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

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Abstract

1. This study involves the clinical determination of whether HR+/HER2− early BC needs chemotherapy and seeks for a more accurate and cheaper method. 2. Select genes related to BC invasion and metastasis, and establish a scoring model for their immunohistochemical results by Cox regression. 3. The scoring model can well judge the prognosis of HR+/HER2− BC patients.

Purpose: Current methods of judging whether HR+/HER2− breast cancer (BC) require adjuvant therapy, such as Ki67 and multigene prognostic tests, cannot balance accuracy with the price most patients can afford. **Methods:** A retrospective analysis of 330 HR+/HER2− BC patients was conducted. Six BC-related genes (Cathepsin L2, MMP11, CyclinB1, Aurora A, Survivin, and Ki67) were screened using univariate and multivariate COX regression, and correlate clinical follow-up with immunohistochemical expression (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. Kaplan-Meier (KM) method was used to analyze Disease-free survival (DFS) and overall survival (OS). Six-IHC score and other factors associated with survival benefit of adjuvant chemotherapy were compared with Ki67 index. **Results:** The receiver operating characteristic curve analysis showed that the patients can be divided into 6-IHC score "High" and "Low" risk groups. The 8-year DFS and OS of 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-breast cancer patients, with additional advantages over using immunohistochemical expression of Ki67.

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Background

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 BC patents.[2] For instance, some of the patents 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, multigene prognostic tests have shown the promising prognostic and predictive value in BC management.[4] Although the benefit of using genetic data is obvious, it also has pitfalls, eg, lack of strong correlation between gene expression and protein levels or activities. 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 the combined IHC analysis results

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; AUC, area under the ROC curve.

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of these 6 genes can achieve a prognostic prediction effect similar to the Oncotype DX test, and can replace using the IHC results of Ki67 alone to predict. 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 BC. 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.

METHODS

Study Selection

We retrospectively evaluated 330 HR-positive and HER2− patients with early BC 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 National Comprehensive Cancer Network (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 primary invasive BC patients with HR+/HER2−; (3) unilateral BC 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. Ethical approval was granted by First Affiliated Hospital Research Ethics Committee (2020-203), and all patients provided written informed consent.

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 no cause of death at the end of the follow-up. Disease-free survival (DFS) was calculated from the date of surgery to the first relapse (determined by physical examination, CT, or MRI) (Local recurrence or distant metastasis) or the time of the most recent follow-up. The last follow-up was in January 2020. The study followed the "Reporting recommendations for tumor marker prognostic studies (REMARK)."[6]

Tissue Microarray and Immunohistochemical

A ready-to-use IHC hypersensitive UltraSensitive SP (Rabbit) IHC (Kit-9706) and DAB (Kit-0031) were purchased from MXB biotechnologies (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.

An 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 tissue microarray. The modified tissue chip wax block was sectioned on a paraffin microtome with a thickness of 4 μm and diameter of 3 mm. 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 antigen retrieval, IHC staining was performed. After the sections were fully rinsed with tap water, they were counterstained, dehydrated, transparent, and mounted.

Under double-blind condition, two experienced pathologists were asked to randomly select 5 different visual fields. A total of 200 BC cells were counted to obtain the average value.

Appearance of brown particles in the nucleus indicates the expression of ER and PR proteins. Any staining of >1% positive tumor cells was considered positive expression of ER and PR.[1,7] Ki67 staining was reported by percentage of positively stained nuclei from the pathology report, and the cut-off value of Ki67 was 14%.[1,7] HER2 stainings were evaluated for membrane staining intensity and percentage of stained tumor cells. Score of 2+ was considered ambiguous and needs to be determined by fluorescence in situ hybridization testing.[8]

The molecular marker expression of Aurora A, Survivin, Cyclin B1, Cathepsin L, and MMP11 were quantifies as average percentage of positive staining cells to the total number of malignant cells and counted in percentages. Except for Ki67, 5 biomarkers are not used for clinical diagnosis, and we uniformly stipulate that >1% is positive (Figure 1a). The histological grading was determined according to the Nottingham histological Scoring System. Positive and negative controls were set for each batch of IHC staining.

