Prognostic Value of an Immunohistochemical Signature in Patients with Bladder Cancer Undergoing Radical Cystectomy

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Abstract

BACKGROUND: This study aimed to assess the prognostic value of a various of diagnostic immunohistochemical (IHC) markers and develop an IHC-based classifier to predict the disease-free survival (DFS) of patients with bladder cancer (BC) undergoing radical cystectomy (RC).

METHODS: IHC was performed on tumor specimens from 366 patients with transitional cell BC. The least absolute shrinkage and selection operator (LASSO) Cox regression model was used to develop a multi-marker classifier for predicting DFS of patients with BC. The Kaplan–Meier estimate was performed to assess DFS, and univariate and multivariate Cox regression models were used to identify independent risk factors to predict DFS of patients with BC.

RESULTS: Based on the LASSO Cox regression model, nine prognostic markers were identified in the training cohort. Patients were stratified into low- and high-risk groups using the IHC-based classifier. In the training cohort, the 10-year DFS was significantly better in low-risk patients (70.7%) compared with high-risk patients (17.9%) (p⊠0.001); in the validation cohort, the 10-year DFS was 85.7% for the low-risk group and 20.4% for the high-risk group (p⊠0.001). Multivariable Cox regression analyses showed that the high-risk group based on the nine-IHC-based classifier was associated with poorer DFS adjusted by clinicopathological characteristics. Finally, a nomogram comprising the nine-IHC classifier and clinicopathological factors was developed for clinical application.

CONCLUSION: The nine-IHC-based classifier is a reliable prognostic tool, which can eventually guide clinical decision making regarding treatment strategy and follow-up scheduling of BC.

Novelty & Impact Statements

The present study generated and validated a nine-IHC-based classifier for prognostic prediction in patients with BC undergoing RC. A prognostic nomogram was also constructed by integrating the IHC classifier and clinicopathological characteristics. Predicting outcomes of patients with accurate prognostic models can eventually guide the clinical decision making regarding treatment strategy and follow-up scheduling of BC.

1. Background

Bladder cancer (BC) is currently the 10th most commonly diagnosed malignancy worldwide, accounting for 549,393 new cases and 199,922 deaths in 2018. [1–3] Transitional cell carcinoma is the predominant histologic subtype of BC, contributing to more than 90% of bladder cancer cases. [4] Approximately, 70% of patients are diagnosed with non-muscle-invasive bladder cancer (NMIBC), whereas the remaining have muscle-invasive bladder cancer (MIBC). For MIBC, radical cystectomy (RC) is considered as the standard treatment choice; neoadjuvant chemotherapy is also used in MIBC to improve the survival of patients. [5] However, despite the aggressive treatment strategy, the 5-year overall survival (OS) rate for MIBC is approximately 50%. [6, 7] Thus, clinicopathological features might not be sufficient to predict prognosis

and identify patients with a high risk of disease progression. Undefined molecular regulatory networks that promotes tumorigenesis and progression of BC still exist.

Immunohistochemistry (IHC) is currently the most widely used pathological technique in the accurate diagnosis of urinary bladder neoplasms. [8] IHC analysis is routinely applied to determine the expression of specific BC-associated molecules involved in several biological pathways. The representative markers comprise oncogenes (HER2, EGFR, VEGF, and CyclinD1), tumor proliferation markers (BAX, BCL2, and Ki67), multidrug resistance (MDR) gene, tumor suppressor genes (p53 and p27), and enzymes (GSTπ and TOPOII). [9–11] The identification and validation of the prognostic IHC signature have been reported in various cancer types and proved to be a promising complement in therapeutic planning and patient management. [12, 13] Also, a large number of IHC markers have been used in predicting the prognosis of BC so far, but none of them have entered clinical practice. [14–16]

This retrospective study assessed the prognostic value of a various of IHC markers representative of different biological pathways and developed nine-IHC-based classifier to predict the disease-free survival (DFS) of patients with BC undergoing RC.

2. Methods

2.1 Patients and clinicopathological information

A total of 366 patients with BC undergoing RC (from January 2008 to December 2015) were recruited from the Department of Urology, Fudan University Shanghai Cancer Center (FUSCC) (Shanghai, China). The clinical and pathological data of each patient were recorded, including age at surgery, sex, depth of tumor invasion (T stage), lymph node metastasis (N stage), grade, vascular invasion, perineural invasion, surgical margin, and tumor size (Table 1). Tissue samples were collected during surgery and preserved in the FUSCC tissue bank. The DFS of patients was calculated from the initiation of surgery until the first recurrence, or first progression, including metastasis or death. To develop and validate the classifier, patients were further randomly stratified into training cohort (n = 256) and validation cohort (n = 110). This study was approved by the institutional ethics committee of FUSCC and written informed consent was obtained from all the patients preoperatively.

Table 1
Demographic and clinical characteristics of patients in discovery and validation cohort.

	Training cohort (n = 256)	Validation cohort (n = 110)	*p* value
Gender			0.649
Male	222 (86.7)	98 (89.1)	
Female	34 (12.3)	12 (10.9)	
Age (year)			0.990
(Mean, SD)	59.36 (9.91)	59.35 (10.21)	
Invasion deep (T stage)			0.744
Tis	4 (1.6)	2 (1.8)	
Ta	14 (5.5)	2 (1.8)	
T1	58 (22.7)	27 (24.5)	
T2	70 (27.3)	33 (30.0)	
T3	74 (28.9)	31 (28.2)	
T4	36 (14.1)	15 (13.6)	
Lymph node metastasis (N stage)			0.416
Negative	198 (77.3)	80 (72.7)	
Positive	58 (22.7)	30 (27.3)	
Grade			0.830
Low grade	22 (8.6)	8 (7.3)	
High grade	234 (91.4)	102 (92.7)	
Vascular invasion			0.894
Absent	178 (69.5)	75 (68.2)	
Present	78 (30.5)	35 (31.8)	
Perineural invasion			0.779
Absent	196 (76.6)	82 (74.5)	
Present	60 (23.4)	28 (25.5)	
Surgical margin			1.000
Negative	232 (90.6)	100 (90.9)	

