



# Identification and evaluation of a risk model predicting the prognosis of breast cancer based on characteristic signatures

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**Background:** Breast cancer (BC) is one of the most common fatal cancers in women. Identifying new biomarkers is thus of great significance for the diagnosis and prognosis of BC.

**Methods:** In this study, 1,030 BC cases from The Cancer Genome Atlas (TCGA) were obtained for differential expression analysis and Short Time-series Expression Miner (STEM) analysis to identify characteristic BC development genes, which were further divided into upregulated and downregulated genes. Two predictive prognosis models were both defined by Least Absolute Shrinkage and Selection Operator (LASSO). Survival analysis and receiver operating characteristic (ROC) curve analysis were used to determine the diagnostic and prognostic capabilities of the two gene set model scores, respectively.

**Results:** Our findings from this study suggested that both the unfavorable (BC1) and favorable (BC2) gene sets are reliable biomarkers for the diagnosis and prognosis of BC, although the BC1 model presents better diagnostic and prognostic value. Associations between the models and M2 macrophages and sensitivity to Bortezomib were also found, indicating that unfavorable BC genes are significantly involved in the tumor immune microenvironment.

**Conclusions:** We successfully established one predictive prognosis model (BC1) based on characteristic gene sets of BC to diagnose and predict the survival time of BC patients using a cluster of 12 differentially expressed genes (DEGs).

**Keywords:** Breast cancer (BC); risk models; characteristic gene set; prediction; prognosis

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## Introduction

Breast cancer (BC) is one of the most common types of cancers worldwide and one of the leading fatal cancers in females, accounting for approximately 14% of all cancer-related deaths (1). While BC is a rare disease in males with

a much lower rate of incidence, the mortality rate of BC male patients is quadruple that of female patients, making it worth attention (2). According to statistics, there were 194,280 new BC cases in the US in 2009 and 40,610 BC-related deaths (3), while a decade later, those numbers

increased to 268,670 and 41,400, respectively (2).

Advancement in the diagnosis of BC may contribute to the rising incidence of BC. Self-inspection of the breasts at an early stage is considered to be effective for the discovery and diagnosis of BC and can prevent BC-related deaths, but there are still numerous BC cases that are only diagnosed at advanced stages due to patient (female) negligence of the importance of self-inspection and regular clinical examination of the breasts. Screening examinations are gradually becoming routine for BC diagnosis, including ultrasound breast imaging, single photon emission computerized tomography, and digital mammography. Mammography is considered the gold standard test for early BC detection (3), although there are concerns, such as radical harm caused by frequent screening examinations, false-positive results and overdiagnosis, which lead to a negative impact on patient quality of life (4). Biomarkers of BC are also used in diagnosis during different stages, including immunohistochemical markers [ER, PR, HER2 (ERBB2), and proliferation marker protein Ki-67 (MKI67)], genomic markers (BRCA1, BRCA2, and PIK3CA), and immunomarkers (tumor-infiltrating lymphocytes and PD-L1) (5). While there has been progress in BC treatment, the mortality rate is not declining, indicating that new biomarkers or models for BC survival prediction and prognosis should be established to help provide more efficient and personalized measurements for managing BC. By 2022, the incidence and mortality of BC in the Chinese population has been predicted to be higher than those

in developed countries (6). Given the lack of an effective survival prediction model for physicians, novel biomarkers or models for BC survival prediction and prognosis should be established to help provide more efficient and personalized measures for managing BC in the early stage.

In our study, we identified two characteristic gene sets of BC development, the BC unfavorable gene set and the BC favorable gene set. Survival analysis and ROC analysis were used to determine the diagnostic and prognostic capabilities of the two gene set model scores, respectively. Both the unfavorable and favorable BC gene set models presented reliable biomarkers for BC diagnosis and independent biomarkers for prediction and prognosis. We present this article in accordance with the TRIPOD reporting checklist (available at <https://tcr.amegroups.com/article/view/10.21037/tcr-22-2444/rc>).

## Methods

### Datasets

The gene expression profiles of BC and healthy breast samples were obtained from The Cancer Genome Atlas (TCGA), including 173 stage I BCs, 597 stage II BCs, 240 stage III BCs, 20 stage IV BCs and 113 normal samples. TCGA is an international database that is publicly accessible and freely available for research and contains a large collection of human cancer genome sequencing data. Previous studies using a group of genes with known alterations in BC to validate TCGA data suggested that TCGA had accurately documented the genomic abnormalities of multiple malignancies (7-9). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

### Identification of differentially expressed genes (DEGs)

Preliminary identification of the characteristic genes promoting or inhibiting the development of BC was performed using differential expression analysis and Short Time-series Expression Miner (STEM) analysis. STEM is a new software package for analyzing short time-series expression data. The software can find statistically significant patterns from short time-series microarray experiments and compare datasets across experiments. STEM presents its analysis of the data in a highly visual and interactive manner (10). STEM's unique algorithm tends to cluster and compare short time-series gene expression data combined

### Highlight box

#### Key findings

- A predictive prognosis model based on characteristic gene sets of breast cancer was established to predict the survival time of breast cancer patients using a cluster of 12 differentially expressed genes.