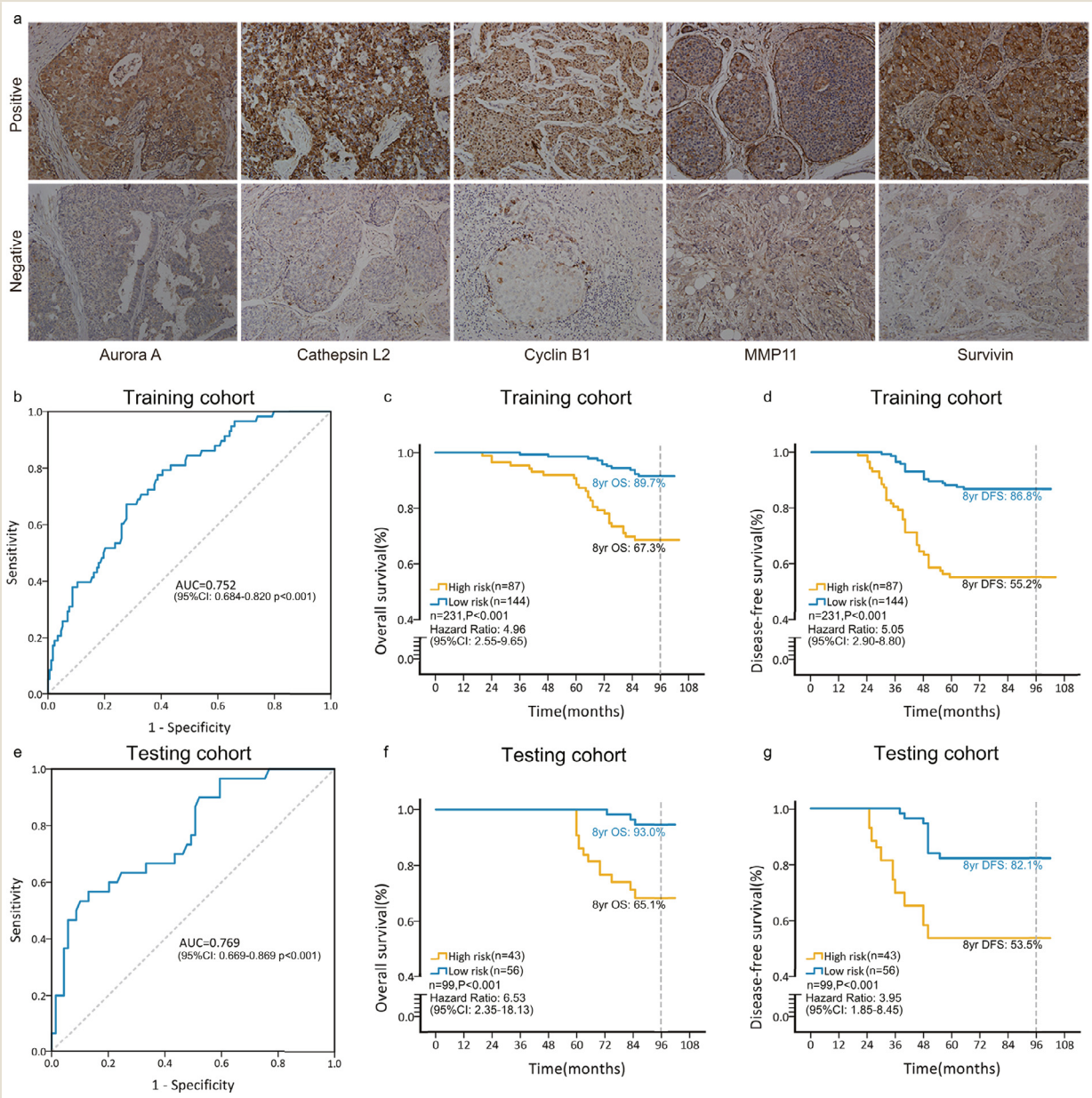
Statistical Analysis

Difference between clinicopathological proportions was determined using the chi square test. Difference between continuous clinicopathological variables was evaluated via a 2-tailed t-test. Univariate Cox regression analysis was used to screen all candidate biomarkers. 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 BC 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). 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. Using univariate and multivariate Cox regression analysis,

