Original Article

Effect of N6-methyladenosine (m6A) regulator-related immunogenes on the prognosis and immune microenvironment of breast cancer

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Background: Breast cancer is one of the most common malignant tumor and the prognosis remains unsatisfying. Various studies demonstrate that m6A modulators are new predictors of prognosis in immune microenvironment. We aimed to identify several m6A regulator-related immunogenes and explore the relationship between m6A regulator-related immunogenes and breast cancer prognosis as well as the tumor immune microenvironment (TIME).

Methods: RNA sequencing data and clinical information on 21 m6A regulators in 1,047 breast cancer samples were downloaded from The Cancer Genome Atlas (TCGA), and immune gene data were downloaded from InnateDB. Kaplan-Meier survival analysis was conducted with log-rank test using the survival package. An m6A-related immunogene-prognostic signature was then constructed, followed by immune infiltration and checkpoint analyses.

Results: A risk prognostic signature of m6A regulator-related immunogenes, including *TOX*, *PSME2*, *MCTS1*, *NFKBIE*, *SH3BP4*, *RSPH1*, *JAK1*, *MLLT4*, and *PTGES3*, was constructed. Furthermore, univariate and multivariate Cox regression analyses suggested that the tumor stage and risk score could be independent prognostic factors for patients with breast cancer. Immune infiltration analysis showed that the infiltration levels of T cells, memory B cells, activated NK cells, and macrophages between the high- and low-risk groups were significantly different. In addition, checkpoint analyses demonstrated that the levels of immune checkpoint genes, such as those of *LAG3*, *PDCD1*, *CTLA4*, and *HAVCR2*, were downregulated in the high-risk group compared to those in the low-risk group.

Conclusions: Our findings suggest that the m6A regulator-related risk prognostic signature can predict the prognosis of breast cancer and that it is related to the immune microenvironment.

Keywords: N6-methyladenosine (m6A); breast cancer; tumor immune microenvironment (TIME); prognosis; checkpoints

Introduction

Breast cancer, one of the most common malignant tumors in women, accounts for 25% of female cancer cases worldwide (1,2). Breast cancer is a very high incidence heterogeneous disease and one of the leading causes of cancer-related diseases (3). Furthermore, recurrence and metastasis are regarded as leading causes of increased tumor-specific death (4). Currently, surgery, radiotherapy, and chemotherapy are conventionally established clinical treatments for breast cancer (5). Moreover, the combination of targeted therapy and immunotherapy has improved therapeutic modalities for breast cancer (6). However, the prognosis of patients with breast cancer remains unsatisfactory (7). Hence, it is important to investigate therapeutic targets and prognostic markers for patients with breast cancer.

The tumor immune microenvironment (TIME) consists of protumor and antitumor immune cells, which can be reprogrammed by tumor-derived factors involved in immune escape and tumor progression (8,9). Accumulating evidence has shown that the TIME plays a critical role in the development of tumors and affects the clinical results of immune checkpoint blockade (ICB) therapies, e.g., those for blocking PD-1/L1 or CTLA-4 (10-14). In recent years, some studies have found a special relationship between the TIME infiltration of immune cells and N6-methyladenosine (m6A) modification (15,16). m6A is the most common type of RNA modification, accounting for approximately 50% of the total methylated nucleotides and occurring in approximately 0.1% and 0.4% of all RNA sequences (17). The dynamic modification of m6A is regulated by three categories of genes: writers, readers, and erasers. Since its discovery in 1974, it has been reported that m6A RNA methylation plays a critical regulatory role in tumor RNA modifications (18). An increasing number of studies have suggested that m6A modulators act as oncogenic promoters or inhibitors in the development of tumors (19-22), and can be used as new predictors of prognosis in different types of malignancies. More importantly, a study mentioned that m6A modification is involved in anticancer immune regulation (23). Hence, the modification of m6A has been a potential immunotherapeutic target and a predictor of ICB responses.

In this study, an m6A-related prognostic signature was constructed for breast cancer and the clinically applicable value of this risk signature was estimated (Figure S1). Furthermore, the relationship between the m6A-related prognostic signature and TIME as well as that with immune checkpoints was explored. The findings of this study provide

new insights into the treatment and prognosis of breast cancer. We present the following article in accordance with the TRIPOD reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-22-1335/rc).