	Training cohort (n = 256)	Validation cohort (n = 110)	*p* value
Positive	24 (9.4)	10 (9.1)	
Tumor size (cm)			0.401
(Mean, SD)	3.74 (1.86)	3.93 (2.03)	

2.2 Immunohistochemistry

All BC tissues were collected from the overall patient cohort, fixed in 10% buffered formalin, and embedded in paraffin. Immunohistochemistry was then performed by the Immunohistochemistry Diagnostic Laboratory of FUSCC to detect the expression of diagnostic biomarkers of BC (Supplementary Table 1). Briefly, the sections were deparaffinized in xylene and rehydrated in graded alcohol washes. Antigen retrieval was performed in citric acid (10 mM, pH 6.0) at 95 °C for 30 min (HER2, EGFR, BAX, BCL2, MDR, and GSTπ), or a Tris-based buffer (pH 8.3) solution at 95 °C for 60 min (VEGF, CyclinD1, Ki67, p53, p27, and TOPOII) with the help of a microwave. The sections were then treated with 0.3% hydrogen peroxide for 30 min to block endogenous peroxide activity. Next, the slides were incubated with primary antibodies at 4°C overnight and then incubated with biotinylated anti- rabbit or anti-mouse IgG secondary antibodies (EnVision Plus; Dako, CA, USA) for 30 min at 37°C. Finally, the sections were stained using a DAB kit (Dako, Agilent Technologists, CA, USA) and counterstained with hematoxylin.

2.3 Evaluation of immunohistochemistry

Immunostaining reactivity was observed by two experienced pathologists blinded to the clinical features independently. The proportion of positively stained cells and the maximum intensity of IHC signal were estimated. The staining score of the surface membrane, cytoplasm, or nucleus of tumor cells was calculated based on the four-point system: IHC0 (negative), IHC1+ (weak), IHC2+ (moderate), and IHC3+ (strong). Notably, the protein expression of Ki67 was scored based on the percentage of positively stained cells in 200 cancer cells, and IHC staining of HER2 was estimated based on a gastric cancer scoring system established by Park et al.. [17]

2.4 Statistical analysis

Demographic characteristics were summarized as count and percentage for categorical variables. Pearson's chi-square test was performed to analyze the distribution of categorical data. The least absolute shrinkage and selection operator (LASSO) Cox regression model was used to develop the multi-marker classifier for predicting DFS of patients with BC in the training cohort. Survival analysis was performed using the Kaplan−Meier method with p values determined by the log-rank test. Univariate and multivariate Cox regression models were used to identify the independent risk factors to predict the DFS of patients with BC. Factors with a p value < 0.1 in the univariate analysis were subjected to multivariate analysis. Receiver operating characteristic (ROC) analysis was used to assess the prognostic

performance of the IHC classifier. Nomogram was developed based on the independent prognostic factors according to the multivariate Cox regression analysis. Calibration curves were employed to compare the nomogram-predicted survival probabilities and the actual survival probabilities.

All statistical assessments were evaluated at a two-sided p value of 0.05. All analyses were conducted using R software 3.5.2 (R Foundation for Statistical Computing, Vienna, Austria). The "maxstat" package was used to determine the cut-off values for continuous variables. The "glmnet" package was used to conduct the LASSO Cox regression model analysis. The "survival" and "survminer" packages were used to perform survival analysis. The "timeROC" package was used to plot the ROC curves and determine the area under the curve (AUC). The "rms" package was used to develop nomogram and calibration curves.

3. Results

3.1 Demographic and clinical characteristics of patients

The demographic and clinical characteristics of 366 patients with BC undergoing RC are presented in Table 1. The mean age of the patients was 59.36 years (9.98), and the median follow-up time was 62 months (range 1–135 months). A majority of patients in both sets were male. In the total patient cohort, 113 (30.9%) patients presented vascular invasion, 88 (24.0%) had perineural invasion, and 34 (9.3%) had histologically positive resection margins. The clinical stage of the patients was determined according to the AJCC 8th edition TNM system. A total of 259 (70.8%) patients had muscle invasion (T2–T4), 88 (24.0%) had lymph node metastasis (N1–N3), and none had distant metastasis (M1).

3.2 Development and validation of the immunohistochemical signature

Based on the LASSO Cox regression model, nine prognostic markers (HER2, EGFR, VEGF, CyclinD1, BAX, MDR, p53, p27, and TOPOII) were identified in the training cohort. The risk score of individual patients was calculated according to the expression of these nine IHC markers and their corresponding coefficients (Fig. 1). Risk score = (0.03338335* HER2) + (0.108497374* EGFR) + (0.027900778* VEGF) + (0.008648065* CyclinD1) + (−0.088330675* BAX) + (−0.0126913* MDR) + (−0.096131019* p53) + (−0.011932695* p27) + (0.311672511* TOPOII). The "maxstat" package was used to determine the cut-off value of the IHC classifier, where the risk score ≤ 0.04311026 represented low risk and the risk score > 0.04311026 represented high risk. Furthermore, an adjusted value (−0.04311026) was added to the final formula to simplify the clinical application (Fig. 2). Based on this IHC prognostic model, 208 (81.3%) patients were stratified into the low-risk group and 48 (18.7%) were stratified into the high-risk group in the training cohort. Patients with higher risk scores had poorer outcomes, with 5-year and 10-year DFS of 71.7% and 17.9%, respectively, compared with patients with lower risk scores (5-year survival probability: 90.9%; 10-year survival probability: 70.7%) (p<0.001, Fig. 3a). The prognostic value of this nine-IHC prognostic model was further examined in the validation cohort. Further, 85 (77.3%) patients were

classified as low risk and 25 (22.7%) as high risk; the 10-year survival probability was significantly better in low-risk patients (85.7%) compared with high-risk patients (20.4%) (p<0.001, Fig. 3b).

