 mutations are distributed in a heterogeneous manner (35). It is necessary to collect evidence regarding p53 accumulation and establish the methodology for clinical practice. Further studies on p53 as a predictive factor for late recurrence and adjuvant chemotherapy are required, along with the findings of previous reports (15,17).

This study's limitations include its retrospective design at a single institution, the heterogeneity of the administered treatments, and the short follow-up period. Nonetheless, a clear statistical significance was identified for the Ki67 labeling index.

Table III. Comparison of clinicopathological parameters between Ki67 labeling index <20 and ≥20% in patients who did not receive chemotherapy (n=138).

Variables	Ki67 labeling index <20% (n=83)	Ki67 labeling index ≥20% (n=55)	P-value
Age, years (median ± SD)	61.1±13.0	62.7±13.1	0.497
pT stage, n (%)			0.00420
T1	68 (81.9)	32 (58.2)	
T2, T3, T4	15 (18.1)	23 (41.8)	
Nodal status, n (%)			0.0236
Negative	83 (100)	51 (92.7)	
Positive	0 (0.0)	4 (7.27)	
Nuclear grade, n (%)			0.739
I,II	66 (79.5)	44 (80.0)	
III	5 (6.02)	5 (9.09)	
Lymphovascular invasion, n (%)			0.296
Negative	22 (26.5)	10 (18.2)	
Positive	58 (69.9)	45 (81.8)	
Progesterone receptor status, n (%)			1.00
Negative	16 (19.3)	11 (20.0)	
Positive	68 (81.9)	44 (80.0)	
p53 status, n (%)			<0.001
Negative	68 (81.9)	25 (45.5)	
Positive	15 (18.1)	30 (54.5)	

SD, standard deviation.

Table IV. Univariate and multivariate analyses of the recurrence-free interval in patients who did not receive chemotherapy (n=138).

	Univariate analysis		Multivariate analysis	
Variables	HR (95% CI)	P-value	HR (95% CI)	P-value
Age (years)				
High	1.03 (0.985-1.07)	0.216		
pT stage				
pT2,3,4	1.66 (0.587-4.63)	0.341		
Nodal status				
Positive	6.84 (1.54-30.5)	0.0116	4.12 (0.872-19.5)	0.0739
Nuclear grade				
III	0.868 (0.113-6.68)	0.892		
Lymphovascular invasion				
Positive	4.10 (0.537-31.3)	0.174		
Progesterone receptor status				
Negative	2.74 (0.970-7.74)	0.0573		
Ki67 labeling index				
≥20%	3.22 (1.10-9.43)	0.0333	2.73 (0.892-8.36)	0.0785
p53 status				
Positive	1.05 (0.357-3.06)	0.935		

HR, hazard ratio; CI, confidence interval.

In conclusion, Ki67 was found to be an independent prognostic factor for patients with ER-positive and HER2-negative breast cancer, and its cut-off values for making a decision regarding adjuvant treatment were validated.

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