#### What is known and what is new?

- Several biomarkers and models have been reported and established for the prognosis of breast cancer, but their efficacy is underestimated.
- A novel prognosis model based on characteristic gene sets of breast cancer has been introduced.

#### What is the implication, and what should change now?

- This model could be used as a tool for the prognosis of patients with breast cancer, and further extensive functional exploration revealed the model was closely related to tumor immune microenvironment, especially M2 macrophage infiltration.

with its visualization capabilities and integration with Gene Ontology, suggesting that STEM is useful in the analysis of data from a significant portion of all microarray studies (10).

The RNA sequencing expression profile of BC was displayed as read counts, which were subsequently normalized by the voom function in the limma package.  $P < 0.01$  adjusted by the false discovery rate (FDR) and  $|\log \text{ fold change (FC)}| > 1.5$  were set as thresholds. In the development of BC, if a DEG was gradually upregulated ( $\log \text{ FC stage I vs. control} < \log \text{ FC stage II vs. control} < \log \text{ FC stage III vs. control} < \log \text{ FC stage IV vs. control}$ ) or gradually downregulated ( $\log \text{ FC stage I vs. control} > \log \text{ FC stage II vs. control} > \log \text{ FC stage III vs. control} > \log \text{ FC stage IV vs. control}$ ), then it was considered to be a characteristic BC development gene.

### *Limma package*

DEGs in the four stages of BC were identified separately using the limma package with RNA sequencing expression profiles of BC in TCGA. The package can now perform both differential expression and differential splicing analyses of RNA sequencing (RNA seq) data. This software provides an integrated data analysis solution, using advanced computational algorithms to deliver reliable performance on large datasets, represent expression data and simplify the user interface (11). Significantly DEGs were selected by using the FDR and log (FC) values. In the development of BC, if the DEGs were gradually upregulated [log (FC) value increased] or gradually downregulated [log (FC) decreased], they were considered to promote or suppress BC development.

### *Cox regression analysis*

Univariate Cox regression analysis was used to identify genes associated with BC prognosis. Multivariate Cox regression analysis was used to test the correlation among our models, current clinicopathological features and BC prognosis.

### *Least Absolute Shrinkage and Selection Operator (LASSO) regression analysis*

We screened the expression levels for significant genes in the favorable and unfavorable groups using univariate Cox regression analysis ( $P < 0.01$ ). Then, these genes were selected for further functional investigation, and a possible risk score was developed using the LASSO Cox regression

algorithm. The most refined prognostic prediction models were constructed by minimal compression using LASSO regression analysis based on two characteristic gene sets of BC development. With a constraint condition in the sum of the absolute terms, LASSO could reduce the dimension of the independent variable and produce a better model fit. It was suitable for analyzing the genetic survival data, identifying more meaningful independent variables and determining the model. The fixed coefficient  $R^2$  indicates the fraction of the variation that the covariate explains. The larger the percentage is, the better the model fit (12).