3.3 Development and internal validation of the nomogram

Based on the univariate and multivariate Cox regression model, older age, advanced T stage, lymph node metastasis, high grade, larger tumor size, vascular invasion, resection margin, and high-risk group based on the IHC classifier were identified as independent risk factors associated with poorer DFS (Table 2). Notably, the 1-year AUC of the IHC classifier based on the time-dependent ROC curve analysis was 0.691, better than that according to the AJCC 8th edition TNM system (AUC = 0.563). Moreover, the combination of the IHC classifier and the AJCC-based prediction model had the best prediction accuracy (AUC = 0.722) (Fig. 4a). The 5-year ROC curve analysis also demonstrated the promising prognostic value of the IHC classifier (Fig. 4b). A prognostic nomogram was then constructed by integrating nine-IHC classifier and multiple clinicopathological prognostic factors independently associated with DFS (Fig. 5). By summing each score of all the selected variables, the 1-, 5-, and 10-year survival probabilities of the individual patients were determined. The C-index of the nomograms to predict DFS was 0.78 (95%CI: 0.65–0.94) and 0.68 (95%CI: 0.60–0.77) for the IHC-classifier-based prediction model and AJCC-based prediction model, respectively. The internal and external calibration curves for 1-, 5-, and 10-year DFS also showed high consistency between the estimates using the IHC-classifier-based nomogram and the actual survival probabilities in the training and validation cohorts (Fig. 6).

Table 2
Univariate and multivariate Cox regression analyses of DFS in BC patients

Covariates	Univariate analysis		Multivariate analysis	
	HR (95%CI)	P value	HR (95%CI)	P value
Age at surgery	**1.05 (1.02–1.07)**	**<0.001***	**1.04 (1.02–1.07)**	**0.001***
Sex (female *vs.* male)	1.29 (0.72–3.09)	0.286		
Invasion depth (MIBC *vs.* NMIBC)	**0.42 (0.24–0.73)**	**0.002***	**0.71 (0.54–0.93)**	**0.015***
Lymph node metastasis (N0 *vs.* N1)	**2.89 (1.81–4.61)**	**<0.001***	**1.89 (1.48–2.41)**	**0.004***
Grade (high grade *vs.* low grade)	**0.11 (0.03–0.44)**	**0.002***	**0.30 (0.12–0.78)**	**0.011***
Vascular invasion (absent *vs.* present)	**2.80 (1.80–4.36)**	**<0.001***	**1.99 (1.14–3.47)**	**0.015***
Perineural invasion (absent *vs.* present)	**1.99 (0.23–3.21)**	**0.005***	1.88 (0.97–3.62)	0.060
Surgical margin (negative *vs.* positive)	**2.66 (1.56–4.51)**	**<0.001***	**2.34 (1.20–4.56)**	**0.013***
Tumor size	**1.13 (1.03–1.24)**	**0.010***	**1.16 (1.01–1.34)**	**0.030***
9-IHC-based classifier (high risk *vs.* low risk)	**0.23 (0.11–0.48)**	**<0.001***	**0.20 (0.09–0.45)**	**<0.001***

BC: bladder cancer, HR: hazard ratio; CI: confidence interval; MIBC: muscle-invasive bladder cancer; NMIBC: non-muscle-invasive bladder cancer; IHC: immunohistochemical

4. Discussion

BC is a biologically heterogeneous disease with complicated molecular alterations during cancer progression and metastasis. [18] Canonical prognostic characteristics, including AJCC TNM system and grade have limited ability to predict the survival of patients BC. Radical cystectomy is the current standard treatment for MIBC, while the potential benefit of RC must be weighed against its risks and impact on the quality of life. [7] Tumor heterogeneity has posed a tremendous challenge to the management of BC. The IHC analysis of biomarker expression is complementary to the evaluation of tumor morphology and important in the accurate diagnosis of BC. [8] The markers that constitute the diagnostic signature have a varied range of ascribed functions. [4, 18] Growing evidence shows that the diagnostic IHC markers can also identify patients at high risk of progression after surgery and improve the disease management of patients in various cancer types. [12, 19]

In this study, a nine-IHC signature was constructed using the LASSO Cox regression model analysis for the prediction of DFS in patients with BC undergoing RC. A significant distinction in prognosis was observed between low-risk and high-risk patients by applying the nine-IHC classifier to the training cohort. Further, the potential value of the signature was validated in the validation cohort, indicating the broad applicability of this classifier. Multivariable Cox regression analyses showed that the high-risk group based on the IHC classifier was an independent risk factor associated with poorer DFS adjusted by clinicopathological characteristics. The time-dependent ROC curve analysis revealed that the IHC signature combined with AJCC staging was a more effective prognostic model than the AJCC staging system alone. Finally, a prognostic nomogram integrating nine-IHC classifier and multiple clinicopathological prognostic factors was developed for clinical application.

The molecular classification of urothelial bladder cancer has taken significant steps forward in the last decade. In 2012, Sjodahl et al. first identified five major subtypes; urobasal A, genomically unstable, urobasal B, squamous cell carcinoma-like, and infiltrated. [20] In 2014, Choi et al. proposed a three-group system; basal, luminal, and p53-like. [21] TCGA developed a four-group system at the same time, and in 2017, Robertson et al. updated the TCGA classification system; luminal-papillary, luminal-infiltrated, luminal, neuronal, and basal-squamous. [22, 23] Numerous researchers then attempted to develop a reliable IHC panel for predicting patient outcome. The panel components were updated regularly; however, only a few have eventually entered clinical practice. [15, 16]

