RESEARCH ARTICLE

A Comprehensive Model for Predicting Recurrence and Survival in Cases of Chinese Postoperative Invasive Breast Cancer

Xianhe Xie[1,2]*, Yanfen Hu[2], Chao Jing[2], Shuimei Luo[1], Yunfu Lv[3], Haitao Yang[1], Lina Li[1], Huijuan Chen[1],Wanzun Lin[1], Weili Zheng[1]

Abstract

We investigated relationships between clinical pathologic data, molecular biomarkers and prognosis of invasive breast cancer based on a Chinese population. Immunohistochemistry (IHC) was used to assess the status of ER, PR, HER-2 and Ki-67, with fluorescence in situ hybridization (FISH) performed to further confirm HER-2 positivity with an equivocal result (IHC 2+). Subsequently, Kaplan-Meier univariate and multivariate COX regression analyses of ER, PR, HER-2, Ki-67, clinical features, therapeutic status and follow-up data were performed according to the establishment principle of the Nottingham prognostic index (NPI). From this study, age, tumor size, lymph node status, ER, HER-2, Ki-67 status were found to be associated with prognosis. Eventually, a prognostic model of (PI= (1.5×age) - size + (0.1×lymph node status) - (0.5×ER) + (2×HER-2) - (0.2×Ki-67)) was established with 288 randomly selected patients and verified with another 100 cases with invasive breast cancer. Pearson correlation analysis demonstrated a significant positive correlation index of 0.376 (P=0.012<0.05) between the prognostic index (PI) and actual prognosis. Remarkably, the consistency with the model predicted recurrence was 93% in the validation set. Therefore, it appears feasible to predict the prognosis of individuals with invasive breast cancer and to determine optimal therapeutic strategy with this model.

Keywords: Breast cancer- ER- HER-2- Ki-67- prognostic model

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Introduction

Worldwide, breast cancer is the most common malignant tumor in women including invasive ductal carcinoma, invasive lobular carcinoma and in situ ductal carcinoma (Wu et al., 2015; Visscher et al., 2016), with a 5-year survival rate of 89% in United States, 87% in Brazil, Finland and Isreal, 86% in Australia, Canada and Italy, 85% in Germany, 84% in Spain, 83% in South Korea, 81% in China and United Kingdom, 79% in Turkey, 76% in Colombia, 71% in Thailand, 60% in Algeria and India, and 53% in South Africa (Healthline Web site). Invasive breast cancer, a malignant epithelial tumor, which frequently invades adjacent tissue with an obvious trend of distant metastasis, is the second leading cause of cancer-related mortality, accounting for 23% of the total new cancer cases and 14% of the total cancer deaths (Jemal et al., 2011) . Invasive breast cancer was diagnosed in approximately 169,000 women each year in China, ranking second place worldwide (Ni L, 2012; Zheng et al., 2013).

So far, multiple prognostic factors for breast cancer have been identified, including clinical pathological features, tumor classification and specific indicators. Several predictive models had been constructed, including the Nottingham Prognostic Index (NPI) which was first established in 1982 by Haybittle et al. (1982) , validated in 1987 and 2001 (Todd et al., 1987; D'Eredita et al., 2001) , updated in 2007 (Blamey et al., 2007; Blamey et al., 2007) , further developed with HER-2 involving in 2012 (Wishart et al., 2012), to provide accurately estimated survival of breast cancer after surgery. ADJUVANT! , a web-based prognostication and treatment benefit tool for breast cancer, which was validated in case cohorts from British Columbia (Olivotto et al., 2005) , the Netherlands (Mook et al., 2009) and the United Kingdom (Campbell et al., 2009) , is now widely used in the United Kingdom to facilitate oncologists and patients determining optimal adjuvant therapy. However, none of the above predictive models has ever been validated with Chinese cohort, the application value of these models in Chinese population remains unclear owing to the regional and ethnic diversity. Therefore, it is essential to establish a predictive model based on Chinese patients.

Additionally, Gene expression biomarker of tumors has become a new paradigm for classifying breast cancer, predicting response to treatment and risk of recurrence.