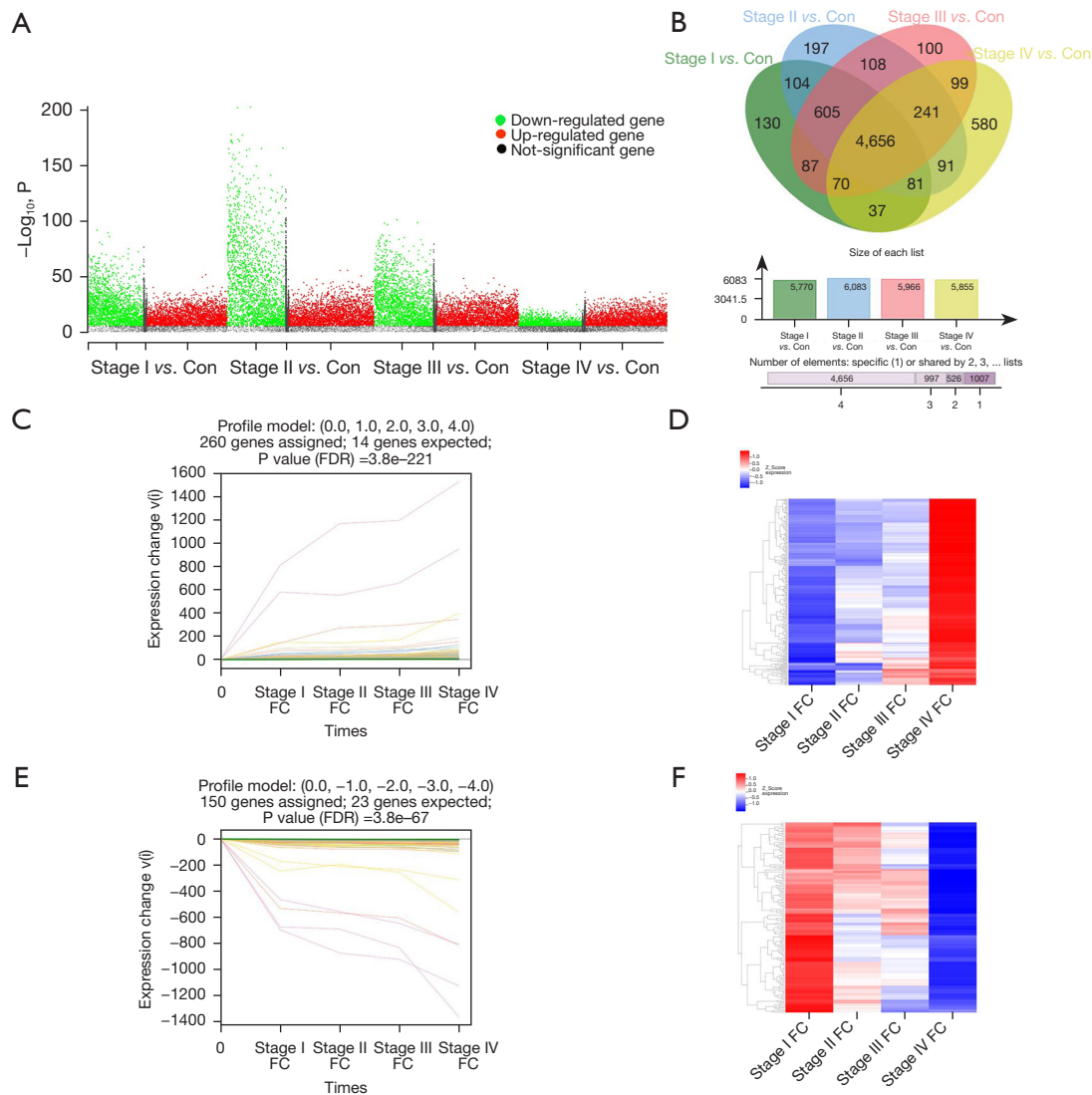
### *Calculation of characteristic BC development Gene Set Variation Analysis (GSVA) Score*

Both models based on upregulated and downregulated characteristic gene sets of BC development were scored. GSVA is a popular method of scoring individual samples for molecular characteristics or gene sets. The GSVA package in R was used to calculate the BC-unfavorable GSVA score and BC-favorable GSVA score for individual samples.

We screened the expression levels for genes in the TCGA data group using univariate Cox regression analysis. Thus, we identified genes that were highly related to survival ( $P < 0.01$ ). We chose these genes for further functional investigation and developed a possible risk score using the LASSO Cox regression algorithm. In detail, the risk score of the TCGA training set was calculated with the linear combination of the signature gene expression weighted by their regression coefficients. Risk score =  $(\text{expr}_{\text{gene1}} \times \text{coefficient}_{\text{gene1}}) + (\text{expr}_{\text{gene2}} \times \text{coefficient}_{\text{gene2}}) + \dots + (\text{expr}_{\text{genen}} \times \text{coefficient}_{\text{genen}})$ . Finally, minimum criteria defined genes and their constants, choosing the perfect penalty parameter  $\lambda$  related to the minimum 10-fold cross-validation in the training set.

### *Statistical analysis*

Receiver operating characteristic (ROC) curve analysis and survival analysis were used to explore the diagnostic and prognostic capabilities of the two scoring systems. In ROC curve analysis, the area under the curve provides an objective parameter of the diagnostic or prognostic accuracy of a test, which is superior to comparing single combinations of sensitivity and specificity values since the influence of the threshold value is eliminated. Because only part of the ROC curve represents clinically relevant combinations of sensitivity and specificity, comparing the



**Figure 1** Identification of DEGs in different stages of BC. (A) Manhattan plot showing DEGs in different stages of BC; (B) Venn diagram showing common DEGs in BC stages I-IV; (C,D) upregulated genes with the development of BC; (E,F) downregulated genes with the development of BC. Con, control; FDR, false discovery rate; FC, fold change; DEGs, differentially expressed genes; BC, breast cancer; STEM, Short Time-series Expression Miner.

ROC curves in the relevant sensitivity or specificity ranges is preferred over comparing the total area under the curve. In addition, ROC analysis can be used to determine the optimal threshold value for tests that generate continuous quantitative data (13). Multivariate Cox regression analysis was used to compare the relative prognostic value of the two scoring systems with that of routine clinicopathological features. Moreover, correlation analysis was used to explore the correlation between these models and the tumor immune microenvironment and drug responses.