The present study developed a risk-stratification algorithm based on an IHC-based classifier with the purpose to facilitate the clinical management of BC. Of the 12 biomarkers tested, nine potential predictors were identified. The biological function of biomarkers included in the signature has been previously reported. [24] EGFR is a receptor tyrosine kinase involved in the pathogenesis of a variety of cancers. The role of EGFR as a strong independent prognostic marker and therapeutic target in BC has been well identified. [25, 26] HER2 is a transmembrane phosphoglycoprotein belonging to the EGFR family and is known as an established therapeutic target in breast carcinomas. [27] HER2 is also overexpressed in a variety of human malignant tumors including BC. Several studies demonstrated that HER2 overexpression was an independent risk factor associated with unfavorable prognosis in BC. [28] VEGF has been considered as an important factor in pathological angiogenesis, and the VEGF level has been identified as a significant predictor of OS and CSS in patients with BC. [29] CyclinD1 is a regulatory protein in the G1/S transition and its aberrant expression can lead to uncontrolled cell proliferation. [30] BAX is an important apoptosis-related molecule that promotes cell apoptosis. Previous studies demonstrated that the expression level of BAX provided the prognostic information of patients with BC. [31] MDR proteins are frequently expressed in untreated BC and confer resistance to a distinct spectrum of drugs. [32] The tumor suppressor gene TP53 is the most commonly mutated gene in human cancer; the mutations of TP53 result in increased p53 nuclear accumulation. The prognostic value of p53 to determine the risk of BC recurrence and progression has been assessed. [33] p27 is a negative cell cycle regulatory gene that potentiates cell cycle arrest in the G1 phase. A decreased p27 protein level has been proved to be associated with the poor prognosis of patients with BC. [34] TOPOII is a DNA gyrase isoform essential in cell cycle. Previous studies have shown its diagnostic value in BC. [35]

Despite promising predictive accuracy of the nine-IHC marker-based nomogram, this study had several limitations derived from its retrospective nature. Moreover, this signature was validated only in an individual cancer center, and thus further validation from multiple centers and across different populations is expected.

In summary, this study generated and validated a nine-IHC-based classifier for prognostic prediction in patients with BC undergoing RC. A prognostic nomogram was also constructed by integrating the IHC classifier and clinicopathological characteristics. Predicting outcomes of patients with accurate prognostic models can eventually guide the clinical decision making regarding treatment strategy and follow-up scheduling of BC.

Declarations

Ethics approval and consent to participate

This study was approved by the institutional ethics committee of Fudan University Shanghai Cancer Center and written informed consent was obtained from all the patients preoperatively.

Consent for publication

Not applicable.

Availability of data and material

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare no conflict of interest.

Funding

Authors' contributions

Jie Wu conceived the idea and was a major contributor in manuscript writing. *Yu-Chen Wang* conducted the data collection and was involved in the manuscript writing. *Jun-Miao Wen* performed all statistical analyses. *Wen-Jie Luo*, *Qi-Feng Wang* and *Hong Lv* designed and performed the experiments. *Bo Dai*, *Ding-Wei Ye* and *Yi-Ping Zhu* critically revised the manuscript. All authors read and approved the final manuscript.

### Acknowledgments

Not applicable.

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Figures
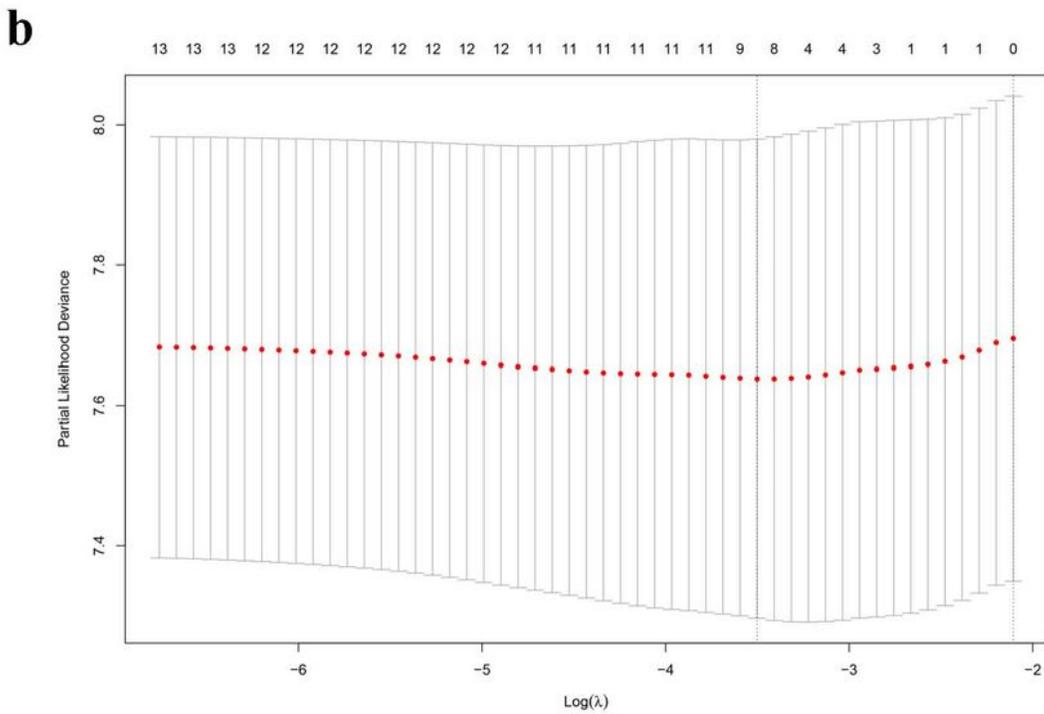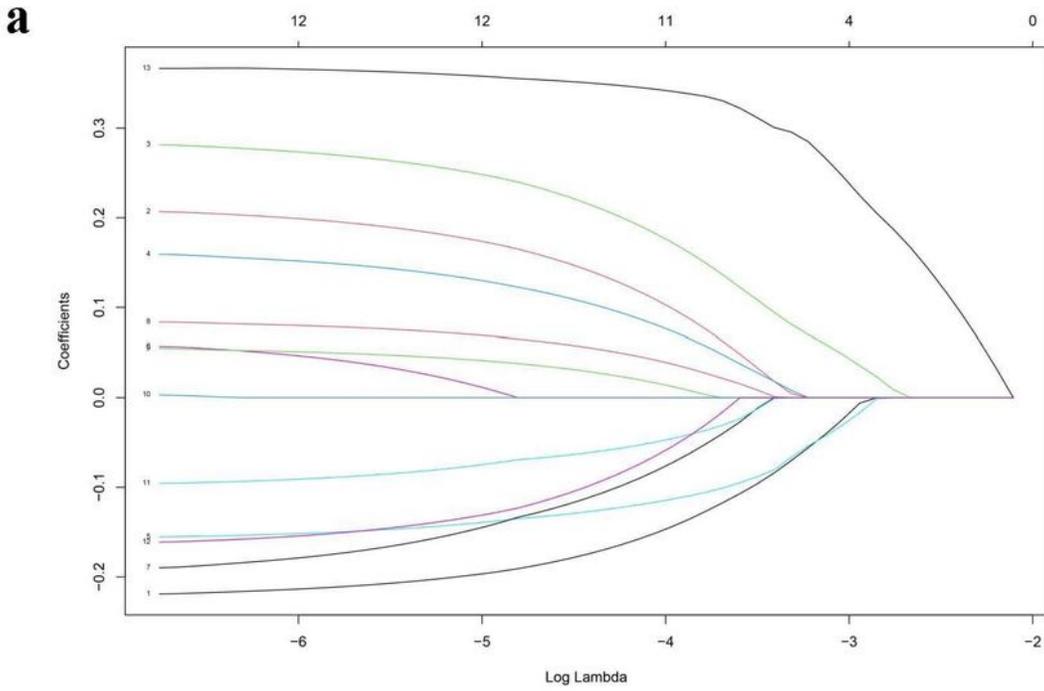
Figure 1

