Omission of Chemotherapy in HR+/HER2- Early Invasive Breast Cancer Based on Combined 6-IHC Score?

Jiaman Lin
China Medical University First Hospital
Zihe Guo
China Medical University First Hospital
Shuo Wang
China Medical University First Hospital
Xinyu Zheng (xyzheng@cmu.edu.cn)
China Medical University Hospital https://orcid.org/0000-0001-8425-3379

Research

Keywords: Hormone receptor positive, Human epidermal growth factor negative, breast cancer, prognosis biomarker, adjuvant chemotherapy, immunohistochemistry

Posted Date: May 26th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-30455/v1

Version of Record: A version of this preprint was published on January 23rd, 2021. See the published version at https://doi.org/10.1016/j.clbc.2021.01.011.

Abstract

Background: Previous randomized studies have assessed the possibility of omission of chemotherapy in some hormone receptor (HR)-positive and HER2-negative (HR+/HER2-) breast cancers (BC) based on gene profiling test, e.g., Oncotype DX. The goal of this study was to evaluate if combination of six proliferation related biomarkers by immunohistochemistry (6-IHC) could be a cost-effective option in determining the necessity of adjuvant chemotherapy in HR+/HER2- BC.

Methods: A retrospective analysis of HR+/HER2- BC patients was conducted in the First Affiliated Hospital of China Medical University from 2010 to 2016. The expression of 6 BC-related proliferation and invasion genes (Cathepsin L2, MMP11, CyclinB1, Aurora A, Survivin and Ki67) from Oncotype DX were analyzed through IHC (designated as 6-IHC). All the included patients were divided randomly at a 7:3 ratio into training and testing cohorts. The cutoff prognosis index (PI) of 6-IHC was determined by multivariate Cox risk regression analysis after calculating the PI of each patient in training cohort and confirmed in testing cohort. The patients were classified into “Low” and “High” risk groups based on the PI value. Kaplan-Meier (KM) method was used to analyze Disease-free survival (DFS) and overall survival (OS). 6-IHC score and other factors associated with survival benefit of adjuvant chemotherapy were compared with Ki67 index.

Results: A total of 330 patients were included and divided into training cohort (n = 231) and validation cohort (n = 99). The receiver operating characteristic (ROC) curve analysis showed that the patients can be divided into 6-IHC score “High” and “Low” risk groups using the cut-off PI of 2.16. The 8-year DFS and OS were 54.6% and 69.2%, respectively in the 6-IHC score “High” risk group; 85.5% and 92.5%, respectively in the 6-IHC score “Low” risk group. The 8-year DFS and OS were 70.8% and 80.9%, respectively in the Ki67 “High” risk group, 77.7% and 87.6%, respectively in the Ki67 “Low” risk group. The KM curves showed that chemotherapy did not significantly improve the DFS in the 6-IHC score “Low” risk group (p = 0.830), but significantly improved the DFS in the 6-IHC score “High” risk group (P = 0.012).

Conclusions: Combined 6-IHC score could be a reliable tool in predicting cancer-specific recurrences and survival in HR+/HER2- BC patients and identifying patients who could benefit from adjuvant chemotherapy regardless of the involvement of axillary lymph node (ALN).

Introduction

Adjuvant chemotherapy (CT) contributed to the reduction of 36% of the 10 years mortality rate for patients with early breast cancer (BC), regardless of age, nodal status, tumor size, differentiation, or ER status [1]. However, chemotherapy may cause overtreatment for some breast cancer patents[2]. For instance, some patents with ER-positive tumors will gain additional benefit from chemotherapy, whereas for other patients with HR-positive and HER2-negative (HR+/HER2-) early invasive BC, endocrine treatment is sufficient and thus chemotherapy can be omitted [3].

Comparing to the traditional clinicopathological parameters, genomic tests have shown the promising prognostic and predictive value in breast cancer[4] management. Although the benefit of using genetic data is obvious, it also has pitfalls, e.g., lack of strong correlation between gene expression and protein levels or activities. The correlation between the genes and proteins and related mortality is also not clear. In addition, although the cost of gene sequencing has decreased dramatically, its price is still unbearable in clinical setting [5].

Oncotype DX® consists of 16 cancer-related genes and 5 reference genes (β-actin, GAPDH, RPLPO, GUS, TFRC). Cancer-related genes can be divided into a proliferation group (Ki67, Aurora A, Survivin, Cyclin B1, MYBL2), an invasion group (MMP11, Cathespsin L2), an ER group (ER, PR, BCL2, SCUBE2), a HER2 group (GRB7, HER2), and a group with related genes (GSTM1, BAG, CD68) [3]. Due to the concordance of gene expression and immunohistochemistry (IHC) profiles, we selected Oncotype DX invasion-related genes MMP11, Cathepsin L2 and proliferation-related genes Aurora A, Survivin, Cyclin B1, and Ki67 for IHC analysis. We hypothesized that IHC analysis of these 6 genes can achieve the same predictive effect on prognosis as Oncotype DX test. To test this hypothesis, we conducted a retrospective analysis on protein expression of these 6 genes through IHC (6-IHC) and constructed the prognostic model for 330 HR+/HER2- patients with early breast cancer. AUC and calibration curves were used to evaluate the predictive accuracy and discriminative ability of 6-IHC score prognostic model in both training and testing cohorts. We finally identified the relationship between the survival and 6-IHC score in HR+/HER2- BC patients and determined the predictive effect of 6-IHC score on adjuvant chemotherapy decision-making.

Materials And Methods

Research Population

We retrospectively evaluated 330 HR-positive and HER2-negative (HR+/HER2-) patients with early breast cancer who underwent surgery in the First Affiliated Hospital of China Medical University from April 2010 to November 2016. Data on patient age, year of diagnosis, menstrual status, tumor size, axillary lymph node (ALN) metastasis, histological grade, chemotherapy information, estrogen receptor (ER) and progesterone receptor (PR), and HER2 and Ki67 expression levels were obtained from the pathological reports. All patients were suggested to treated with hormone therapy (HT) and radiotherapy if tumor size was ≥5 cm and/or ALN metastasis was ≥4, or lumpectomy was performed in accordance with the NCCN guidelines after surgery. Chemotherapy was performed under the condition of tumor size >2 cm, age <40 year-old, Ki67 index > 14% and/or ALN metastasis ≥1. Types of treatment and follow-up status were collected from clinical reports. Inclusion criteria were as follows: (1) female patients; (2) early breast cancer patients with primary HR+/HER2- and at least three-year adjuvant HT; (3) unilateral breast cancer with no indication of distant metastasis or skin involvement at presentation; (4) no other diagnosed cancer; and (5) no radiotherapy, neoadjuvant chemotherapy, endocrine therapy, or targeted therapy before surgery. Exclusion criteria were: (1) carcinoma in situ; (2) inflammatory breast cancer or distant metastasis at diagnosis; (3) history of acute or chronic liver disease at presentation before surgery; and (4) combination with serious heart, kidney, and/or lung diseases. The criteria for determining HR+/HER2-

included ER and PR positive status (immunohistochemical staining positive cells were ≥1%). HER2- were considered when HER2 was negative or (+), and HER2 was (++) by IHC without confirmation using fluorescence in situ hybridization (FISH). HER2 (+++) or (++) comfirmed by FISH indicates overexpression of HER2 (HER+). Ethical approval of informed consent exemption due to retrospective setting was granted by First Affiliated Hospital Research Ethics Committee (2020-203).

Follow Up

The data in the database was continuously followed up every year, and the follow-up content included the time of death (accurate to month) and survival status. Overall survival (OS) was calculated as the time from the day of surgery to the day of death due to any cause. Disease-free survival (DFS) was calculated as no further recurrence from the day of surgery until the tumor was treated (by way of physical examination, CT or MRI, etc.) or the time of death. The last follow-up was in January 2020.