*[1]Department of Chemotherapy, The First Affiliated Hospital of Fujian Medical University, Fuzhou, Fujian, [2]Department of Internal Medicine Oncology, [3]Surgery Department, Hainan General Hospital, Haikou, Hainan, China. *For Correspondence: xiexianhe@yahoo.com. Xianhe Xie and Yanfen Hu have equal contribution in this study.*

Estrogen receptors (ER) and progesterone receptors (PR) are critical biomarkers for the prognosis of endocrine therapy (Ba et al., 2014) while HER-2 is recognized as the target of trastuzumab. Meanwhile, breast cancer with HER-2 over-expression was characterized by relapsing and metastasizing with short period of survival (Santos et al., 2013) . Increasing studies (Joensuu et al., 2013; Rasmy A et al., 2016; Yamashita Het al., 2016) showed that Ki-67 over-expression was associated with early recurrence and progression. Currently, ER, PR, HER-2 and Ki-67 status have been deemed to the essential biomarker in determining adjuvant therapy aelevand predicting prognosis. Furthermore, these biomarkers are routinely tested in all invasive breast cancer.

Therefore, establishing a prognostic model with a comprehensive analysis of pertinent factors to accurately predict the survival and risk of recurrence is urgently needed. Except for clinical features, additional predictive molecular markers, such as ER, PR, HER-2 and Ki-67, should be taken into account.

The first procedure of this study was to develop a prognostic model to predict overall survival (OS) and recurrence from a large cohort of Chinese patients (288 cases) diagnosed in Hainan Provincial General Hospital from January 2008 to December 2012, utilizing clinical data, follow-up data, the status of ER, PR, HER-2 and Ki-67. The Cox regression analysis was conducted in multivariate analysis according to the principle of Nottingham Prognostic Index to study the impact of prognostic factors and to establish a prognostic model for invasive breast cancer. The second procedure was to validate the model with another 100 cases in the same cohort.

Materials and Methods

Patients

We retrospectively evaluated 577 cases with invasive breast cancer, who underwent treatment in Hainan General Hospital from January 2008 to December 2012 and approved by the institutional review board. All patients were confirmed by postoperative pathologic examination with archival paraffin-embedded tissue. Information acquired from the database, including age, operation type, TNM stage, histological grade, tumor size, lymph node status, neoadjuvant therapy, adjuvant therapy, was showed in Table 1. Afterwards immunohistochemistry (IHC) test was performed to detect the ER, PR, HER-2 and Ki-67 status of the paraffin-embedded tissue, respectively. When HER-2 was equivocal (IHC 2+), fluorescence in situ hybridization (FISH) was used to further confirm HER-2 status. The ER, PR, HER-2 and Ki-67 were evaluated conforming to IHC and FISH guideline of the breast cancer receptor detection (Wolff et al., 2007; Hammond et al., 2010) and the results were judged according to the standards reported by Wei-liang Z (2012). Patients with non-invasive breast cancer or functional disorder of critical organs such as heart, brain, kidney and lung or systemic immune disease were excluded.

Molecular Markers Analysis

Immunohistochemistry Assay (IHC)

Firstly, paraffin-embedded tissue was sectioned at 4 μm thickness, dewaxed and washed with distilled water. This was followed with antigen retrieval using citrate buffer (PH 6.0) for 2.5 minutes in autoclave at 200 degrees centigrade and incubation in H_2O_2 after cooling. Then, these slices were washed in PBS buffer consecutively for 3 times and processed thereafter. All further processing for ER, PR, HER-2 and Ki-67 IHC was performed according to the instructions in PV - 9000 universal LDPE-G-NVP detection kit. Briefly, it consisted of the sequential application of the primary antibody (mouse monoclonal anti-human ER-antibody, PR-antibody, HER-2-antibody (Zhongshan Jinqiao Biological Technology, Inc., Beijing, China) and Ki-67 monoclonal antibody (Abcam, British) for 60 min, followed by incubation for 60 min and thereafter sequentially added Polymer Helper and bridging antibody (Polymers peroxidase anti-rabbit antibody) (Zhongshan Jinqiao Biological Technology, Inc., Beijing, China) with a second incubation for 20 min. All incubation was performed at 37 degrees centigrade in thermotank (Chengshun Instrument, Inc., Shanghai, China). The sites of immune precipitate formation were identified by DAB chromogenic agent (Zhongshan Jinqiao Biological Technology, Inc., Beijing, China). In addition, a series of sectioned slices was treated with PBS buffer instead of the primary antibody as negative controls. Specimens which showed positive results with the primary antibodies failed to show positive reaction with the PBS buffer. Tumor specimens were categorized into positive or negative by ER or PR status and low- or high-expression by Ki-67 status through estimation on screening wide areas within each tissue section: ER or PR negative, <5% stained cells (Fig.1A); ER or PR positive, ≥5% stained cells (Fig.1B); Ki-67 low-expression, <14% stained cells (Fig.1C) ; Ki-67 high-expression, ≥14% stained cells (Fig.1D), compared with the total of tumor cells. However, with regard to HER-2 status (Fig.1E and Fig.1F), tumor specimens displaying >10% positively stained cells was evaluated as +, ++, or +++ based on the staining intensity: + representing for the weakest staining, ++ for mild to moderate staining and +++ for the most intense staining.