Figure 1 Immunohistochemical staining images and validation of training cohort and testing cohort. (a) Representative immunohistochemical staining images of BC tissues were quantifies as average positive staining area percentage (H&E, × 20) and negative control. (b) ROC curve of the training cohort (AUC = 0.752, 95%CI: 0.684-0.820 $P < 0.001$). (c) KM curves of OS in training cohort (n = 231). (d) KM curves of DFS in training cohort (n = 231). (e) ROC curve of the testing cohort (AUC = 0.769, 95%CI: 0.669-0.869 $P < 0.001$). (f) KM curves of OS in testing cohort (n = 99). (g) KM curves of DFS in testing cohort (n = 99).



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, progesterone receptor, chemotherapy). Finally, the prognostic model is verified by Kaplan-Meier (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 $P < 0.05$.

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
Unknown 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.

Results

Patient Characteristics

Randomization was performed by computer-generated random numbers, and each case was assigned with a random number. These numbers were then arranged from large to small according to their value 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. 127 patients (55.0%) in the training cohort and 55 patients (55.6%) in the training cohort had lymph node metastasis. 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 (Table 1). Among the pathological types, invasive ductal carcinoma accounted for 87%. Chi-square analysis showed that there was no significant difference between the 2 cohorts. 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 ≦0.006). Multivariate Cox regression analysis showed that MMP, Cyclin B1, and Aurora A were associated with DFS ($P < 0.05$; Table 2). 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

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

Abbreviations: CI = confidence interval; HR = hazard ratio.
Cox regression analysises are based on continuous variables with a percentage of positive expression of 6 IHCs.

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 data range of IHC expression of each biomarker was 0% to 100%, and that of PI was 0 to 7.8.

The 6 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 largest AUC was 0.752 and the highest sensitivity and specificity was 67.2% and 72.3%, respectively (Figure 1b). The cutoff point of 6-IHC score was established using the point with the largest Youden index (sensitivity+specificity−1) on the ROC curve of training cohort (n = 231) with the 5-years DFS as an endpoint. In addition, based on the cut-off value (PI = 2.16), 231 BC patients in the training cohort were divided into 2 groups: a "High" risk group (PI ≥2.16, n = 87) and a "Low" risk group (PI < 2.16, n = 144). KM curves showed that the OS (Figure 1c) and DFS (Figure 1d) 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 1e). 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; Figure 1b). 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 1f: 65.1% vs. 93.0%, $P < .001$) and DFS (Figure 1g: 53.5% vs. 82.1%, $P < .001$) 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 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,[9-11] 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 (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 ($P = 0.572$; 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. Shown in Figure 2a, based on all 330 BC cases, the generated AUC value was 0.754 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 for

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 (Unknown 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 (Unknown 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**

Abbreviations: CI = confidence interval; HR = hazard ratio.
Ki67 in Table 4 is a 2-category variable that is classified as low risk (Ki67 <14%) and high risk (Ki67 ≥14%) by 14%, which is different from Table 2.