Tissue Microarray

A ready-to-use IHC hypersensitive UltraSensitive^{TM} SP Rabbit IHC Kit-9706 and DAB Kit-0031 were purchased from MXB biotechnologles (MXB, Fuzhou, China). Antibodies of Aurora A, Survivin, Cyclin B1, Cathepsin L, MMP11, and Ki67 were purchased from Abcam (Cambridge, MA USA). Specimens of all patients' postoperative lesions were collected and fixed with formalin. Fixed samples were dehydrated and sections were obtained with a thickness of 4μm. Sections were processed with HE staining and diagnosis was confirmed with a microscope. Immunohistochemical labeling was performed strictly in accordance with the SP method. Tissue sections were dehydrated with xylene dewaxing gradient, hydrated with 3% hydrogen peroxide, followed by incubating at room temperature and rinsing with PBS. Normal sheep serum was used for blocking at room temperature. Primary antibody was added, followed by rinsing with PBS. Secondary antibody was added and incubated in 37 ° C incubator. After rinsing with PBS, horseradish peroxidase-labeled streptavidin working solution was added and incubated in 37 °C incubator. After rinsing with PBS, conventional DAB was added. The sections were rinsed, counterstained with hematoxylin and mounted with neutral gum.

An 8 mm inner hollow tube was used to puncture the tissue at the mark and numbered, and then discharged into a blank wax block to make a microarray tissue chip. According to the experimental purpose, the tissue microarray (TMA) arrangement was designed. A total of 44 points were made for each chip, and one blank point was left for labeling. A tissue chip sampler of the corresponding specification and a suitable blank recipient wax block were selected. According to the HE positioning results, the tissue points of the site to be measured were sampled with the sampler to obtain a tissue column of the donor. Subsequently, the donor tissue column points were injected into the corresponding recipient wax block holes in sequence according to the predetermined sequence. The tissue chip fusion device was used to donor column and recipient wax block. Multiple repeated fusions were performed to make the two completely merge into a tissue chip wax block. The modified tissue chip wax block was sliced on a paraffin microtome with a thickness of 4 μm. The sections were floated on 40 °C warm water of the

spreader to flatten the tissue. The tissue was picked up with a glass slide and placed in a 60 °C oven to bake slices. After the water-dried wax was baked, it was taken out for use in IHC experiments. Paraffin sections were routinely dewaxed and were incubated in a 3% Hydrogen Peroxide Block for 10-15 minutes, followed by washing for twice (5 min/each). Tissue sections were placed in a repair box filled with citric acid antigen retrieval buffer (PH = 6) or EDTA antigen retrieval buffer in a high pressure water bath in a pressure cooker for 2.5 minutes after boiling. After the section was slightly dried, a circle was drawn surrounding the tissue with a histochemical pen (to prevent the antibody from flowing away), and goat serum was added within the circle to cover the tissue evenly and block at room temperature for 20 minutes. Primary antibody working solution was added dropwise and incubated overnight at 4 °C. HRP-conjugated secondary antibody was added and incubated at room temperature for 30 minutes. Streptomycin avidin peroxidase was added and incubated at room temperature for 15 minutes. After the sections were slightly dried, 1-2 drops of DAB with Chromogen were added in the circle and incubated for 3-15 minutes. After the sections were fully rinsed with tap water, they were counterstained, dehydrated, transparent, and mounted.

Immunohistochemical Analysis

Under double-blind condition, two experienced pathologists were asked to randomly select 5 different visual fields. A total of 200 breast cancer cells were counted to obtain the average value. Appearance of brown particles in the nucleus indicates the expression of ER and PR proteins. According to the Consensus of St. Gallen 2011, ASCO 2014, and CAP guidelines, the cut-off value of ER and PR positivity was 1%. Any staining of >1% of cells was considered positive [1,6]. For HER2 staining, only membrane staining intensity and pattern were evaluated. Circumferential membrane staining that was complete, intense, and within > 10% of tumor cells resulted in a score of 3+. Circumferential membrane staining that was incomplete and/or weak/moderate and within >10% of tumor cells or complete and circumferential membrane staining that was intense and within 10% of tumor cells was scored 2+. Incomplete membrane staining that was faint or barely perceptible and within >10% of tumor cells was scored 1+. When no staining was observed or membrane staining was incomplete and was faint/barely perceptible and within 10% of tumor cells, the assigned score was 0. A positive test was defined as staining score of 3+. The score of 2+ was interpreted as equivocal. Additionally, a negative test was defined as a score of 0/1+. Score of 2+ was considered ambiguous and needs to be determined by FISH testing. When the FISH result was amplified, the result was strongly positive; if the FISH result was not amplified, the patient was negative. Ki67 staining was reported by percentage of positively stained nuclei from the pathology report, and the cut-off value of Ki67 was 14% [1,6].

The expression of Aurora A, Survivin, Cyclin B1, Cathepsin L, and MMP11 was quantified as the average percentage of positively stained cells in the total number of malignant cells (Figure 1). In this experiment, these six biomarkers were used to make TMA and the expression of samples were analyzed by immunohistochemical staining together, which would be more convenient to record if the standards were unified. On the one hand, these six biomarkers were not used for clinical diagnosis, and there was no clear criterion for the assessment of positivity. This method can reduce the error caused by uneven dying

depth. Moreover, continuous variables were more convenient to construct Cox regression model. To unify the criteria, the values of the 6-IHC score (including Ki67 index) used in the Cox regression model were the average percentage of positively stained cells of malignant cells. The histologic grade was determined in accordance with the Nottingham Histologic Scoring system.

Statistical Analysis

Difference between clinicopathological proportions was determined using the chi square test. Difference between continuous clinicopathological variables was evaluated via a two-tailed t-test. Univariate Cox regression analysis was used to screen all candidate biomarkers. If $P < 0.1$, multivariate Cox risk regression analysis was included to evaluate the role of biomarkers as independent prognostic factors for patient survival. Multivariate Cox risk regression model was used to calculate the risk score prognosis index (PI) of each patient. A 6-IHC prognosis risk score was established based on a linear combination of the regression coefficient derived from the multivariate Cox regression model (β) multiplied with its expression level. The patient's PI value can be obtained according to the following formula:

PI = (β_1*expression level of Cathepsin L2) + (β_2*expression level of MMP11) + (β_3*expression level of CyclinB1) + (β_4*expression level of Aurora A) + (β_5*expression level of Survivin) + (β_6*expression level of Ki67).

From this formula, the smaller PI value indicated better prognosis, while the larger PI value would indicate the poorer prognosis. The 6-IHC score prognostic model predicts that the optimal threshold for DFS in the subtype of breast cancer patients was determined by the receiver operating characteristic curve (ROC), when the sensitivity, specificity, and accuracy were calculated. The optimal cut-off values were based on the maximum point of the sum of sensitivity and specificity (Youden index). The specific value after adjusting the number of decimal places according to clinical experience determine the optimal threshold for distinguishing the “High” from “Low” risk of the 6-IHC prognostic model, and divide the patient into high risk group and low risk group. The determination of the threshold value converts the numerical variable into a categorical variable for subsequent statistical analysis. We used the Kaplan–Meier method to describe OS and DFS. The log-rank test was utilized to determine the differences in OS and DFS between groups. The area under the ROC curve (AUC) was used to evaluate the 6-IHC score prognostic model. AUC between 0.7 and 0.9 indicates higher authenticity. Using univariate and multivariate Cox regression analysis, we determined the predictive power of the 6-IHC score prognostic model independent of other clinical variables of BC (including age, menopausal status, tumor size, lymph node metastasis, histological grade, ER, PgR, chemotherapy). Finally, the prognostic model is verified by KM and ROC analysis.

Statistical analysis was performed using statistical software SPSS 23.0 software (SPSS Inc., Chicago, IL, USA). All reported P values were bilateral and the hazard ratio (HR) and 95% confidence interval were calculated. The test level was set at $p < 0.05$.

Results

Patient Characteristics

Randomization is performed by computer-generated random numbers, and each case is assigned a random number, arranged according to the size of the random number and divided into a training cohort (n=231) used to construct the model and a testing cohort (n=99) used for verification according to ratio of 7:3. The basic clinicopathological characteristics for both training cohort and testing cohort were shown in Table 1. The median age of patients were 50 and 52 years old in the training and testing cohorts, respectively. Postmenopausal patients accounted for 70.6% (163/231) and 67.7% (67/99) of the training cohorts and testing cohorts, respectively. 179 patients (77.5%) in the training cohort and 80 patients (80.8%) in the testing cohort had a large tumor size (> 2 cm). 127 patients (55.0%) in the training cohort and 55 patients (55.6%) in the training cohort had lymph node metastasis. 196 cases (84.8%) in the training cohort and 89 patients (89.9%) in the testing cohort were in histological grade II and III. 225 cases (97.4%) and 95 cases (96.0%) were ER positive in the training and testing cohort, respectively. 198 cases (85.7%) and 88 cases (88.9%) had PR positive tumors in the training and testing cohorts, respectively. 141 cases (61.0%) and 68 cases (68.7%) had Ki67 positive tumors, and 178 cases (77.1%) and 81 cases (81.8%) underwent postoperative chemotherapy in the training and testing cohorts, respectively (see Table 1). Among the pathological types, invasive ductal carcinoma accounted for 87%, while the rest were invasive lobular carcinoma, medullary carcinoma, and other types. Chi-square analysis showed that there was no significant difference between the two cohorts. Finally, the characteristics of all 330 patients were compiled and analyzed. The median follow-up time was 95 months (range: 20-104 months). During follow-up, there were 89 patients who developed recurrence and 56 patients died. The OS and DFS for all 330 cases were 83.3% and 73.3%, respectively.