(a) LASSO coefficient profiles of the selected IHC markers. (b) The tuning parameter (λ) selection used 10-fold cross-validation via minimum criteria. Partial likelihood deviance was plotted versus log(λ).
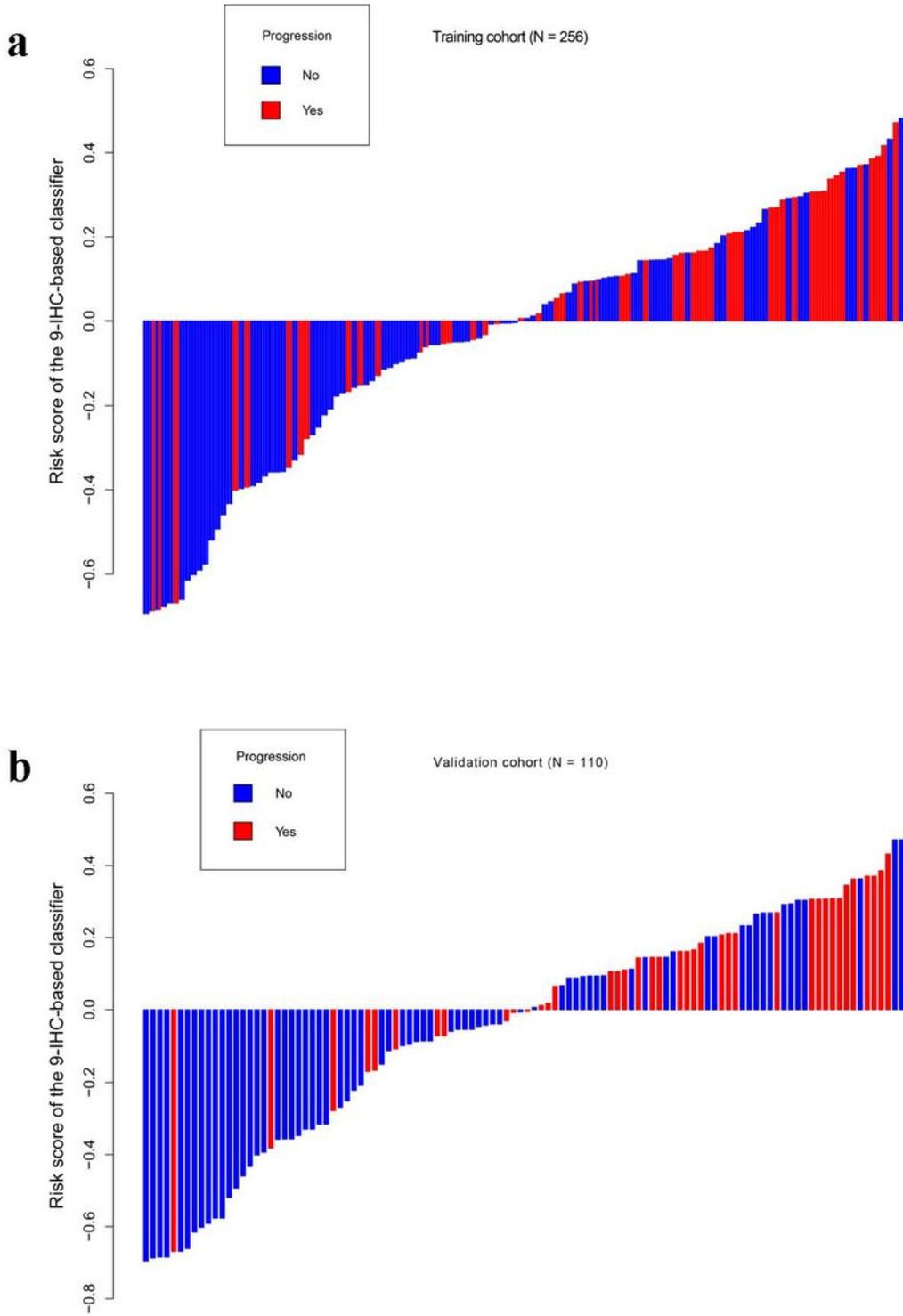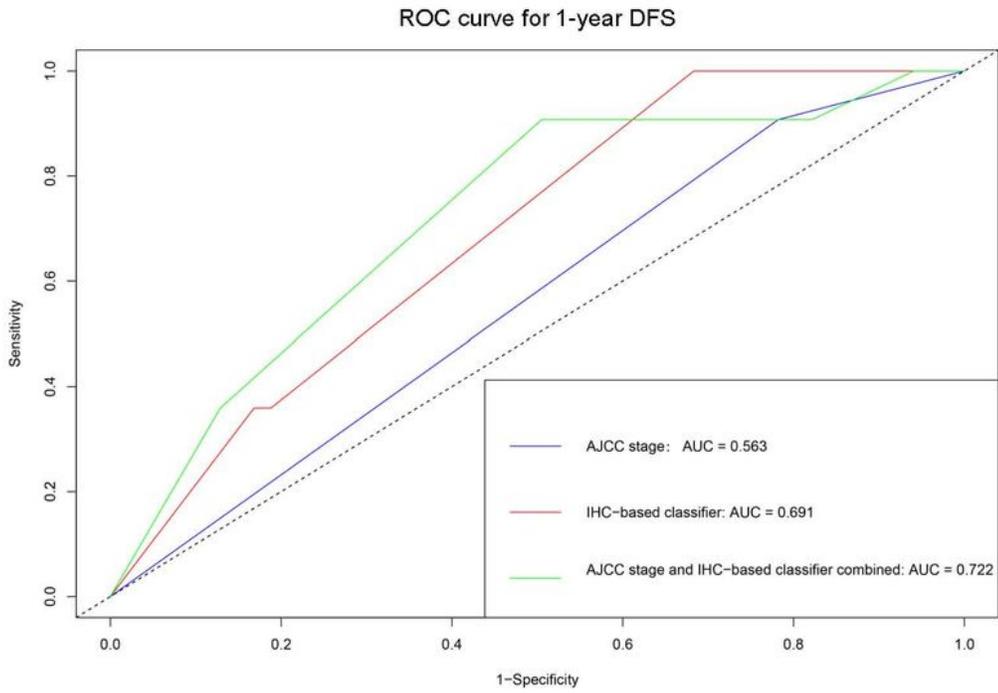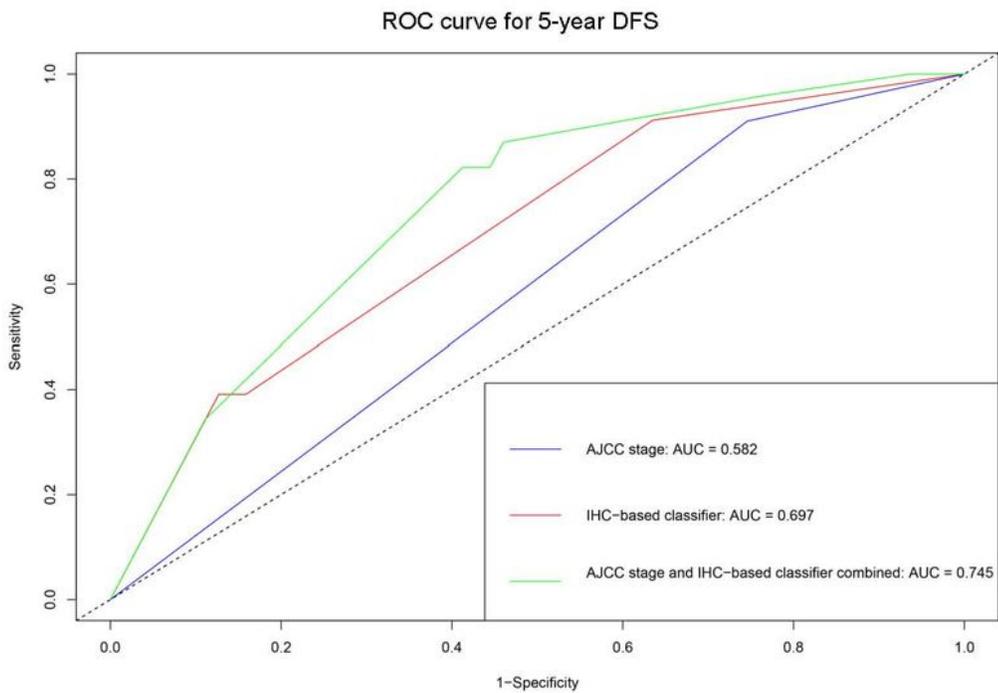
Figure 2