## Results

### Identification of gradually up/downregulated genes

Compared to normal breast tissue samples, a total of 5,770 DEGs were identified in stage I BCs, 6,083 DEGs in stage II BCs, 5,966 DEGs in stage III BCs, and 5,855 DEGs in stage IV BCs, with a total of 4,656 common DEGs among all stages (Figure 1A,1B). We summarized 10 major variation tendencies of gene expression by STEM analysis in BC gene expression profiles in TCGA (Figure S1). Based on these

tendencies, DEGs were classified into two groups: one group (BC1 cluster) contained 476 genes that were gradually upregulated with the development of BC from stage I to stage IV (Figure 1C,1D), indicating the progression of BC; this group was named the BC unfavorable gene set. The second group (BC2 cluster) contained 262 genes that were gradually downregulated with the development of BC (Figure 1E,1F), which indicated a good prognosis, named the BC favorable gene set.

To identify BC DEGs associated with prognosis, we first used univariate Cox regression analysis to assess the association between the expression levels of each DEG and found that 26 genes were significantly associated with BC prognosis in the BC unfavorable gene set (Table S1;  $P < 0.01$ ) and 14 genes in the BC favorable gene set (Table S2;  $P < 0.01$ ).

#### ***Establishment and evaluation of a prognostic model based on BC DEGs***

LASSO regression analysis was performed to establish risk models concerning BC prediction and prognosis based on the two characteristic gene sets of BC development. The results showed that a total of 12 DEGs (Figure 2A-2D) in the BC unfavorable gene set and 7 DEGs (Figure 2E-2H) in the BC favorable gene set were significantly associated with BC prognosis. On this basis, two risk models were obtained, consisting of 12 genes and 7 genes, named the BC unfavorable model (BC1 model) and BC favorable model (BC2 model), respectively.

ROC analysis was performed to evaluate the prognostic efficacy of the two risk models. In the BC1 model, the AUC was 0.80 (Figure 2I), presenting great capability of the model for BC prognosis, while in the BC2 model, the AUC was 0.66 (Figure 2J). Compared with the BC2 model, the BC1 model was more efficient in prognostic capability. ROC analysis of BC patients at 1, 2, and 3 years after diagnosis was also performed, and the AUCs were 0.80, 0.77, and 0.75, respectively, in the BC1 model (Figure 2K) and 0.66, 0.69, and 0.74 in the BC2 model (Figure 2L). The results showed that both the BC1 and BC2 models could accurately predict the survival rate of BC patients, especially the BC1 model.

Additionally, the efficacy of the risk score, age, stage, T stage, M stage and N stage for BC prognosis was compared. In the BC1 model, the AUC of the risk score was 0.80, which was the highest among these clinical characteristics, illustrating that the BC1 model was more efficient than other clinical factors for prognosis (Figure 2M). In the BC2

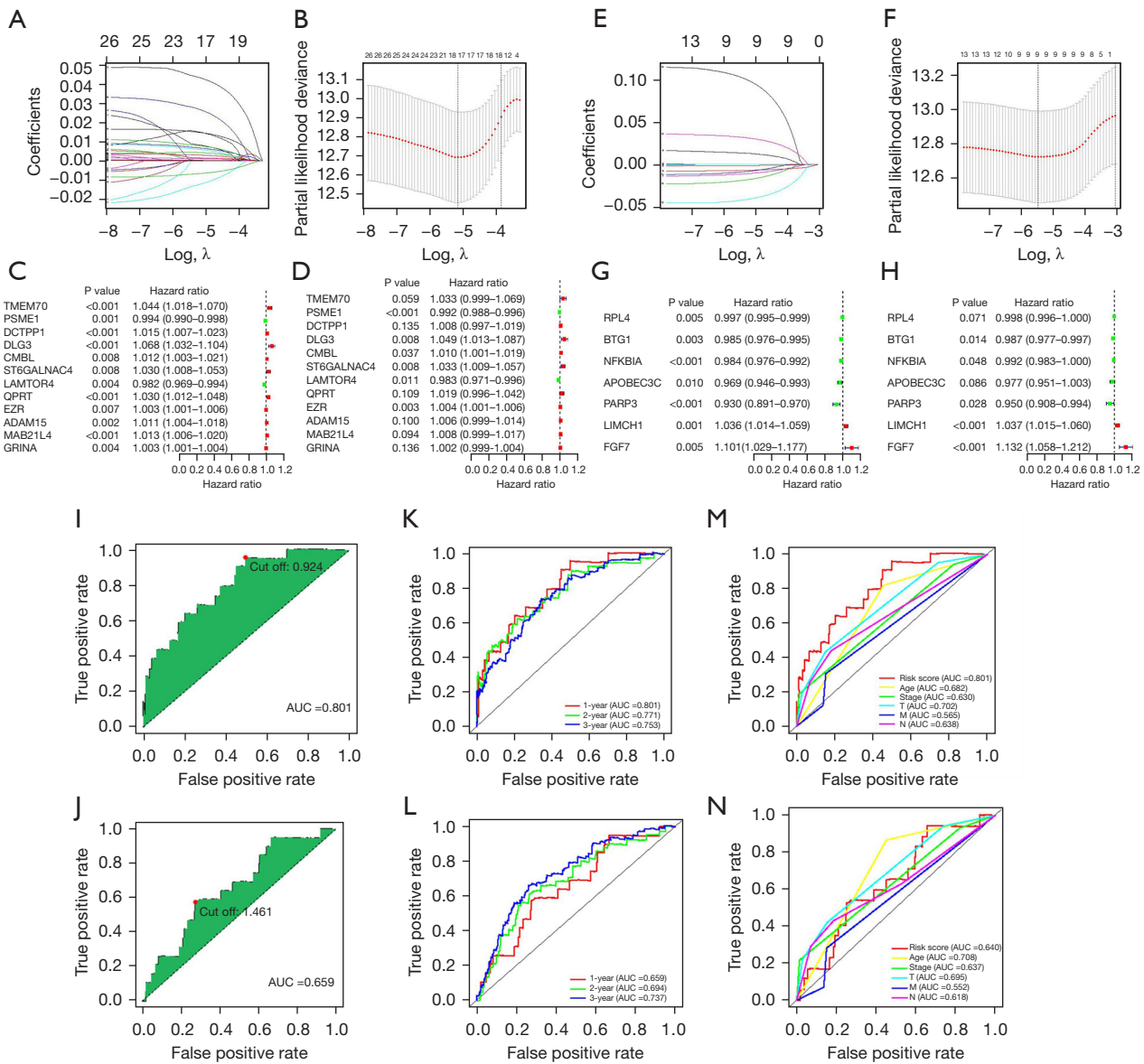
model, the AUC of the risk score was 0.64, which was lower than that of the clinical characteristics, including 0.71 for age, 0.64 for stage, 0.70 for T stage, 0.55 for M stage and 0.62 for N stage (Figure 2N).