Establishment and Validation of the 6-IHC Prognostic Models

Univariate Cox regression analysis showed that all six biomarkers were associated with DFS ($P \leqq 0.006$). Multivariate Cox regression analysis showed that MMP, Cyclin B1, and Aurora A were associated with DFS ($P < 0.05$) (Table 2). These results indicated that higher expression of 6-IHC was associated with shorter DFS. Thus, a combined 6-IHC prognosis risk score was established based on a linear combination of the regression coefficient derived from the multivariate Cox regression model (β) multiplied by its expression level. The prognostic score formula was:

PI = (1.2*expression level of Cathepsin L2) + (1.3*expression level of MMP11) + (1.4*expression level of Cyclin B1) + (1.3*expression level of Aurora A) + (1.2*expression level of Survivin) + (1.4*expression level of Ki67).

The expression level in the above formula was the percentage of immunohistochemical positive staining of each biomarker.

The six independent prognostic factors (6-IHC) were used to establish a model to predict the 8-year DFS and OS rates of HR+/HER2- BC patients. The prediction ability of the 6-IHC score was evaluated by calculating the AUC of the ROC curve. The largest AUC was 0.752 and the highest sensitivity and specificity was 67.2% and 72.3%, respectively (Figure 2A). The cut-off value for the combined 6-IHC score was 2.16, which was the highest Youden index in the ROC curve based on 231 BC patients in training cohort. In addition, based on the cut-off value (PI = 2.16), 231 breast cancer patients in the training cohort were divided into two groups: a “High” risk group (PI ≥2.16, n = 87) and a “Low” risk group (PI <2.16, n = 144). Kaplan-Meier curves showed that the OS (Figure 2B) and DFS (Figures 2C) were significantly different between the “High” and “Low” risk groups (training cohort: 67.3% vs 89.7%, P <0.001 for OS, and 55.2% vs 86.8%, P <0.001 for DFS), indicating a good prediction ability of the 6-IHC prognostic model using 2.16 as the PI cut-off value.

To further verify the prediction value of the 6-IHC prognostic model and test the cut-off value (PI = 2.16), 99 BC patients in testing cohort were divided into “High” risk group (PI≥2.16, n = 43) and “Low” risk group (PI <2.16, n = 56) and analyzed by ROC curve (Figure 2D). The AUC in the testing cohort was 0.769 (95% CI: 0.669–0.869), which was comparable to that in the training cohort (0.752) (95% CI: 0.684–0.820) (see Figure 2A). The sensitivity and specificity in the testing cohort was 66.7% and 66.8%, respectively. KM curves showed that there was significant difference of OS (Figure 2E: 65.1% vs 93.0%, P <0.001) and DFS (Figure 2F: 53.5% vs 82.1%, P <0.001) between the “High” risk and “Low” risk groups in the testing cohort based on cut-off PI of 2.16.

Combined 6-IHC Score Prognostic Model Was Independent of Prognostic Clinical Factors

Univariate and multivariate Cox regression analysis were conducted to assess the independent predictive value of the 6-IHC prognostic model for the 330 breast cancer patients. Univariate Cox regression analysis showed that patients' tumor size, lymph node metastasis, histological grade, and 6-IHC score had prognostic value, while the age, menstrual status, ER status, PR status, chemotherapy information, and Ki67 status were not associated with DFS and OS (Table 3). Therefore, we included patients' tumor size, lymph node metastasis, histological grade, and 6-IHC score in multivariate Cox regression analysis. The results showed that tumor size (P = 0.017, HR: 3.46, 95% CI: 1.25~9.58), lymph node metastasis (P < 0.001, HR: 5.83, 95% CI: 3.05~11.14), and 6-IHC score (P < 0.001, HR: 3.51, 95% CI: 2.24~5.50) were independent prognostic factors associated with DFS. The tumor size (P = 0.071, HR: 3.75, 95% CI: 0.89~15.69), lymph node metastasis (P < 0.001, HR: 7.52, 95% CI: 2.96~19.20), and 6-IHC score (P < 0.001, HR: 4.18, 95% CI: 2.30~7.62) were also independent prognostic factors associated with OS (see Table 3).

Comparison of 6-IHC Score with Ki67 to Predict the Benefit of Chemotherapy in Hormone Receptor-positive Breast Cancer

A retrospective analysis was performed for all 330 cases as shown in Table 4. We categorized 6-IHC scores at the threshold of PI = 2.16 and Ki67 at the cutoff of 14% to distinguish “Low” from “High” risk of recurrence and survival. Based on the NCCN and St. Gallen International Expert Consensus [7–9],

Chemotherapy was usually recommended under the clinical condition of tumor size ≥2 cm, ALN-positive, or Ki67 >14% before 2015 and Ki67 >20% after 2015. Our results showed that the percentage of patients with tumor size ≥2 cm (86.9% vs 72.5%) and ALN+ (63.8% vs 49.5%) in “High” risk group was significantly higher than that in the “Low” risk group based on 6-IHC score (P = 0.002 and p = 0.011, respectively). In contrast, there was no significant difference between “Low” and “High” risk groups based on Ki67 index (see Table 4). All patients in this study received HT. The percentage of patients with chemotherapy or without chemotherapy in the “High” risk group was not significantly different from that in the “Low” risk group based on either 6-IHC score (P = 0.174) or using Ki67 index (p = 0.572)(see Table 4). The tumor recurrence rates in the “Low” risk and “High” risk groups were 13.2% and 44.8%, respectively, with an accuracy rate of 71.0% based on 6-IHC score. The tumor recurrence rates in the “Low” risk and “High” risk groups were 24.4% and 25.5%, respectively, with an accuracy rate of 45.0% based on Ki67 index. Based on all 330 BC cases, the generated AUC value was 0.754 (Figure 3) for 6-IHC score (95% CI 0.698-0.811, with a sensitivity of 67.1% specificity of 70.7% and accuracy = 69.7%), however, the AUC value was 0.574 (see Figure 3) for Ki67 index (95% CI 0.505-0.643, with sensitivity = 65.9% specificity = 28.5% accuracy = 38.5%). AUC was significantly different between 6-IHC score and Ki67 index (P <0.001) (see Figure 3). These results indicated that the 6-IHC score was more powerful than Ki67 index alone in the prediction of tumor recurrence and DFS in the target subtype of BC patients.

The 8-year DFS and OS of all the 330 HR+/HER2- BC patients were 73.3% and 83.3% respectively. The 8-year DFS and OS were 54.6% and 69.2%, respectively in the 6-IHC score “High” risk group, whereas the 8-year DFS and OS were 85.5% and 92.5%, respectively in the 6-IHC score “Low” risk group. The 8-year DFS and OS were 70.8% and 80.9%, respectively in the Ki67 “High” risk group, whereas the 8-year DFS and OS were 77.7% and 87.6%, respectively in the Ki67 “Low” risk group. The KM curves for DFS revealed that chemotherapy did not significantly improve the prognosis (Figure 4A, p = 0.911) in the 6-IHC score “Low (PI <2.16)” risk group in which 152 (76%) patients received chemotherapy. In contrast, in the 6-IHC score “High” risk group, chemotherapy significantly improved the prognosis (Figure 4B, P = 0.010). However, The KM curves for DFS showed that chemotherapy did not significantly improve the prognosis either in the Ki67 “Low” (Figure 4C, p = 0.933) or “High” (Figure 4D, p = 0.187) risk groups. These results indicated that chemotherapy could be omitted in the 6-IHC score “Low” risk group, while in 6-IHC score “High” risk group, survival benefit could be obtained from chemotherapy, indicating that the 6-IHC score, but not Ki67 index alone, might be an indicator that can distinguish the benefit of chemotherapy for HR+/HER2- BC patients.