Fluorescence in situ hybridization Assay (FISH)

Thirty four micron thick paraffin-embedded sections from paraffin-embedded tissue was utilized. The slides were dewaxed with dimethylbenzene, gradient ethanol and washed with distilled water. This was followed by denaturation with Protease K and processed thereafter. All further processing for HER-2 FISH was performed according to the instructions in Fluorescence in-situ hybridization kit. All reagents used for HER-2 FISH were purchased from Jinpujia Medical Technology, Inc., Beijing, China. The slides then required a manual probe step and probe mixture was added to the target area of each slide. Immediately, a glass coverslip was applied over the probe and sealed with rubber cement. Subsequently, the slides were hybridized overnight and counterstained with hematoxylin thereafter. Finally, thirty cells were counted by two independent observers for each case with

10×objective lens to evaluate the reproducibility and inter-operator variability. All cases were scored according to the 2007 ASCO/CAP breast cancer guidelines (Wolff et al.,2007) . A HER-2: CEP17 ratio of < 1.8 was classified as negative for amplification, 1.8–2.2 as equivocal and > 2.2 as amplified as shown in (Figure 1G and Figure 1H).

Follow-up and statistical analysis

Among 577 cases, 436 cases (75.56%) were followed-up mainly through out-patient review and telephone call for a median duration of 33 months. Statistical analysis was conducted using SPSS19.0. Enumeration data were calculated using the Chi-square test and *P* values < 0.05 was considered as statistically significant. Postoperative progression free survival (PFS) of invasive breast cancer was analyzed using the Kaplan-Meier method or life-table method. The disease-free survival rate was defined as prognostic indicators. Univariate analysis of relevant prognostic factors was conducted. Multivariate COX regression analysis of factors that affected the prognosis were performed. Eventually, the prognostic mathematical model was established and verified based on the results of multivariate analysis and NPI index principle.

The hierarchy and coding of general clinical data of individuals were listed in Table 2.

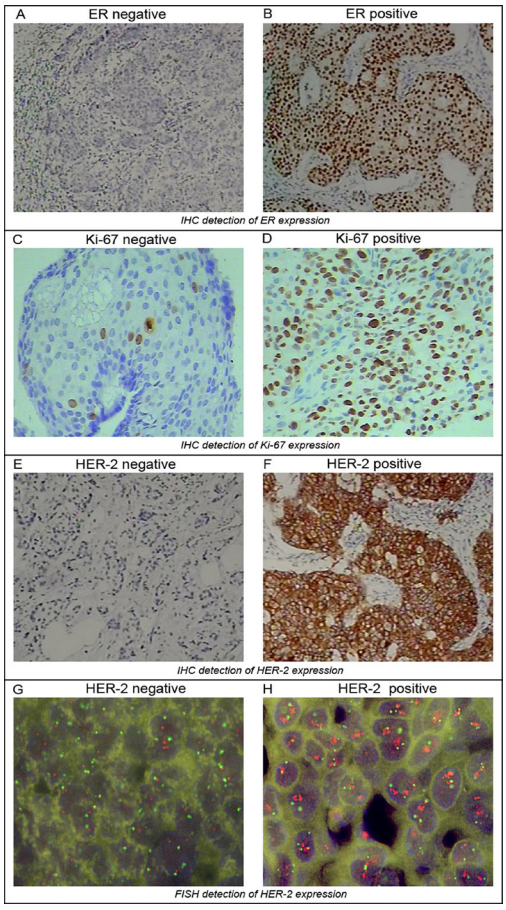

Figure 1. IHC and FISH Detection of Molecular Biomarkers: A. ER negative (IHC detection); B. ER positive (IHC detection); C. Ki-67 negative (IHC detection); D. Ki-67 positive (IHC detection); E. HER-2 negative (IHC detection); F. HER-2 positive (IHC detection); G. HER-2 negative (FISH detection); H. HER-2 positive (FISH detection)