Survival analysis was performed to evaluate the survival possibility of BC patients divided into a high-risk group and a low-risk group based on these two models. In the BC1 model, the results of survival analysis showed that the survival possibility of the high-risk group was lower than that of the low-risk group, and the gap between the two groups became much wider as the survival years increased ( $P < 0.001$ ; Figure 3A). The final survival time of the high-risk group was less than 22 years, which was shorter than that of the low-risk group (23.5 years). In the scatter graphs, we ranked BC patients by the risk scores evaluated with our model from low scores to high scores on the horizontal axis. The vertical axis represented their survival time; green scatter represents living patients, and red represents patients who died. The graphs demonstrate that more patients with high risk scores died, and more patients with low risk scores were living. The survival time of patients with low risk scores was longer than that of patients with high risk scores. As shown in Figure 3B, the survival time in the high-risk group was significantly lower than that in the low-risk group using the BC1 model. In the BC2 model, the results were similar (Figure 3C,3D), indicating that these two models can accurately predict the survival rate of BC patients.

#### ***Association of risk score from BC models with clinical characteristics***

Multivariate Cox regression analysis and univariate Cox regression analysis were used to identify factors independently associated with BC prognosis. For both the BC1 and BC2 models, the P values for age, N stage and risk score were all less than 0.001, suggesting that age, lymphatic metastasis and the risk score were all independently associated with BC prognosis. These findings demonstrated that the risk score derived from our model was an independent prognostic predictor of BC patients (Figure 3E-3H).