Discussion

The treatment of breast cancer is increasingly shifting towards personalized medicine, particularly for HR+/HER2- subtype. Current guidelines often classify this subtype of breast cancer to the intermediate-risk group. And hormonal therapy (HT) might be enough and chemotherapy could be omitted in some patients of this subtype. Based on some clinical trials [10–13], several commercial tests,such as the 21-gene recurrence score (Oncotype DX®), the 70-gene signature (MammaPrint®), the PAM50 (Prosigna®),

and the 11-gene assay (EndoPredict®) are currently available in some countries for systemic treatment decision-making. Among those, Oncotype DX has been recommended by the National Comprehensive Cancer Network (NCCN), American Society of Clinical Oncology (ASCO), European Society for Medical Oncology (ESMO), and the St. Gallen International Expert Consensus guidelines[7,9,14]. However, use of Oncotype DX in routine practice is hampered in some countries such as European countries and China because these molecular profiling tests are costly and not covered by the National Health Insurance System[15]. Cost and technical barriers make it difficult to be applied in most developing countries. In addition, there are about 30% of the tumors that do not belong to any of the molecular subtypes[16] based on microarray analysis of even thousands of genes. In China, although some biological companies can do the test, there is no authoritative scientific testing institutions, hence the lack of standardization and monitoring of the test. Additionally, the test takes time.

Though the IHC-based and the gene expression profile-based classifications are not completely overlapped[17]，IHC techniques have subsequently been developed to identify 4 subtypes of invasive BC clinically: basal-like, HER2-enriched, luminal A, and luminal B, due to the concordance of gene expression and IHC profiles. The 2011 St Gallen Breast Cancer Conference Expert Panel proposed to use IHC criteria to evaluate the gene expression-based classification and then allow its use in clinical practice[1]. The percentage of Ki67 positive tumor cells, or the Ki67 index, is used clinically to distinguish Luminal A from Luminal B subtypes[18]. Ki67 index is also considered as a predictor of pathological complete response[19–21], response to chemotherapy[22,23], and likelihood of relapse[24,25]. However, use of Ki67 index has some disadvantages. First, universally-accepted guidelines for analysis and clinical interpretation of Ki67 staining is not available[26,27]. Second, there are no well-designed prospective clinical trials that specifically focuse on Ki67 and demonstrate that Ki67 index can differentiate Luminal A from B subtypes. Thus, the St. Gallen Expert Panel recommended a Ki67 cut-point of 14% in 2011 according to the vote, but subsequently withdrew their recommendation of the cut-point due to the variability across different centers[28]. Third, even there was consensus regarding to the cut-off values and scoring methods, inter-observer variability in manual scoring still exists, even among pathologists[29–32]. Similar to Oncotype DX®, combined 6 IHC multi-biomarkers should be more reliable than Ki67 alone. Our results demonstrated that combined 6-IHC could overcome the shortcomings, e.g., lacking analytical validity, less repeatability, and different cut-off values for Ki67 index.

Two prospective clinical trials (the TAILORx[13] and the Phase III Plan B West German Study Group Trial[33]) showed good 5-year DFS rate in the low-risk group without chemotherapy. However the cut-off value in the trials for the low-risk group was lower than previously described, which is similar to the IHC methodology. In addition, another shortcoming of the TAILORx trial was that it targeted patients with lymph node pN0, and large numbers of patients with positive lymph nodes were excluded. The data in this study showed that 63.0% of patients in 6-IHC score “High” risk group had ALN metastasis, while only 49.5% of patients in the 6-IHC score “Low” risk group had ALN metastasis ($p = 0.011$), indicating that 6-IHC score might also predict ALN metastasis and ALN+ was one of the independent prognostic factors. 6-IHC score might be a good substitute for Oncotype DX, and could be immediately obtained from routine

pathology report together with Ki67 result. Studies of the prognostic value of any new marker should also examine the extra benefit of including Ki67 when traditional clinico-pathological variables are also included.

The Ki67 index cut-off value was set at 14% [1] by St. Gallen Expert Panel until 2015 [28]. The patients in our study were diagnosed between 2010 and 2016, and the patients diagnosed before 2015 accounted for the majority of the total subjects. We still chose 14% as the cut-off value of Ki67 index in this retrospective analysis because chemotherapy decision was made mainly based on this level. However, KM curve for DFS showed that chemotherapy did not significantly improve the prognosis either in the Ki67 “Low (<14%)” ($p = 0.834$) or “High (≥14%)” ($p = 0.194$) risk groups. In contrast, the combined 6-IHC score can effectively distinguish patients who are at high risk of recurrence and require chemotherapy for HR+/HER2- invasive early stage breast cancer. In 2015, a majority of the Panel of St. Gallen conference voted to accept a cut-off value of Ki67 within the range of 20%–29% to distinguish ‘luminal B-like’ disease [28]. Criscitiello et al investigated 1241 patients with Luminal B early stage breast cancer with 1-3 axillary positive nodes and found that patients with high Ki67 expression (>32%) could benefit from adjuvant chemotherapy in addition to HT[34]. Kang et al. reported that patients with high Ki67 expression had poorer RFS and OS compared with those with low Ki67 expression (10% of cutoff) only when the PR was low[35]. Unfortunately, we did not test the cohort at this Ki67 level. However, the strength of the current study lies in 1) the long-term follow-up and detailed clinical information; 2) the unique largest IHC multi-biomarker data set available for this cohort; and 3) the comprehensive analytical approaches.

To the best of our knowledge, we showed for the first time that combined 6-IHC score, together with tumor size, lymph node metastasis, was independent prognostic factor. Combined 6-IHC score is positively associated with recurrences, increased risk of death, as well as predictive benefit effect of chemotherapy. Most importantly, we demonstrated that patients with low 6-IHC expression might not benefit from adjuvant chemotherapy, indicating that hormonal therapy might be sufficient and chemotherapy can be omitted in HR+/HER2- subtype BC if 6-IHC score was under the cut-off value of 2.16.

The major limitations of this study were its retrospective nature; performed in a single center, and a relatively small sample size. Because we do not have Oncotype DX data for this cohort, direct comparison of 6-IHC score with “21-gene recurrence score” of Oncotype DX was not performed. We did not get the positive association between 6-IHC score and OS rate (data not shown), which is probably duo to the quite low number of death in this cohort. This study was a single-center study, and the predictive power of the prediction model still needs to be verified by larger sample size and multi-center evidence-based medical study in order to control the confounding factors more effectively and establish a more accurate prediction model. In addition, there is not a high degree of consistency between gene-based and IHC-based approaches, so it is difficult to determine if one approach might be an appropriate alternative to the other. The well-designed prospective studies with more patient size are required to confirm our results.

Conclusions

Combined 6-IHC score predicted cancer recurrence and survival in HR+/ her2-bc patients to a certain extent, and identified patients who could benefit from adjuvant chemotherapy without considering axillary lymph node (ALN) metastasis to reduce overtreatment

List Of Abbreviations

HR:hormone receptor

IHC: immunohistochemistry

BC:breast cancers

PI：prognosis index

KM：Kaplan-Meier

DFS：Disease-free survival

OS：overall survival

ROC：receiver operating characteristic

ALN：axillary lymph node

CT：chemotherapy

PgR：progesterone receptor

HT：hormone therapy

TMA：tissue microarray

FISH：fluorescence in situ hybridization

AUC：area under the ROC curve

NCCN：National Comprehensive Cancer Network

ASCO：American Society of Clinical Oncology

CAP：College of American Pathologists

ESMO：European Society for Medical Oncology

Declarations

Ethics approval and consent to participate

Ethical approval was granted by First Affiliated Hospital Research Ethics Committee in accordance with the principles of the Declaration of Helsinki.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors have declared no conflicts of interest.

Funding

This work was supported by grants from the Natural Science Foundation of Liaoning Province (No. 20180551215), Science and Technology Plan Project of Liaoning Province (2013225585).The funding sources no such involvement other parts of the article.

Authors' contributions

JL designed the research, conducted the data collection and analysis, constructed the model and interpreted the data, and was a major contributor to the manuscript.ZG and SW conducted data acquisition and analysis and supervised part of the research.XZ designed and oversaw the study and made critical revisions to the manuscript.All authors read and approved the final manuscript.