Results

Testing results of molecular markers

Among 416 patients, 178 cases were ER negative (42.79%), 238 ER positive (57.21%), 198 PR negative, 218 PR positive, 188 Ki-67≤ 14% (45.19%) and 228 Ki-67>14% (54.81%). 124 of 373 patients were HER-2 positive, accounting for 33.24%, and the other 249 (66.76%) HER-2 negative.

Disease-free survival rate of invasive breast cancer

Randomly selected 288 patients from above mentioned 416 cases. Three years and five years disease-free survival cases were 258 (89.58%) and 244 (84.72%), respectively. The disease-free survival curve was showed in Figure 2.

Univariate analysis of prognostic predictors and interventions for patients with invasive breast cancer

The Kaplan-Meier univariate analysis of clinic-pathologic features and adjuvant therapy for the 288 randomly selective patients, exhibited that age, PR expression, with or without neoadjuvant chemotherapy and radiotherapy had no significant correlation with prognosis (P>0.05). Interestingly, significant correlation of prognosis with TNM stage, ER, HER-2, Ki-67 expression, lymph node status, histological grade, cycles

Table 1. Characteristics of the Included Patients

	Characteristics	Cases
Age (years)		24-81, mean (47.62±10.05)
Operation	BCS	37
	modified radical/radical mastectomy	540
TNM stage	I-II	462
	III-IV	115
HG	I	39
	II	291
	III	247
Tumor size	≤2cm	123
	>2cm	454
LNS	no node metastasis	296
	1 to 3 nodes metastasis	146
	more than 3 nodes metastasis	135
NAT	NCT	42
	None	535
AT	PCT<6 cycles	149
	PCT≥6 cycles	267
	PET	86
	PCT	69

LNS, lymph node status; HG, histological grade; NCT, neoadjuvant chemotherapy; PCT, postoperative chemotherapy; PET, postoperative endocrinotherapy; NAT, Neoadjuvant therapy; NT, Adjuvant therapy; BCS, breast-conserving surgery

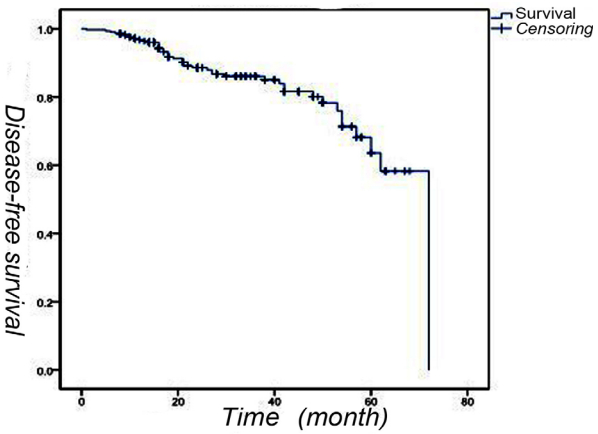

Figure 2. Disease-Free Survival Rate Curve of 288 Cases of Patients with Invasive Breast Cancer

Table 2. the Hierarchy and Coding of General Clinical Data of Patients

Items	Hierarchy	Code
Age (years)	≤35	1
	>35	2
Surgical type	Radical operation	1
	BCS	2
Tumor size (cm)	≤2	1
	>2	2
TNM stage	I- II	1
	III- IV	2
Lymph node status	I	1
	II	2
	III	3
Histological grade	I	1
	II	2
	III	3
ER	Positive	1
	Negative	2
PR	Positive	1
	Negative	2
HER-2	Positive	1
	Negative	2
Ki-67	≤14%	1
	>14%	2
Neoadjuvant chemo-therapy	No	1
	Yes	2
Postoperative chemo-therapy	≤6 cycles	1
	>6 cycles	2
Postoperative endocrine therapy	No	1
	Yes	2
Radiotherapy	No	1
	Yes	2