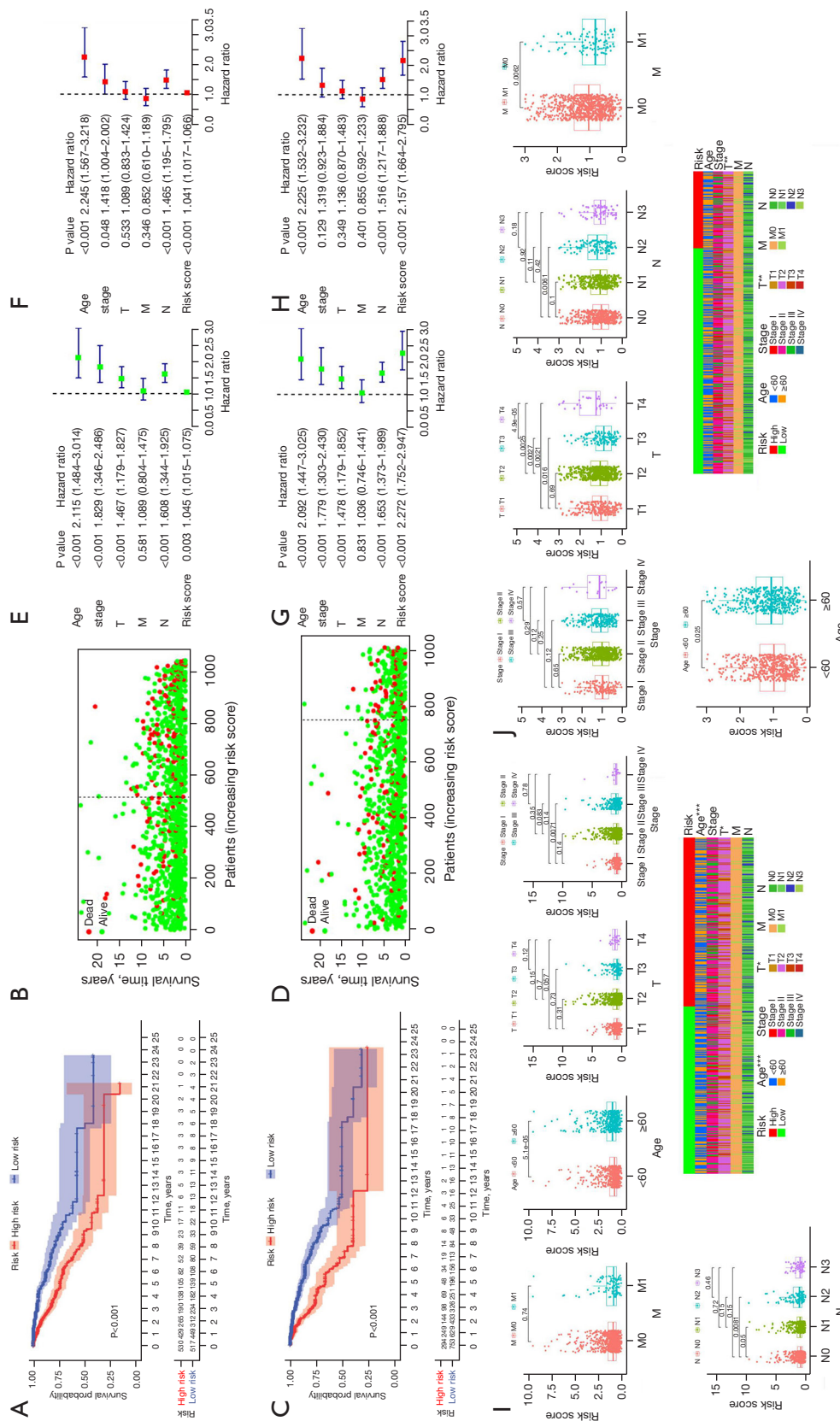
The performance of the risk scores was tested with each clinical characteristic. Significant differences were observed between risk score and age ( $P < 0.0001$ ; Figure 3I) in the BC1 model, while risk score and tumor topography were significantly correlated ( $P < 0.001$ ; Figure 3J) in the BC2 model.



**Figure 2** Establishment and evaluation of a prognostic model based on BC DEGs. (A,B) Essential parameters for the establishment of the BC1 model. (C,D) Forest plots of significant DEGs in the BC1 model using univariate and multivariate Cox regression analyses. (E,F) Essential parameters for the establishment of the BC2 model. (G,H) Forest plots of significant DEGs in the BC2 model using univariate and multivariate Cox regression analyses. (I) Evaluation of prognostic efficacy in the BC1 model by ROC analysis. (J) Evaluation of prognostic efficacy in the BC1 model on the survival rate at 1, 2, and 3 years by ROC analysis. (K) Comparison of the prognostic efficacy of the BC1 model and clinical variables by ROC analysis. (L) Evaluation of prognostic efficacy in the BC2 model by ROC analysis. (M) Evaluation of prognostic efficacy in the BC2 model on the survival rate at 1, 2, and 3 years by ROC analysis. (N) Comparison of the prognostic efficacy of the BC2 model and clinical variables by ROC analysis. AUC, area under the curve; T, tumor; N, node; M, metastasis; BC, breast cancer; DEGs, differentially expressed genes; ROC, receiver operator characteristic.

In the heatmap, there were obviously more patients ( $\geq 60$  years) with high risk scores, yet there was no distinct regularity between risk scores and other features, which was

consistent with the results of multivariate Cox regression analysis that age was an independent factor correlated with the BC1 model (Figure 3I). In the BC2 model, a marked



**Figure 3** Association between BC models and clinical characteristics. (A) ROC analysis for the survival rate of BC patients using the BC1 model. (B) Scatterplot of risk scores derived from the BC1 model to evaluate the survival rate. (C) ROC analysis for the survival rate of BC patients using the BC2 model. (D) Scatterplot of risk scores derived from the BC2 model to evaluate the survival rate. (E,F) Univariate Cox regression analysis (E) and multivariate Cox regression analysis (F) for the BC1 model to explore the association between clinical characteristics and BC prognosis. (G,H) Univariate Cox regression analysis (G) and multivariate Cox regression analysis (H) for the BC2 model. (I,J) Comparison and heatmap of the distribution of clinicopathological features between the low-risk and high-risk groups with the BC1 model (I) and BC2 model (J). \*,  $P < 0.01$ ; \*\*,  $P < 0.001$ ; \*\*\*,  $P < 0.0001$ . T, tumor; N, node; M, metastasis; BC, breast cancer; ROC, receiver operator characteristic.

difference was observed between tumor topography and the risk score (Figure 3f).