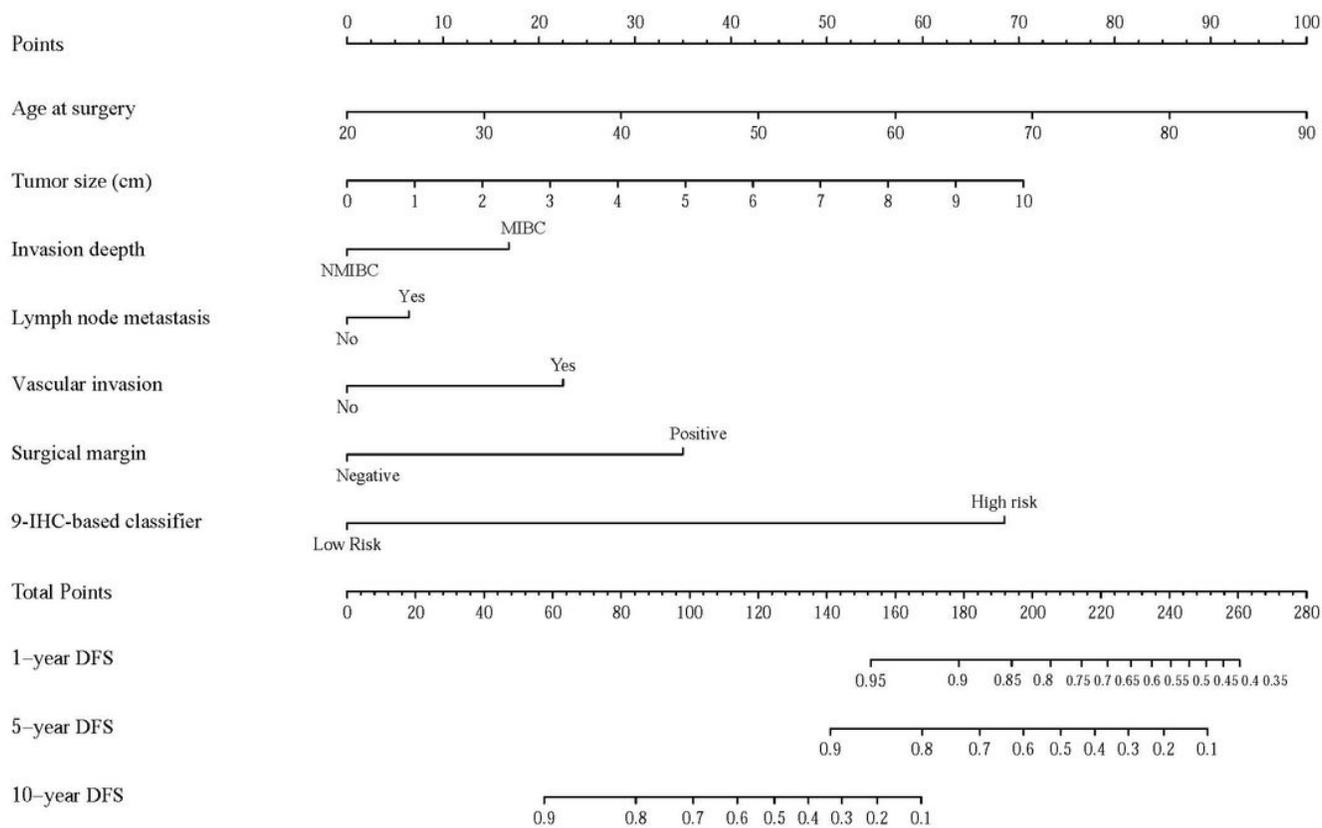Distribution of risk score based on 9-IHC-based classifier. (a) Training