BCS, breast conserving surgery

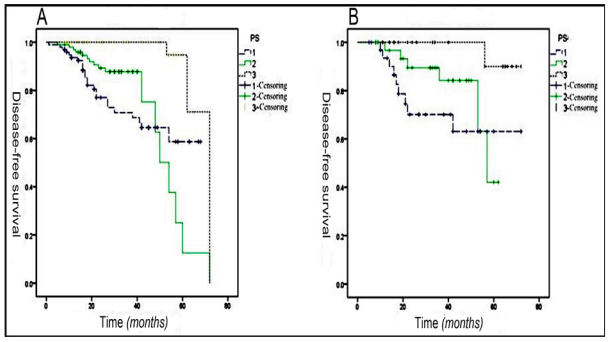

Figure 3. A. Correlation between the PS Value and Prognosis of Patients in Modeling Set; B. Correlation between the PS Value and Prognosis of Patients in Validation Set

of postoperative chemotherapy, endocrinotherapy was observed (P<0.05) (Table 3).

Multivariate COX regression analysis of relevant prognostic factors

Univariate log-rank test showed that TNM stage, ER, HER-2, Ki-67 expression, lymph node status, histological grade, cycles of postoperative chemotherapy and endocrinotherapy had significant impacts on prognosis (P<0.05). Accordingly, previous studies (Yang XR et al., 2011; Eichler et al., 2008; Joensuu et al., 2013; Nishimura et al., 2014) revealed that these clinicopathological characteristics were critical indexs in predicting the prognosis of breast cancer, thereby, these variables were included in Multivariate COX regression. Univariate log-rank test showed that age, PR expression, with or without neoadjuvant chemotherapy and radiotherapy had no significant correlation with prognosis (P>0.05). However, previous studies displayed that PR expression (Yang XR et al., 2011) was a key factor for predicting the efficacy and prognosis of hormone-dependent breast cancer after delivering hormone therapy; younger age at diagnosis (Colzani E et al.,2011, Langlands AO et al.,1979) tended to be suffered from a more invasive histological type of breast cancer and a worse prognosis; neoadjuvant chemotherapy (Li S et al., 2013) had been the standard option for locally advanced breast cancer; JY Chen's study demonstrated that radiotherapy was effective for breast cancer with isolated local-regional recurrence after mastectomy and recommed that radiotherapy could be applied to predict the prognosis (Chen JY et al.,2009). Thus, age, PR, neoadjuvant chemotherapy and radiotherapy were also included in Multivariate COX regression. The data of age, tumor size, lymph node status, TNM stage, histological grade, ER, PR, HER-2, Ki-67 expression, neoadjuvant chemotherapy, postoperative chemotherapy, endocrine therapy and radiotherapy were introduced into the analysis model. The results showed that age, lymph node status, HER-2 expression and neoadjuvant chemotherapy were independent prognostic risk factors (P<0.05) for patients with invasive breast cancer (Table 4).

Establishment and validation of the prognostic model
Establishment of the prognostic model

Table 3. The Correlation Analysis of Prognostic Predictors and Intervention with Prognosis