### **Immune infiltration analysis and drug susceptibility**

To further explore the immune-related mechanism involved in BC patients, we performed immune infiltration analysis using the two BC prognosis models. M2 macrophages were significantly correlated with the BC1 and BC2 models (Figure 4A,4B). Moreover, macrophage M2 infiltration was remarkably higher in the high-risk group than in the low-risk group (Figure 4C,4D).

For BC chemotherapy, bortezomib is a first-in-class selective and reversible proteasome inhibitor that targets the 26S proteasome (14). Previous studies have observed that bortezomib combined with antiestrogen therapy might have therapeutic advantages in the management of early-stage BC (15-17). Our study also found that patients in the high-risk group were more sensitive to bortezomib than those in the low-risk group (Figure 4E,4F).

To further test these two models in cohorts from different populations, we assessed the testing power of both models of BC development-related gene sets in GSE4922. The AUC of the BC1 model was 0.79, which was higher than that of the BC2 model, implying that the testing power of the BC1 model was stronger than that of the BC2 model (Figure 4G,4H).

## **Discussion**

Worldwide, especially for women, BC is the main cause of cancer-related death (18). Even with gradually progressing diagnosis and surgical treatments, the recurrence rate and cancer-related death rate of BC are still very high. Hence, it is of great urgency to explore new biomarkers that can precisely diagnose BC and predict prognosis for the treatment and management of BC. In this study, we identified two prognostic models (BC1 and BC2) using bioinformatics analysis on the basis of BC-related DEGs and further explored the prognostic value of BC survival time and the mechanism involved. One model (BC1) was observed to derive a better prognostic risk factor, which classified the survival time in BC patients into low- and high-risk categories.

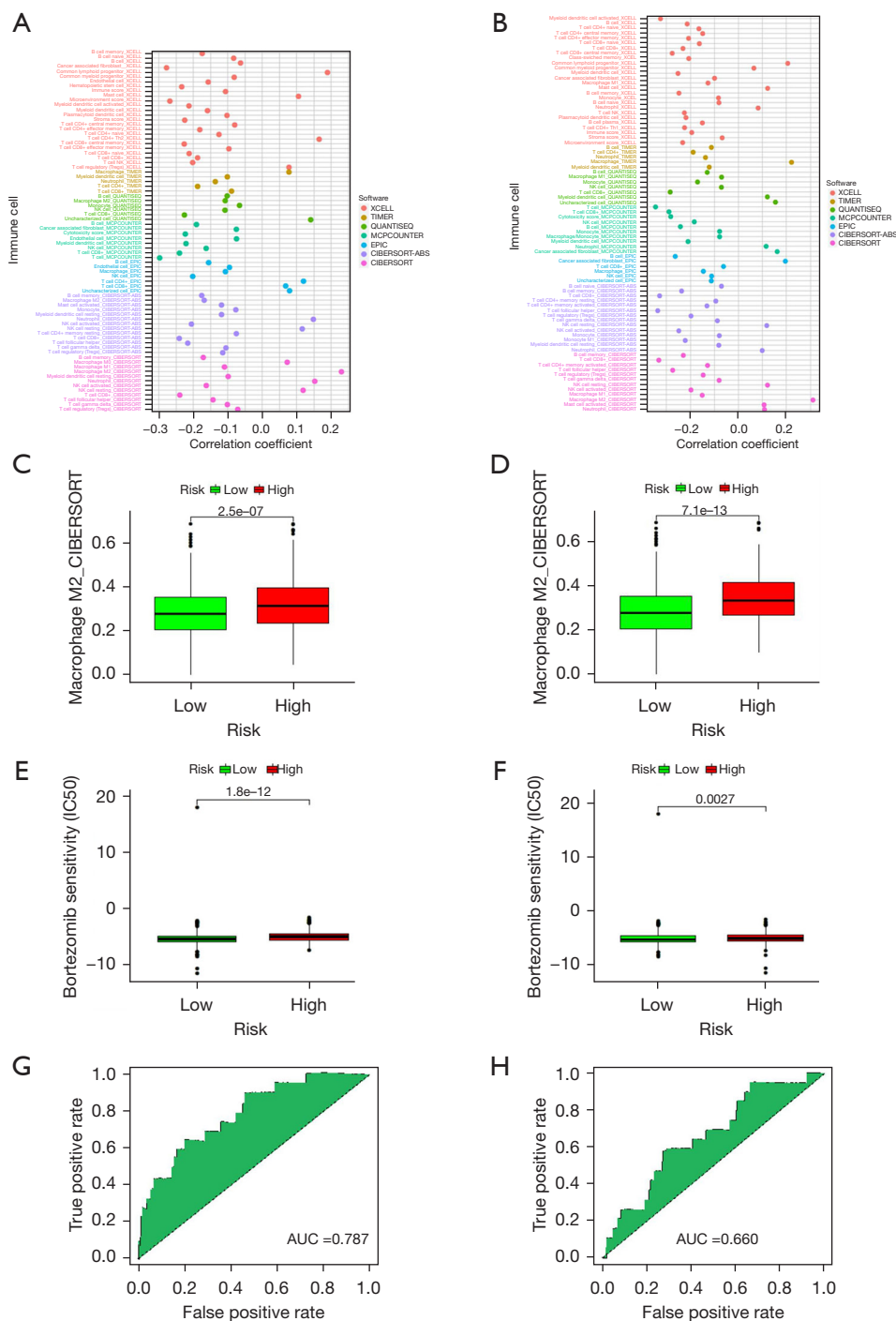
A number of previous studies have shown that abnormal expression of genes in BC is closely related to disease prognosis and can be used as a potential biomarker of prognosis (19,20). In the present study, a number of genes