Acknowledgements

Not applicable

References

1. Goldhirsch A, Wood WC, Coates AS, Gelber RD, Thürlimann B, Senn HJ. Strategies for subtypes-dealing with the diversity of breast cancer: Highlights of the St Gallen international expert consensus on the primary therapy of early breast cancer 2011. Ann Oncol. 2011;22:1736–47.
2. Gnant M, Steger GG. Fighting overtreatment in adjuvant breast cancer therapy. Lancet. 2009. p. 2029–30.
3. Alexandre M, Maran-Gonzalez A, Viala M, Firmin N, D'hondt V, Gutowski M, et al. Decision of adjuvant systemic treatment in HR+ HER2-early invasive breast cancer: Which biomarkers could help? Cancer

Manag. Res. 2019. p. 10353–73.

4. Winslow S, Leandersson K, Edsjö A, Larsson C. Prognostic stromal gene signatures in breast cancer. Breast Cancer Res. 2015;17.
5. Klimov S, Rida PC, Aleskandarany MA, Green AR, Ellis IO, Janssen EA, et al. Novel immunohistochemistry-based signatures to predict metastatic site of triple-negative breast cancers. Br J Cancer [Internet]. 2017;117:826–34. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28720841
6. Hammond ME, Hayes DF, Dowsett M, Allred DC, Hagerty KL, Badve S, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer (unabridged version). Arch Pathol Lab Med [Internet]. 2010;134:e48-72. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20586616
7. Gradishar WJ, Anderson BO, Balassanian R, Blair SL, Burstein HJ, Cyr A, et al. Breast Cancer, Version 4.2017, NCCN Clinical Practice Guidelines in Oncology. J Natl Compr Canc Netw [Internet]. 2018;16:310–20. Available from: http://www.ncbi.nlm.nih.gov/pubmed/29523670
8. Senkus E, Kyriakides S, Ohno S, Penault-Llorca F, Poortmans P, Rutgers E. Primary breast cancer: ESMO Clinical Practice clinical practice guidelines. Ann Oncol. 2015;26.
9. Harris L, Fritsche H, Mennel R, Norton L, Ravdin P, Taube S, et al. American society of clinical oncology 2007 update of recommendations for the use of tumor markers in breast cancer. J. Clin. Oncol. 2007. p. 5287–312.
10. Mamounas EP, Tang G, Fisher B, Paik S, Shak S, Costantino JP, et al. Association between the 21-gene recurrence score assay and risk of locoregional recurrence in node-negative, estrogen receptor-positive breast cancer: results from NSABP B-14 and NSABP B-20. J Clin Oncol [Internet]. 2010;28:1677–83. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20065188
11. Tang G, Shak S, Paik S, Anderson SJ, Costantino JP, Geyer Jr. CE, et al. Comparison of the prognostic and predictive utilities of the 21-gene Recurrence Score assay and Adjuvant! for women with node-negative, ER-positive breast cancer: results from NSABP B-14 and NSABP B-20. Breast Cancer Res Treat [Internet]. 2011;127:133–42. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21221771
12. Cardoso F, van't Veer LJ, Bogaerts J, Slaets L, Viale G, Delaloge S, et al. 70-Gene Signature as an Aid to Treatment Decisions in Early-Stage Breast Cancer. N Engl J Med [Internet]. 2016;375:717–29. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27557300
13. Sparano JA, Gray RJ, Makower DF, Pritchard KI, Albain KS, Hayes DF, et al. Prospective Validation of a 21-Gene Expression Assay in Breast Cancer. N Engl J Med [Internet]. 2015;373:2005–14. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26412349
14. Senkus E, Kyriakides S, Ohno S, Penault-Llorca F, Poortmans P, Rutgers E, et al. Primary breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol [Internet]. 2015;26 Suppl 5:v8-30. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26314782

15. Vissio E, Metovic J, Osella-Abate S, Bertero L, Migliaretti G, Borella F, et al. Integration of Ki-67 index into AJCC 2018 staging provides additional prognostic information in breast tumours candidate for genomic profiling. Br J Cancer [Internet]. 2020;122:382–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/31780778
16. Pusztai L, Mazouni C, Anderson K, Wu Y, Symmans WF. Molecular classification of breast cancer: limitations and potential. Oncologist [Internet]. 2006;11:868–77. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16951390
17. Guiu S, Michiels S, Andre F, Cortes J, Denkert C, Di Leo A, et al. Molecular subclasses of breast cancer: how do we define them? The IMPAKT 2012 Working Group Statement. Ann Oncol [Internet]. 2012;23:2997–3006. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23166150
18. Geyer FC, Rodrigues DN, Weigelt B, Reis-Filho JS. Molecular classification of estrogen receptor-positive/luminal breast cancers. Adv Anat Pathol [Internet]. 2012;19:39–53. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22156833
19. Kurozumi S, Inoue K, Takei H, Matsumoto H, Kurosumi M, Horiguchi J, et al. ER, PgR, Ki67, p27(Kip1), and histological grade as predictors of pathological complete response in patients with HER2-positive breast cancer receiving neoadjuvant chemotherapy using taxanes followed by fluorouracil, epirubicin, and cyclophosphamide conco. BMC Cancer [Internet]. 2015;15:622. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26345461
20. Sinn HP, Schneeweiss A, Keller M, Schlombs K, Laible M, Seitz J, et al. Comparison of immunohistochemistry with PCR for assessment of ER, PR, and Ki-67 and prediction of pathological complete response in breast cancer. BMC Cancer [Internet]. 2017;17:124. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28193205
21. Yoshioka T, Hosoda M, Yamamoto M, Taguchi K, Hatanaka KC, Takakuwa E, et al. Prognostic significance of pathologic complete response and Ki67 expression after neoadjuvant chemotherapy in breast cancer. Breast Cancer [Internet]. 2015;22:185–91. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23645542
22. Kim KI, Lee KH, Kim TR, Chun YS, Lee TH, Park HK. Ki-67 as a predictor of response to neoadjuvant chemotherapy in breast cancer patients. J Breast Cancer [Internet]. 2014;17:40–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24744796
23. Brown JR, DiGiovanna MP, Killelea B, Lannin DR, Rimm DL. Quantitative assessment Ki-67 score for prediction of response to neoadjuvant chemotherapy in breast cancer. Lab Invest [Internet]. 2014;94:98–106. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24189270
24. Pathmanathan N, Balleine RL. Ki67 and proliferation in breast cancer. J. Clin. Pathol. 2013. p. 512–6.
25. Vincent-Salomon A, Hajage D, Rouquette A, Cedenot A, Gruel N, Alran S, et al. High Ki67 expression is a risk marker of invasive relapse for classical lobular carcinoma in situ patients. Breast [Internet]. 2012;21:380–3. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22531230
26. Dowsett M, Nielsen T O, A'Hern R, Bartlett R, Coombes R C, Cuzick J, Ellis M, Henry N L, Hugh J C, Lively T, McShane L, Paik S, Penault-Llorca F, Prudkin L, Regan M, Salter J, Sotiriou C, Smith I E, Viale

G, Zujewski J A HDF. Assessment of Ki67 in Breast Cancer. J Nati Cancer Inst. 2011;103:1656–64.