Items		Cases	3-year DFS (%)	5-year DFS (%)	$\chi 2$	P
Age (years)	≤35	26	21 (80.77)	19 (73.08)	2.994	>0.05
	>35	262	237 (90.46)	225 (85.88)		
Tumor size (cm)	≤2cm	84	79 (94.05)	77 (91.67)	4.418	<0.05
	>2cm	204	179 (87.74)	167 (81.86)		
TNM stage	I- II	230	212 (92.17)	210 (91.30)	38.227	<0.01
	III- IV	58	46 (79.31)	34 (58.62)		
ER status	positive	165	157 (95.15)	148 (89.70)	8.606	<0.01
	negative	123	101 (82.11)	92 (78.05)		
PR status	positive	151	142 (94.04)	132 (87.42)	2.633	>0.05
	negative	137	116 (84.67)	112 (81.75)		
HER-2 status	positive	96	77 (80.21)	73 (76.04)	10.683	<0.01
	negative	192	181 (94.27)	171 (89.06)		
Ki-67 status	≤14%	130	121 (93.08)	117 (90.0)	5.099	<0.05
	>14%	158	137 (86.71)	127 (80.38)		
LNS	Stage I	148	145 (97.97)	143 (96.62)	36.628	<0.01
	Stage II	73	61 (83.56)	57 (78.08)		
	stage III	67	52 (77.61)	44 (65.67)		
	Grade I	19	19 (100.0)	19 (100.0)	12.452	<0.01
HG	Grade II	145	136 (93.79)	130 (89.66)		
	Grade III	124	103 (83.06)	95 (76.61)		
NCT	None	259	234 (90.35)	220 (84.94)	0.096	>0.05
	Yes	29	24 (82.76)	24 (82.76)		
PCT	≤6 Cycles	202	177 (87.62)	165 (81.68)	4.827	<0.05
	>6 Cycles	86	81 (94.19)	79 (91.86)		
PET	None	228	201 (88.16)	188 (82.46)	4.342	<0.05
	Yes	60	57 (95.00)	56 (93.33)		
RT	None	240	215 (89.58)	202 (84.17)	0.343	>0.05
	Yes	48	43 (89.58)	42 (87.50)		

LNS, lymph node status; HG, histological grade; NCT, neoadjuvant chemotherapy; PCT, postoperative chemotherapy; PET, postoperative endocrinotherapy; RT, radiotherapy

Table 4. Multiple-Factor Analysis of Prognosis of Patients with Invasive Breast Cancer

Items	B	SE	Wald	P	OR	95.0% CI	
						LL	UL
Age	1.52	0.653	5.415	0.02	4.571	1.271	16.443
Tumor size	-0.875	0.733	1.426	0.232	0.417	0.099	1.753
LNS	0.128	0.535	8.906	0.012	1.136	0.398	3.241
TNM stage	-0.41	0.511	0.644	0.422	0.664	0.244	1.807
HG	0.357	0.574	0.388	0.823	1.43	0.464	4.407
ER status	-0.515	0.584	0.779	0.377	0.597	0.19	1.875
PR status	0.767	0.618	1.542	0.214	2.153	0.642	7.223
HER-2 status	1.905	0.445	18.311	0	6.719	2.808	16.078
Ki-67 status	-0.17	0.585	0.084	0.772	0.844	0.268	2.656
NCT	-3.829	0.82	21.782	0	0.022	0.004	0.109
PCT	0.015	0.591	0.001	0.98	1.015	0.319	3.234
PET	1.147	0.747	2.354	0.125	3.148	0.727	13.621
RT	0.494	0.747	0.436	0.509	1.638	0.379	7.087

LNS, lymph node status; HG, histological grade; NCT, neoadjuvant chemotherapy; PCT, postoperative chemotherapy; PET, postoperative endocrino-therapy; RT, radiotherapy; LL, lower limit; UL, upper limit

Table 5. The Comparison of Recurrence of Actual Situation and Models Predict Relapse in 100 Cases of the Validation Set

Actual relapse	Predict relapse	
	Yes	No
Yes	9	5
No	2	84

Based on the results of univariate analysis and multivariate COX regression analysis, 6 indicators, including age, tumor size, lymph node status, ER, HER-2, Ki-67 status were introduced into the establishment of the prognostic model according to Nottingham prognostic index (NPI) principles and the impact degree of these indicators on prognosis. The simplified prognostic mathematical model was established with B values in Table 4. The established prognostic mathematical model was showed as follows: Prognostic index (PI) = (1.5×age) - size + (0.1×lymph node status) - (0.5×ER) + (2×HER-2) - (0.2×Ki-67).

Validation of the prognostic model

Two cutoffs, 2.4 and 4.4, were obtained when substituting the clinical data into the established model. The assignment of the results based on the cutoffs was as follows: When PI≤2.4, assigned Predictive score (PS) =1; When 2.4<PI≤4.4, assigned PS=2; When PI>4.4, assigned PS=3. The Pearson correlation analysis of PI with prognosis obtained a correlation index of 0.376 (P=0.012<0.05). When PS was 1, 2, 3, the 3-year disease-free survival of invasive breast cancer was 79%, 90.41%, 100%, respectively (Figure 3A). The clinical data of 100 cases who had not been used for the establishment of model were substituted into the PI model to validate the correlation of PS with prognosis. When PS was 1, 2, 3, the 3-year disease-free survival was 77.78%, 90.91%, 100%, respectively (Figure 3B). The consistency between actual and model predicted recurrence was 93% (Table 5).