were differentially expressed in BC at different stages. This indicated that gene expression patterns varied along with BC development. Compared to normal breast tissue, a gene may be differentially expressed in early BC but not in the advanced stage. A total of 738 characteristic BC development genes were identified, including 476 genes that were gradually upregulated and 262 genes that were gradually downregulated with BC development. The development of BC results from interactive effects of multiple genes. Prominently, not all characteristic BC development genes were associated with the prognosis of BC, some of which may result from selection bias or analytical error. Furthermore, DE genes that were upregulated or downregulated may represent entirely different mechanisms involved in BC development. The BC unfavorable gene set comprised 26 gradually upregulated DEGs, and the BC favorable gene set contained 14 gradually downregulated DEGs. Unsurprisingly, previous studies have suggested that some of these genes are associated with BC development (21-23). These results confirmed the possibility that the BC unfavorable gene set and BC favorable gene set could be used as a prognostic model for BC.

In previous studies, a gene coefficient was often obtained from a Cox regression analysis or other method in the training set (24-26). However, due to the limitations of the sample size and the heterogeneity of the tumor, we may never know the true coefficient of a gene. Therefore, GSVA was used to score individual samples against the gene sets (BC unfavorable gene set and BC favorable gene set) in our study. ROC curve analysis suggested that both the BC unfavorable and BC favorable GSVA scores exhibited strong prognostic capacity for BC, which was verified in two other independent datasets. Univariate and multivariate Cox regression analyses suggested that the BC unfavorable and BC favorable gene sets and the risk score systems were independent prognostic factors for BC. This result was also verified in an independent dataset. However, further studies are needed to investigate and validate the functions of these genes, and the synergy between the genes of these two gene sets in BC development still requires molecular experimental validation. Their application in the clinic still needs to wait for further decline in sequencing costs.

In addition, the interaction between the immune microenvironment and cancer cells is important for tumor progression (27). Both the BC development-promoting gene model and BC development inhibitory gene model were associated with high infiltration of M2 macrophages.





**Figure 4** Immune infiltration analysis and drug susceptibility to bortezomib. (A,B) Immune infiltration analysis using the BC1 (A) and BC2 (B) models. (C,D) Quantitative analysis of M2 infiltration between low-risk and high-risk groups using the BC1 (C) and BC2 (D) models. (E,F) Bortezomib susceptibility test of low-risk and high-risk groups using the BC1 (E) and BC2 (F) models. (G,H) Validation of the prognostic efficacy of the BC1 (G) and BC2 (H) models in GSE4922. AUC, area under the curve; BC, breast cancer.

Both the BC development-promoting gene model and the BC development-suppressing gene model were related to patient drug responsiveness to bortezomib. Naturally, studies aimed at revealing the exact mechanisms and DEGs in BC are advocated. The potential of molecularly targeted immunotherapy to intervene in BC may be promising.

## Conclusions

In conclusion, this study explored and validated one prognostic model (BC1) to better diagnose and predict the survival of BC patients using a cluster of 12 DEGs, and extensive functional exploration revealed that the model was closely related to the tumor immune microenvironment, especially M2 macrophage infiltration. Our study provides new insights into further studies in BC, which requires further research.

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## Footnote

*Reporting Checklist:* The authors have completed the TRIPOD reporting checklist. Available at <https://tcr.amegroups.com/article/view/10.21037/tcr-22-2444/rc>

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*Conflicts of Interest:* All authors have completed the ICMJE uniform disclosure form (available at <https://tcr.amegroups.com/article/view/10.21037/tcr-22-2444/coif>). The authors have no conflicts of interest to declare.

*Ethical Statement:* The authors are accountable for all aspects of the work and in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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