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 ($P < 0.001$). 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 2b, 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 2c, $P = 0.010$). However, The KM curves for DFS showed that chemotherapy did not significantly improve the prognosis either in the Ki67 "Low" (Figure 2d, P= 0.933) or "High" (Figure 2e, 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

Based on some clinical trials,[12-15] several commercial tests, such as the 21-gene recurrence score (Oncotype DX), and the 70-gene signature (MammaPrint) are currently available in some countries for systemic treatment decision-making. Among those, Oncotype DX has been recommended by the NCCN, ASCO, ESMO, and the St. Gallen International Expert Consensus guidelines.[9-11,16] 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.[17] Technical barriers or lack of standardization of the test 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 based on microarray analysis of even thousands of genes.[18]

Though the IHC-based and the gene expression profile-based classifications are not completely overlapped.[19] IHC techniques

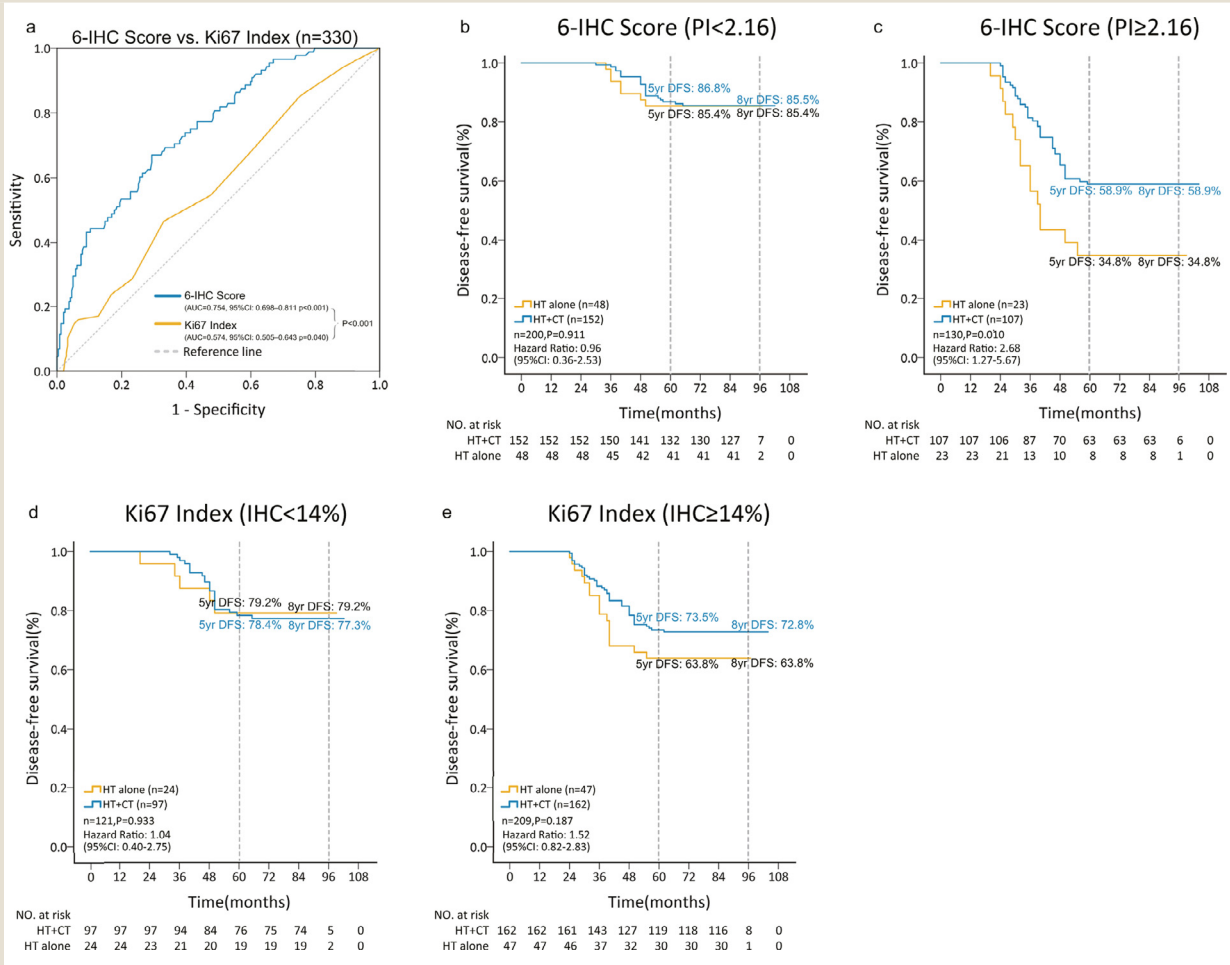
Table 4 Correlation Between 6-IHC Prognostic Model and Ki67 With Clinicopathological Characteristics in all HR+HER2− Early Invasive Breast Cancer Cases (n = 330).