Discussion

Based on the identification of significant prognosis related biomarkers, ER, PR, HER-2 and Ki-67, we established this simplified prognostic index model using comprehensive clinical data and the biomarkers of Chinese population. Multiple-regression analysis of prognostic factors and survival in a series of 288 patients with invasive breast cancer showed three clinical factors (age, tumor size, lymph node status) and three biomarkers (ER, HER-2, Ki-67 status) were critical indicators of prognosis. A prognostic index model was established applying the six factors: PI= (1.5×age) - size + (0.1×lymph node status) - (0.5×ER) + (2×HER-2) - (0.2×Ki-67). Subsequently, this PI model was verified by the clinical data of another 100 cases with invasive breast cancer, which had not been used to establish the model. When PS was 1, 2, 3, the model predicted 3-year DFS was 77.78%, 90.91%, 100%, respectively. Meanwhile, actual 3-year DFS of 100 cases in validation set was 86% , which was most consistent with 90.91% predicted by the PI model (PS=2). Accordingly, a coincidence rate of 93% between theoretical and actual prognosis was obtained which confirmed the accuracy and effectiveness of this PI model in predicting the prognosis of Chinese invasive breast cancer.

Currently, a series of predictive models have been developed, such as NPI which was first established in 1982 (Haybittle et al.,1982) . Compared with NPI model, our PI model shared several similarities as following: firstly, this PI model was established and verified according to the same NPI index principle (Haybittle et al.,1982; Wishart et al., 2012); secondly, we recruited a similar sample size in a single research center as that of NPI model (Haybittle et al., 1982) when it was initially established and verified in 1982; however, some distinctions were indicated between this PI model and NPI model: Except for the tumor size and lymph node status in NPI, age was found to be a key prognostic factor and involved in our PI model; furthermore, compared with NPI model in 2012 (Wishart et al., 2012) , we took Ki-67 into account; eventually, NPI model was a well-known prognostic scoring system which had been prospectively validated in a second Nottingham dataset (Todd et al., 1987), as well as in other centers (D'Eredita et al., 2001). However, none of the above predictive models has ever been validated with a Chinese cohort, the application value of these models in Chinese population remains unclear. Therefore, it is essential to establish a predictive model based on Chinese population.

In China, the prognostic mathematical model of lymph node negative breast cancer was firstly established in 2003 (Fang-Ming et al., 2003). Subsequent prognostic model involved in six factors, consisting of PR, p53, EGFR, C atheps in D, PCNA, HER-2, was established in 2006 (Yue et al., 2006). Compared with them, our study possessed some unique features: firstly, we had no restriction only to the clinical features, such as lymph node negative breast cancer, so it might be universally applied; secondly, biomarkers, such as ER, PR, HER-2, Ki-67, are routine test indexes but p53, C atheps in D, PCNA are not. Thus, the factors in our model are easily available; thirdly, this PI model possessed a higher coincidence rate of DFS between model predicted and actual situation (93%) than that of Fang-Ming's model (77.78%) and that of Yue's model (80.0%).

Our study has a limitation for recruiting a small cohort of patients in the establishment and validation from only one research center. Therefore, a large-scale multicenter study is needed to further validate.

Summarily, this simplified prognostic model was potentially feasible to predict the prognosis of individuals with invasive breast cancer and to determine optimal therapeutic strategy.

Disclosure of Potential Conflicts of Interest

Xianhe Xie and Yunfu Lv were employees of Hainan General Hospital; Xianhe Xie currently is employed at The First Affiliated Hospital of Fujian Medical University; Yanfen Hu and Chao Jing were undergraduate students of Hainan General Hospital. Xianhe Xie received a research funding from Health and Family Planning Commission of Hainan Province and Hainan General Hospital. The other authors have no conflict of interest. All authors had

full access to all of the data in the study and had final responsibility for the decision to submit for publication.

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