cohort. (b) Validation cohort.
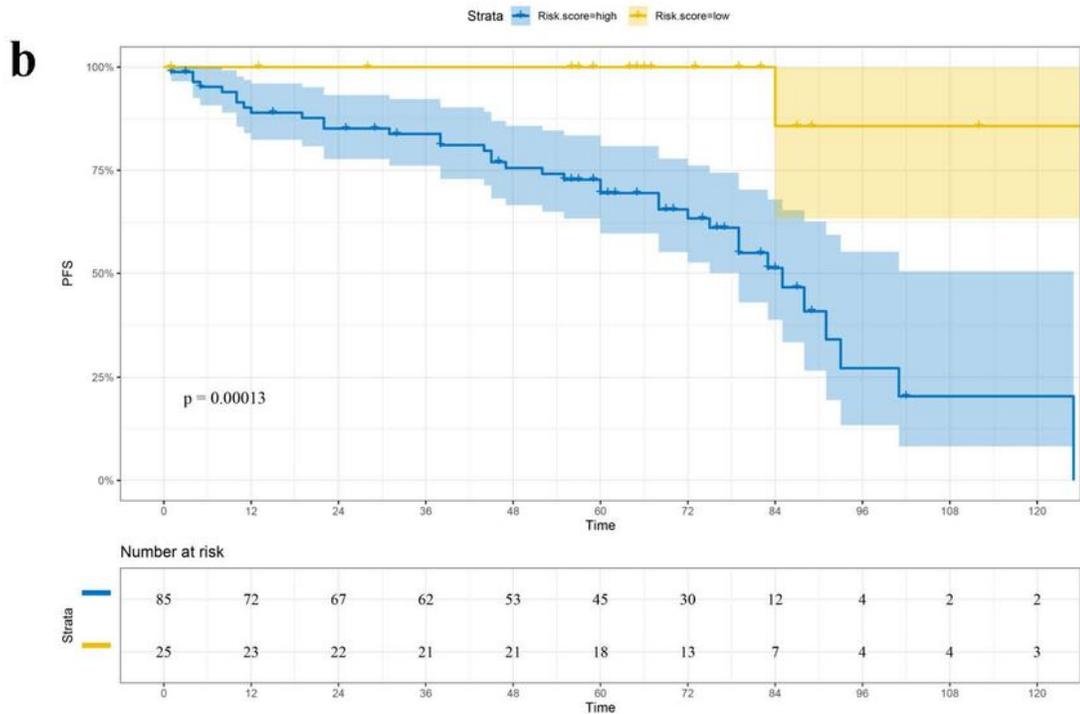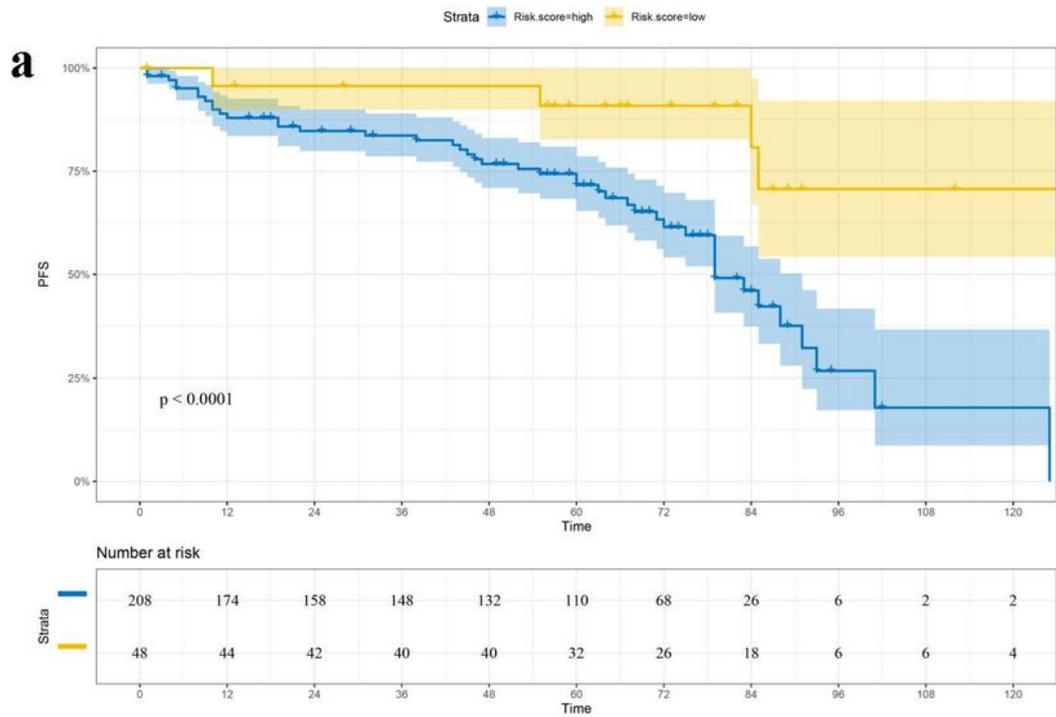
Figure 3