Methods

Data source

RNA sequencing data and clinical data (phenotype) were downloaded from The Genomic Data Commons (GDC) data portal of the Cancer Genome Atlas (TCGA) Database (24). A total of 1,047 tumor samples were included in the follow-up analysis after excluding samples from patients with a survival of less than 1 month. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Identification of m6A regulator-related immunogens

A total of 21 m6A regulators, including eight writers (METTL3, METTL14, METTL15, WTAP, RBM15, RBM15B, KIAA1429, and ZC3H13), two erasers (FTO and ALKBH5), and eleven readers (RBMX, YTHDC1, YTHDC2, IGF2BP1, IGF2BP2, IGF2BP3, YTHDF1, YTHDF2, YTHDF3, HNRNPA2B1, and HNRNPC) were analyzed. Furthermore, the immune genes (n=3,537) included in the Immunology Database and Analysis Portal (ImmPort) were downloaded from InnateDB (25). The expression levels of the 21 m6A regulators and 3,879 immune genes were then obtained from TCGA database. The Pearson correlation coefficient of expression levels between m6A regulators and obtained immune genes was calculated using the cor function of R package, with a threshold of correlation coefficient greater than 0.3 and P value less than 0.05.

Construction of an m6A-related immunogene-prognostic signature

According to the median expression of m6A-related immunogenes, the samples were divided into high- and low-expression groups. A survival analysis using Kaplan-Meier (KM) was conducted with log-rank test using the survival package (Version 3.2-7, http://bioconductor.org/packages/survival/). P<0.05 was selected as the threshold of statistical significance to screen for genes in the high- and low-expression level groups that might lead to significant survival differences. Subsequently, the samples were

randomly divided into a training dataset (523 cases) and a validation dataset (524 cases). In the training dataset, immune genes related to prognosis were screened using univariate and multivariate Cox regression analyses, based on a threshold of P<0.05. Finally, a prognostic risk model was constructed using the obtained prognostic genes, and the calculation formula was defined as follows:

$$\text{Risk score} = \sum \text{Coef immuno gene} \times \text{Exp immuno gene} \qquad [1]$$

where Coef is the prognostic coefficient of the multivariate Cox regression analysis of immune genes, and Exp is the immune gene expression from TCGA dataset.

Validation of the m6A-related prognostic signature

To further verify the prognostic risk model, we explored whether there was a significant difference in the prognosis of patients in the high- and low-risk groups in the validation and entire datasets (including both the training and validation datasets). Furthermore, association analysis between the risk score and clinical features (including TNM stage, tumor stage, age, and sex) was conducted using ggstatsplot (version 0.5.) followed by a chi-squared test.

Univariate and multivariate Cox analyses were performed successively to identify whether the m6A-related prognostic signature could be an independent prognostic factor in patients with breast cancer. Meanwhile, nomogram plots of independent prognostic factors were drawn, and the C-index was calculated to evaluate the predictive power of the nomogram.

Immune infiltration and checkpoints analyses

To determine the effect of the m6A-related prognostic signature on TIME, immune infiltration analyses were performed on 22 immune cells using the CIBERSORT algorithm (26).

Additionally, the expression of immune checkpoint genes, such as *PD1* (PDCD1), *PD-L1* (CD274), *CTLA-4* (CTLA4), *TIM3* (HAVCR2), *LAG3*, and *B7-H4* (VTCN1), in the high- and low-risk groups was analyzed, followed by a Student's *t*-test.

Functional annotation of the m6A-related prognostic signature

The GSVA R package (27) (version 1.36.2) for enrichment

analysis was utilized. The scores of each KEGG pathway in each sample were calculated using the c2.cp.kegg. v7.2.symbols.gmt gene set. The differential analysis of KEGG pathways between the high- and low-risk groups was conducted using limma of R package, with a threshold of P<0.05 and |logFC| >0.263.

Statistical analysis

All the data analyses and plotting were conducted in R 4.2.1 software. The log-rank test was used to assess statistical significance of KM analysis. The hazard ratio (HR) was calculated by univariate and multivariate cox regression analysis. For comparisons, Pearson correlation analysis, Log-rank test, chi-squared test, and Student's *t*-test were performed as indicated. A P value of <0.05 was considered statistically significant. All P values are not corrected for multiple comparisons.

Results

m6A regulator-related immunogens

After analyzing the correlation between the expression of 3,879 immune and 21 m6A-related genes, 6,649 co-expression pairs, including 1,841 immune and 21 differential m6A genes, were screened. A heatmap of the correlation between the expression levels of these key immune genes in the prognostic signature and m6A-related genes is shown in *Figure 1*.