27. Jang MH, Kim HJ, Chung YR, Lee Y, Park SY. A comparison of Ki-67 counting methods in luminal Breast Cancer: The Average Method vs. the Hot Spot Method. PLoS One [Internet]. 2017;12:e0172031. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28187177
28. Coates AS, Winer EP, Goldhirsch A, Gelber RD, Gnant M, Piccart-Gebhart MJ, et al. Tailoring therapies-improving the management of early breast cancer: St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2015. Ann Oncol. 2015;26:1533–46.
29. Klauschen F, Wienert S, Schmitt WD, Loibl S, Gerber B, Blohmer JU, et al. Standardized Ki67 Diagnostics Using Automated Scoring–Clinical Validation in the GeparTrio Breast Cancer Study. Clin Cancer Res [Internet]. 2015;21:3651–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25501130
30. Polley MY, Leung SC, McShane LM, Gao D, Hugh JC, Mastropasqua MG, et al. An international Ki67 reproducibility study. J Natl Cancer Inst [Internet]. 2013;105:1897–906. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24203987
31. Shui R, Yu B, Bi R, Yang F, Yang W. An interobserver reproducibility analysis of Ki67 visual assessment in breast cancer. PLoS One [Internet]. 2015;10:e0125131. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25932921
32. Varga Z, Diebold J, Dommann-Scherrer C, Frick H, Kaup D, Noske A, et al. How reliable is Ki-67 immunohistochemistry in grade 2 breast carcinomas? A QA study of the Swiss Working Group of Breast- and Gynecopathologists. PLoS One [Internet]. 2012;7:e37379. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22662150
33. Gluz O, Nitz UA, Christgen M, Kates RE, Shak S, Clemens M, et al. West German Study Group Phase III PlanB Trial: First Prospective Outcome Data for the 21-Gene Recurrence Score Assay and Concordance of Prognostic Markers by Central and Local Pathology Assessment. J Clin Oncol [Internet]. 2016;34:2341–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26926676
34. Criscitiello C, Disalvatore D, De Laurentiis M, Gelao L, Fumagalli L, Locatelli M, et al. High Ki-67 score is indicative of a greater benefit from adjuvant chemotherapy when added to endocrine therapy in luminal B HER2 negative and node-positive breast cancer. Breast [Internet]. 2014;23:69–75. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24314620
35. Kang YJ, Lee HB, Kim YG, Han J, Kim Y, Yoo TK, et al. Ki-67 Expression is a Significant Prognostic Factor Only When Progesterone Receptor Expression is Low in Estrogen Receptor-Positive and HER2-Negative Early Breast Cancer. J Oncol [Internet]. 2019;2019:7386734. Available from: http://www.ncbi.nlm.nih.gov/pubmed/31975992

Tables

Table 1. Baseline characteristics of the study population

Characteristics	Case(n=330)	Training cohort(n=231)	Testing cohort(n=99)	X^2	P-value
Age year					
Median (range)	53(28-82)	50(28-82)	52(31-78)		
Mean ± SD	52.5±11.4	52.3±11.3	52.9±11.6		
Distribution, *n* (%)				0.68	0.41
≤60	253	180(77.9)	73(73.7)		
>60	77	51(22.1)	26(26.3)		
Menopausal status, *n*(%)				0.27	0.60
Premenopausal	100	68(29.4)	32(32.3)		
Postmenopausal	230	163(70.6)	67(67.7)		
Size, n (%)				0.57	0.45
<2cm	71	53(22.9)	19(19.2)		
≥2cm	259	178(77.1)	80(80.8)		
Node Status, n (%)				0.01	0.92.
Positive	182	127(55.0)	55(55.6)		
Negative	148	104(45.0)	44(44.4)		
Histological grade, n (%)				1.50	0.22
Unkwon or I	45	35(15.2)	10(10.1)		
II or III	285	196(84.8)	89(89.9)		
ER status, n (%)				<0.001	1.00
Positive	320	224(97.0)	96(97.0)		
Negative	10	7(3.0)	3(3.0)		
PgR status, n (%)				0.60	0.44
Positive	286	198(85.7)	88(88.9)		
Negative	44	33(14.3)	11(11.1)		
Ki67 status, n (%)				0.93	0.34
<14%	121	90(39.0)	31(31.3)		
≥14%	209	141(61.0)	68(68.7)		
Chemotherapy after surgery, n (%)				0.93	0.34
No	71	53(22.9)	18(18.2)		
Yes	259	178(77.1)	81(81.8)		

* Meet any of the following conditions to be considered menopausal: 1. After bilateral ovariectomy. 2. Age≥60 years. 3. Age<60 years, natural menopause≥12 months, FSH and estradiol levels in the postmenopausal range without chemotherapy, tamoxifen, toremifene, or ovarian castration in the past year. 4. Patients <60 years of age who are taking tamoxifen or toremifene, FSH and estradiol levels are in the postmenopausal range.

Table 2. Univariable and Multivariable Cox regression analysis of expression of 6 IHCs.

Marker	Univariate		Multivariate	
	HR(95% CI)	P-value	HR(95% CI)	P-value
CathepsinL2	1.020(1.009~1.032)	<0.001	1.012(1.000~1.025)	0.091
MMP11	1.018(1.007~1.029)	0.001	1.013(1.001~1.024)	0.037
CyclinB1	1.022(1.012~1.032)	<0.001	1.014(1.002~1.025)	0.020
AuroraA	1.024(1.013~1.035)	<0.001	1.013(1.001~1.026)	0.035
Survivin	1.015(1.004~1.026)	0.006	1.012(1.000~1.023)	0.063
Ki67	1.020(1.006~1.034)	0.005	1.014(0.999~1.029)	0.058

CI：confidence interval；HR：hazard ratio

Cox regression *analysises are based on continuous variables with a percentage of positive expression of 6 IHCs.*

Table 3. Univariate and multivariate Cox regression analyses for DFS and OS in all HR+HER2- early invasive breast cancer cases (n=330).

Variables	Univariate Cox regression			Multivariate Cox regression		
	HR	95% CI	P-value	HR	95% CI	P-value
DFS						
Age at diagnosis (≤60 vs. >60 years)	1.463	0.925~2.313	0.104	-	-	-
Menopausal status(Pre vs. Post)	1.116	0.702~1.774	0.642	-	-	-
Size(<2cm vs. ≥2cm)	6.794	2.491~18.528	**<0.001**	3.459	1.249~9.584	**0.017**
Node Status(Positive vs. Negative)	7.217	3.834~13.583	**<0.001**	5.830	3.051~11.138	**<0.001**
Histological grade(unkwon or I vs. II or III)	3.708	1.360~10.111	**0.010**	1.088	0.388~3.048	0.873
ER status(Positive vs. Negative)	0.653	0.240~1.782	0.406	-	-	-
PgR status(Positive vs. Negative)	1.278	0.662~2.469	0.465	-	-	-
Ki67 status(<14% vs. ≥14%)	1.437	0.913~2.261	0.117	-	-	-
Chemotherapy after surgery(No vs. Yes)	0.757	0.467~1.227	0.259	-	-	-
6–IHC prognostic model(Low risk vs. High risk)	4.061	2.601~6.340	**<0.001**	3.510	2.241~5.499	**<0.001**
OS						
Age at diagnosis (≤60 vs. >60 years)	1.413	0.789~2.529	0.244	-	-	-
Menopausal status(Pre vs. Post)	1.169	0.646~2.116	0.606	-	-	-
Size(<2cm vs. ≥2cm)	8.212	2.001~33.702	**0.003**	3.746	0.894~15.689	**0.071**
Node Status(Positive vs. Negative)	9.723	3.875~24.395	**<0.001**	7.519	2.945~19.198	**<0.001**
Histological grade(unkwon or I vs. II or III)	4.546	1.108~18.658	**0.036**	1.144	0.269~4.858	0.855
ER status(Positive vs. Negative)	1.734	0.240~12.535	0.585	-	-	-
PgR status(Positive vs. Negative)	1.310	0.561~3.059	0.532	-	-	-
Ki67 status(<14% vs. ≥14%)	1.615	0.892~2.924	0.114	-	-	-
Chemotherapy after surgery(No vs. Yes)	0.838	0.450~1.560	0.576	-	-	-
6–IHC prognostic model(Low risk vs. High risk)	5.011	2.765~9.079	**<0.001**	4.184	2.299~7.617	**<0.001**

CI：confidence interval；HR：hazard ratio

Ki67 in Table4 is a two-category variable that is classified as low risk (Ki67<14%) and high risk (Ki67≥14%) by 14%, which is different from Table2.