Comparison of DFS in low-risk and high-risk groups stratified by IHC signature. (a) Training cohort. (b) Validation cohort.
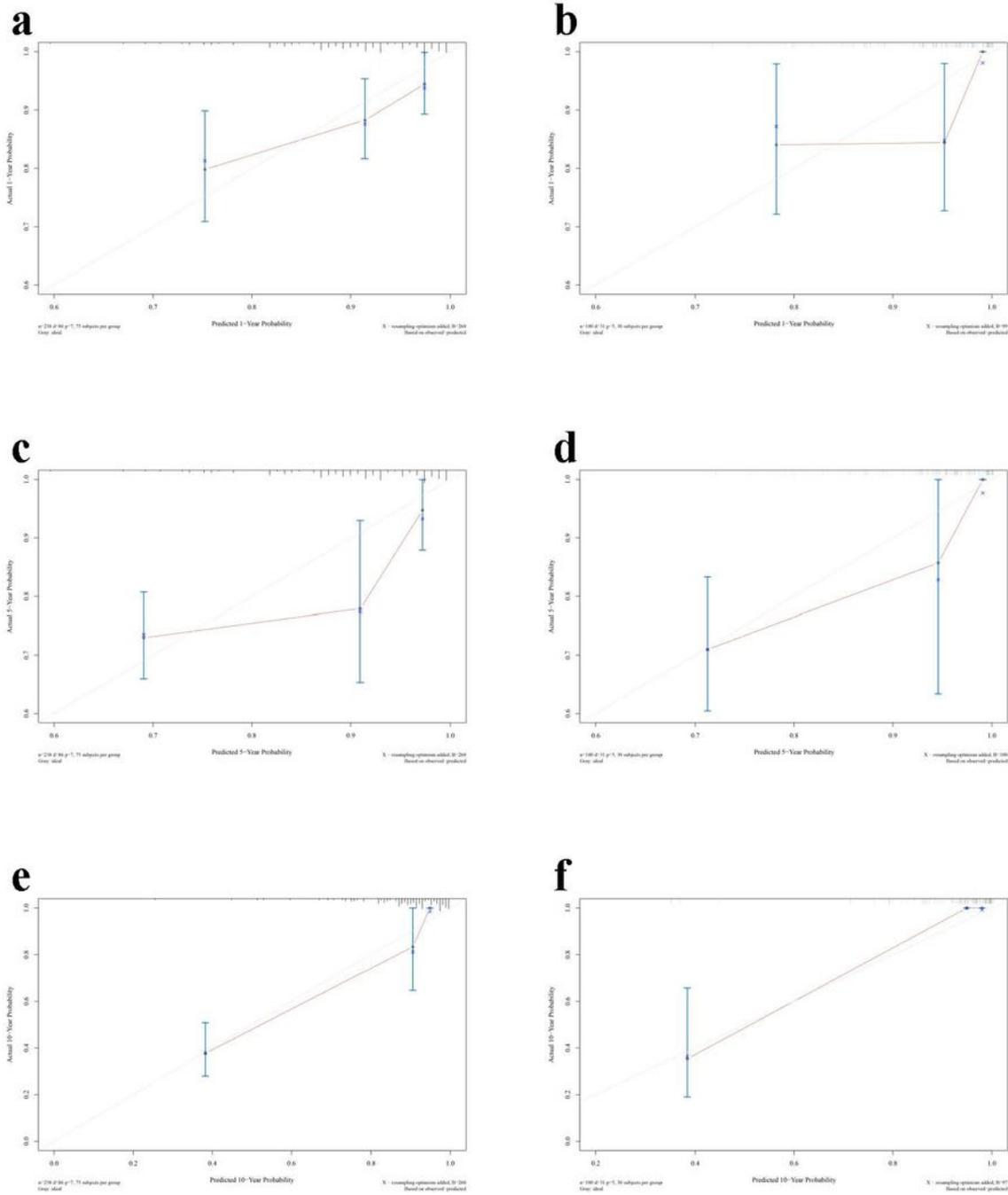
Figure 4

ROC curve analyses of the prognostic value of IHC-based classifier and AJCC stage. (a) 1-year DFS. (b) 5-year DFS.

Figure 5

The nomogram for predicting 1-, 5-, and 10-year DFS of BC patients receiving RC.

Figure 6

Calibration curves of (a, c, and e) 1-, 5-, and 10-year DFS for training cohort. (b, d, and f) 1-, 5-, and 10-year DFS for validation cohort.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable1.docx