	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
Unknown 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)	

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.[20] Ki67 index is also considered as a predictor of pathological complete response,[21-23] response to chemotherapy,[24,25] and likelihood of relapse.[26,27] However, use of Ki67 index has some disadvantages. First, universally-accepted guidelines for analysis and clinical interpretation of Ki67 staining is not available.[28,29] Second, there are no well-designed prospective clinical trials that specifically focus 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.[30] 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.[31-34] 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.

Two prospective clinical trials (the TAILORx[15] and the Phase III Plan B West German Study Group Trial[35]) 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. Six-IHC

Figure 2 Comparison of 6-IHC Score with Ki67. (a) 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− BC. (b) HT alone vs. HT + CT in 6-IHC score prognostic model low risk group. (c) HT alone vs. HT + CT in 6-IHC score prognostic model high risk group. (d) HT alone vs. HT + CT in Ki67 low risk group. (e) HT alone vs. HT + CT in Ki67 high risk group.



score might be a good substitute for Oncotype DX, and could be immediately obtained from routine pathology report together with Ki67 result.

The Ki67 index cut-off value was set at 14%[1] by St. Gallen Expert Panel until 2015.[30] The patients in our study were mostly diagnosed before 2015. 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 BC. Ki67 also predicts risk with other values such as 20%[30] and 10%[36] as cut off values, and we did not test the queue 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. The well-designed prospective studies with more patient size are required to confirm our results.

Compared with the routine pathology that the hospital can review the entire slice, TMA can only select part of the tissue, which has certain limitations. Tumor cells are heterogeneous and there is no guarantee that the part selected by TMA contains the tumor cells that express the markers. Thus, bias and false negative cannot be completely avoided. We used a diameter of 3mm. Compared with the usual size of TMA (0.6-1 mm), the wider core size can ensure that the TMA is stronger, and more tumor cell immunohistochemical staining can be observed.

Conclusions

Combined 6-IHC score predicted cancer recurrence and survival in HR+/ her2-bc patients to a certain extent, reduces overtreatment and better identify patients who will benefit from adjuvant chemotherapy, and hopefully replaces the Ki67 immunohistochemical prediction approach.

Clinical Practice Points

1. Clinical use of Ki67 immunohistochemical results combined with multigene prognostic tests to determine the prognostic risk of HR+/ HER2−BC patients determines whether chemotherapy is required for subsequent treatment, in order to reduce the overtreatment caused by inaccurate Ki67 immunohistochemical judgment, and to replace expensive multigene prognostic tests with cheap immunohistochemical method.
2. Multiple genes including Ki67 related to breast cancer invasion and metastasis were selected from the multigene prognostic tests to determine the prognosis of HR+/ HER2−BC patients, and their immunohistochemical results were combined to establish a prognostic scoring model, which was found to be able to effectively determine whether patients needed chemotherapy through retrospective analysis.
3. Based on immunohistochemical method, this method is easier to be widely used than the multigene prognostic tests, and the combined determination of multiple biomarkers is more accurate than Ki67 immunohistochemical method, which reduces bias and can reduce overtreatment and medical cost if it can be widely used in clinical practice.

Disclosures

The authors declare no conflict of interest with the content of this paper.

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Authors' contributions

JL designed the research, conducted the data collection and analysis, constructed the model and interpreted the data, contributed to the experimental part, 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.

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 (2020-203).

Consent for publication

Not applicable.

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N/A.

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