Table 4. Correlation between 6-IHC prognostic model and Ki67 with clinicopathological characteristics in all HR+HER2-early invasive breast cancer cases (n=330)

	6-IHC prognostic model			Ki67		
	Low risk (n=200)	High risk (n=130)	P-value	<14% (n=121)	≥14% (n=209)	P-value
Age year						
Median (range)	49(28-78)	53(31-82)		54(28-78)	49(31-82)	
Mean ± SD	52.2±11.0	53.0±12.0		53.8±12.0	51.8±11.0	
Distribution, *n* (%)			0.657			0.069
≤60	155(77.5)	98(75.4)		86(71.1)	167(79.9)	
>60	45(22.5)	32(24.6)		35(28.9)	42(20.1)	
Menopausal status, *n*(%)			0.557			0.679
Premenopausal	63(31.5)	37(28.5)		35(28.9)	65(31.1)	
Postmenopausal	137(68.5)	93(71.5)		86(71.1)	144(68.9)	
Size, n (%)			0.002			0.699
<2cm	55(27.5)	17(13.1)		25(20.7)	47(22.5)	
≥2cm	145(72.5)	113(86.9)		96(79.3)	162(77.5)	
Node Status, n (%)			0.011			0.391
Positive	99(49.5)	83(63.8)		63(52.1)	119(56.9)	
Negative	101(50.5)	47(36.2)		58(47.9)	90(43.1)	
Histological grade, n (%)			0.063			0.406
Unkwon or I	33(16.5)	12(9.2)		14(11.6)	31(14.8)	
II or III	167(83.5)	118(90.8)		107(88.4)	178(85.2)	
ER status, n (%)			0.489			0.380
Positive	195(97.5)	125(96.2)		11(95.9)	204(97.6)	
Negative	5(2.5)	5(3.8)		5(4.1)	5(2.4)	
PgR status, n (%)			0.825			0.964
Positive	174(87.0)	112(86.2)		105(86.8)	181(86.6)	
Negative	26(13.0)	18(13.8)		16(13.2)	28(13.4)	
Chemotherapy after surgery, n (%)			0.174			0.572
No	48(24.0)	23(17.7)		24(19.8)	47(22.5)	
Yes	152(76.0)	107(82.3)		97(80.2)	162(77.5)	

Figures

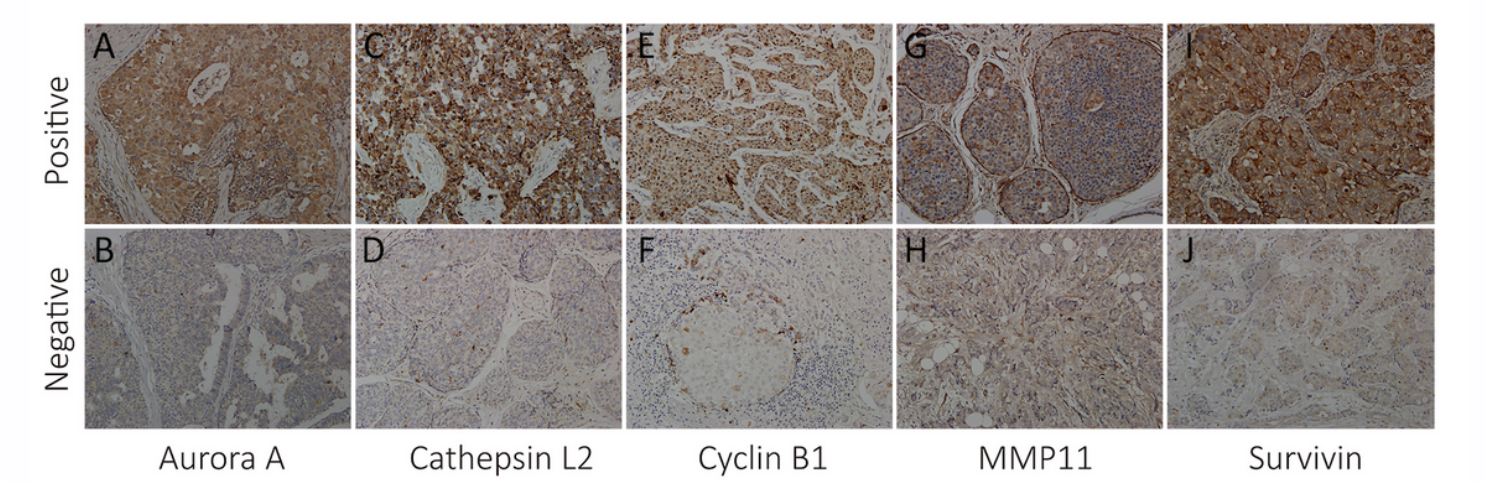

Figure 1

Immunohistochemical staining images. Representative immunohistochemical staining images of breast cancer tissues were quantifies as average positive staining area percentage (H&E, X20) and negative control.

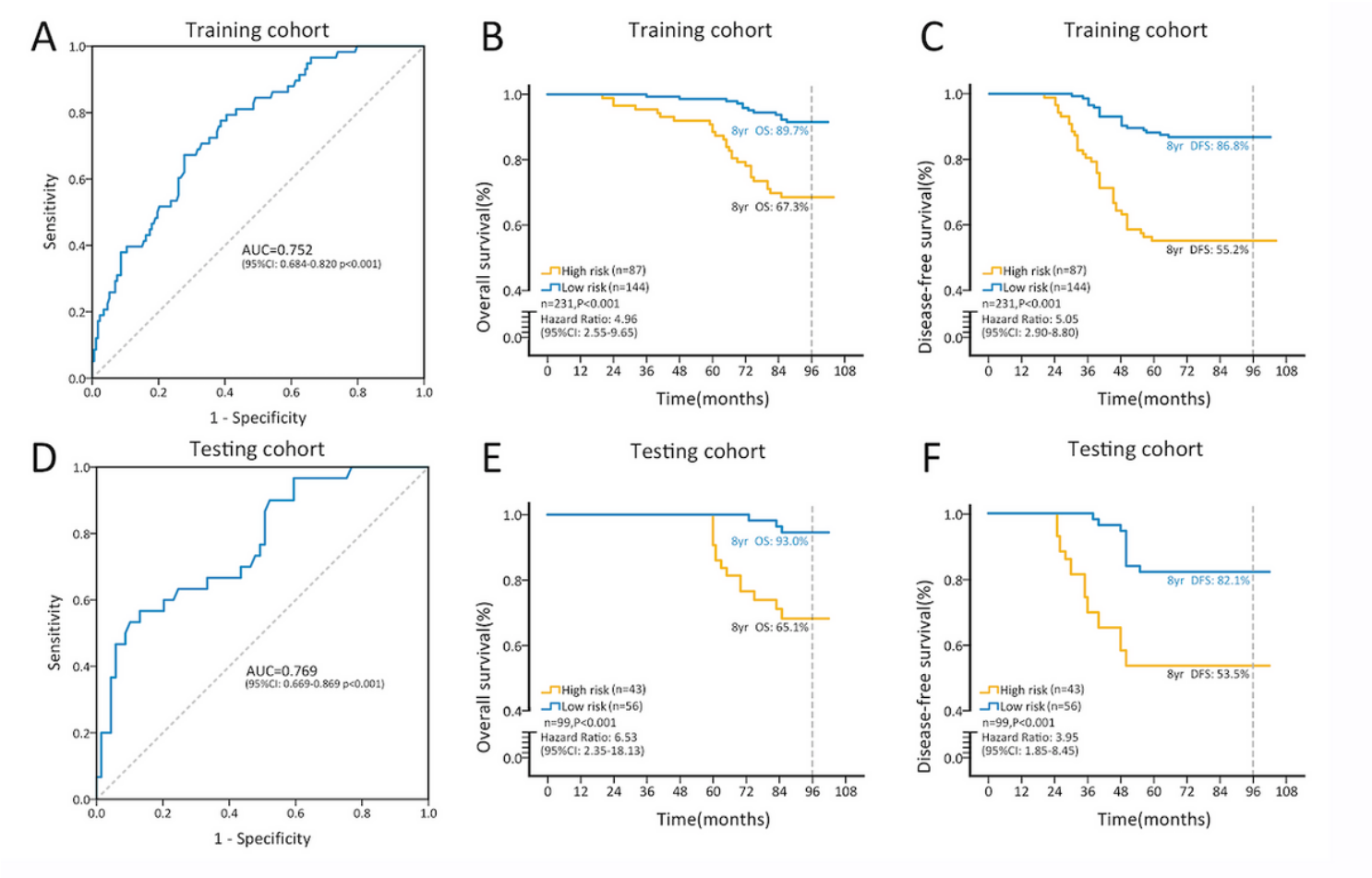

Figure 2

ROC curve and Kaplan-Meier (KM) curves of training cohort and testing cohort. Optimal cutoff point of prognosis index (PI) determined by ROC curves and cancer-specific survival DFS/OS by KM curves between the high risk group (PI≥2.16) and low risk group (PI<2.16) in training and testing cohort. A, ROC curve of the training cohort (AUC=0.752, 95%CI: 0.684–0.820 p<0.001). B, KM curves of OS in training cohort (n=231). C, KM curves of DFS in training cohort (n=231). D, ROC curve of the testing cohort (AUC=0.769, 95%CI: 0.669–0.869 p<0.001). E, KM curves of OS in testing cohort (n=99). F, KM curves of DFS in testing cohort (n=99).

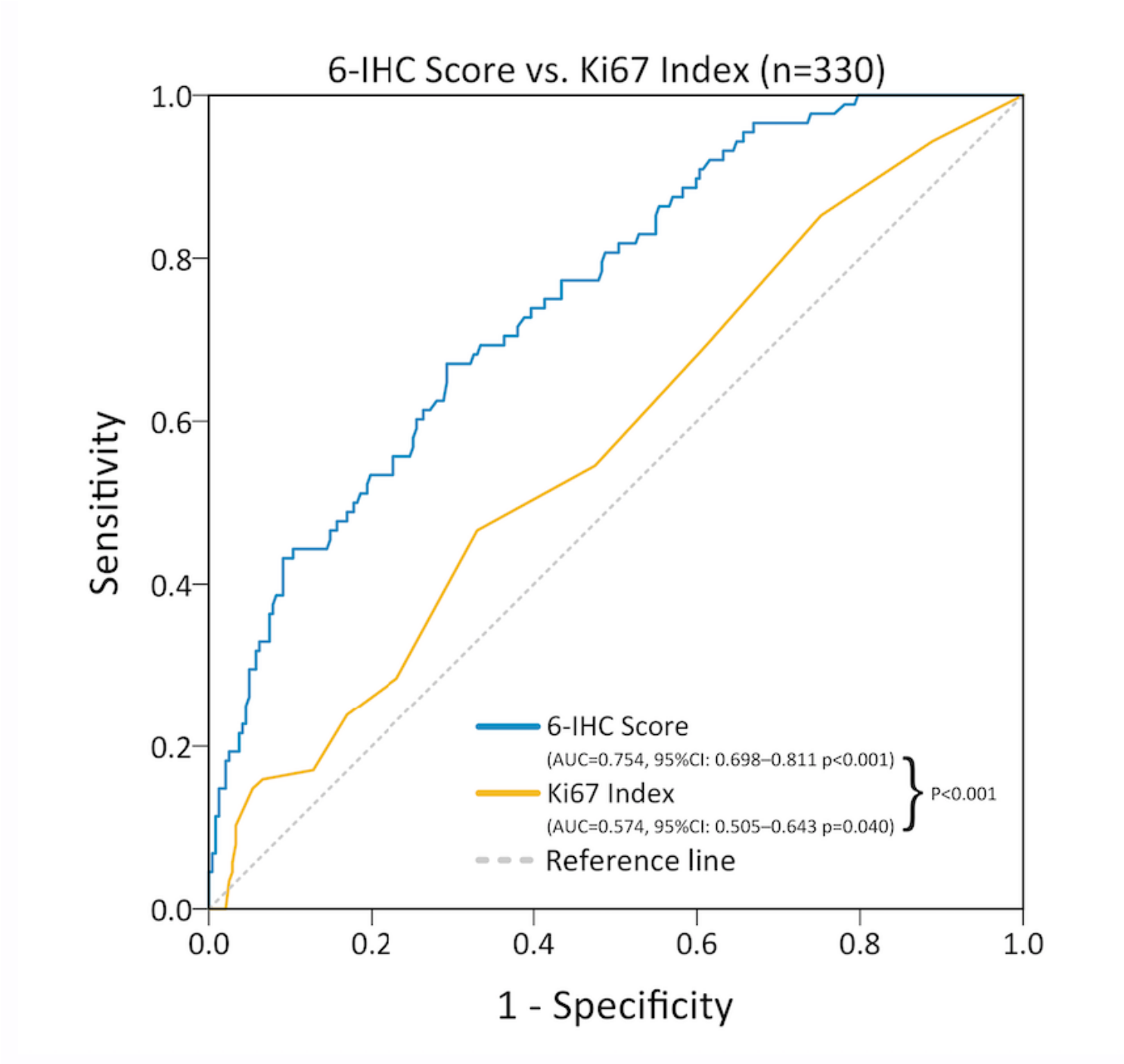

Figure 3

ROC curve of 6-IHC score and Ki67 index. ROC analysis of DFS by the 6-IHC score prognostic model (AUC=0.752, 95%CI: 0.684–0.820 p<0.001) and Ki67 index (AUC=0.576, 95%CI: 0.490–0.662 p=0.084) in all 330 HR+/HER2- breast cancer.

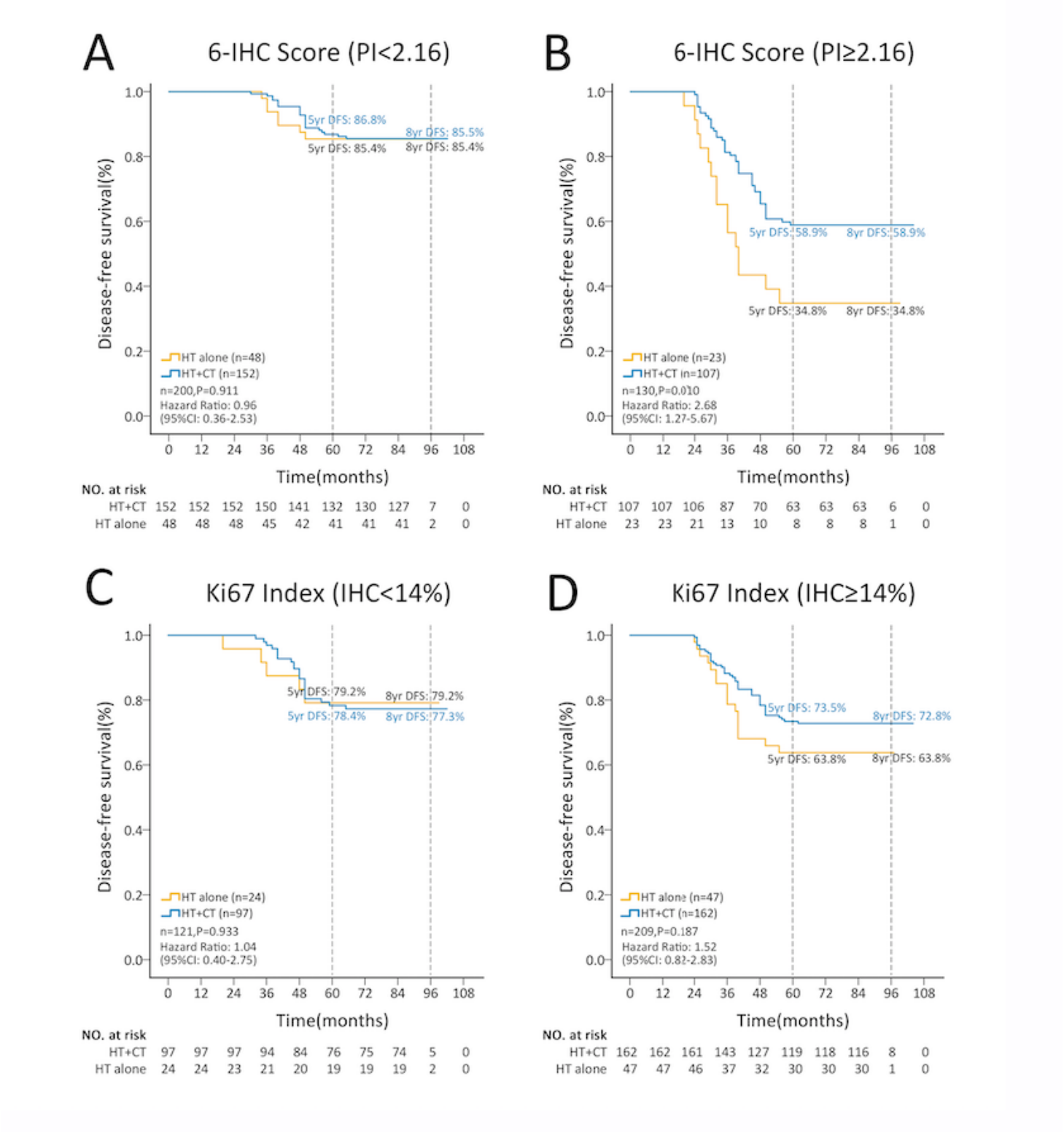

Figure 4

Kaplan–Meier analysis of DFS in low-risk and high-risk patients determined by 6-IHC score/Ki67 index. A, HT alone vs HT+CT in 6-IHC score prognostic model low risk group. B, HT alone vs HT+CT in 6-IHC score prognostic model high risk group. C, HT alone vs HT+CT in Ki67 low risk group. D, HT alone vs HT+CT in Ki67 high risk group.