Prognostic risk model

To develop a prognostic risk model for breast cancer, KM survival analysis was conducted on the key 1,841 immune gene, and 163 genes were found to be significantly associated with survival (log-rank P<0.05). Next, from these 163 genes, we identified 47 immune genes significantly related to prognosis by performing a univariate Cox regression analysis with the training set. In addition, according to a multivariate Cox regression analysis, 9 immune genes (P<0.05) were independent prognostic factors for patients with breast cancer (*Table 1*). Finally, based on these 9 immune genes, the m6A-related prognostic risk signature was constructed using the risk score formula [1]:

Risk score = −0.536 × *TOX* − 0.239 × *PSME2* + 0.853 × *MCTS1* − 0.152 × *NFKBIE* + 0.366 × *SH3BP4* − 0.381 × *RSPH1* - 0.484 × *JAK1* + 0.313 × *MLLT4* + 0.231 ×
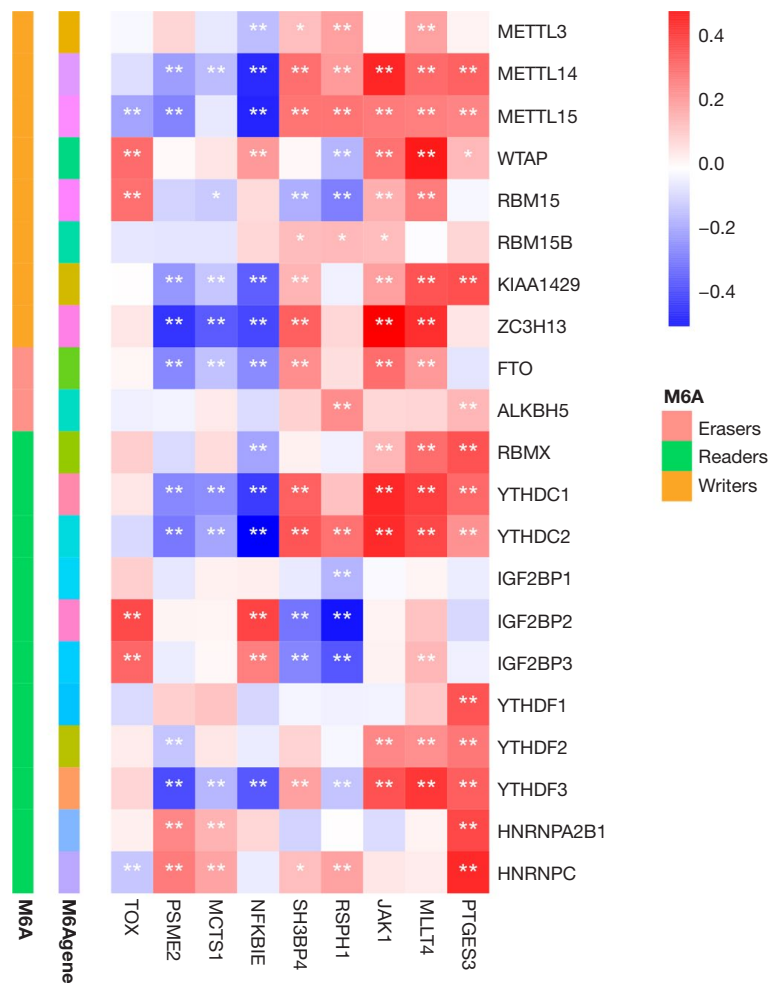
Figure 1 Heatmap showing the correlation between the expression levels of key immune genes in prognostic signature and m6A-related genes. *, P<0.05; **, P<0.01.

Table 1 Multivariate analyses of the immune genes of the training cohort used for predicting prognosis of breast cancer

Gene	HR (95% CI for HR)	P value
RSPH1	0.469 (0.32–0.687)	0.000101
JAK1	0.349 (0.177–0.689)	0.0024
PTGES3	3.57 (1.46–8.75)	0.00537
TOX	0.282 (0.104–0.765)	0.0129
NFKBIE	0.385 (0.175–0.845)	0.0174
MCTS1	2.55 (1.07–6.08)	0.0354
MLLT4	1.61 (1.02–2.53)	0.0387
PSME2	0.508 (0.262–0.983)	0.0444
SH3BP4	1.46 (1.01–2.11)	0.0453

HR, hazard ratio.

PTGES3 (*Figure 2A*).

Furthermore, the risk model was constructed for the training, validation, and entire datasets. The samples were divided into high- and low-risk groups based on the median risk scores. KM analysis showed that the high-risk group had a worse prognosis than the low-risk group in all datasets (all P<0.05; *Figure 2B*). Moreover, the prognostic characteristics of immune genes in the risk model were investigated using KM curves. As shown in *Figure 2C*, the patients who had highly expressed genes with a HR <1 (*PSME2*, *NFKBIE*, *RSPH1*, *JAK1*, and *TOX*) had better outcomes than those with low expression levels. Conversely, patients with high expression levels of genes and a HR >1 (*PTGES3*, *MLLT4*, *SH3BP4*, and *MCTS1*) had a worse prognosis than those with low expression levels.
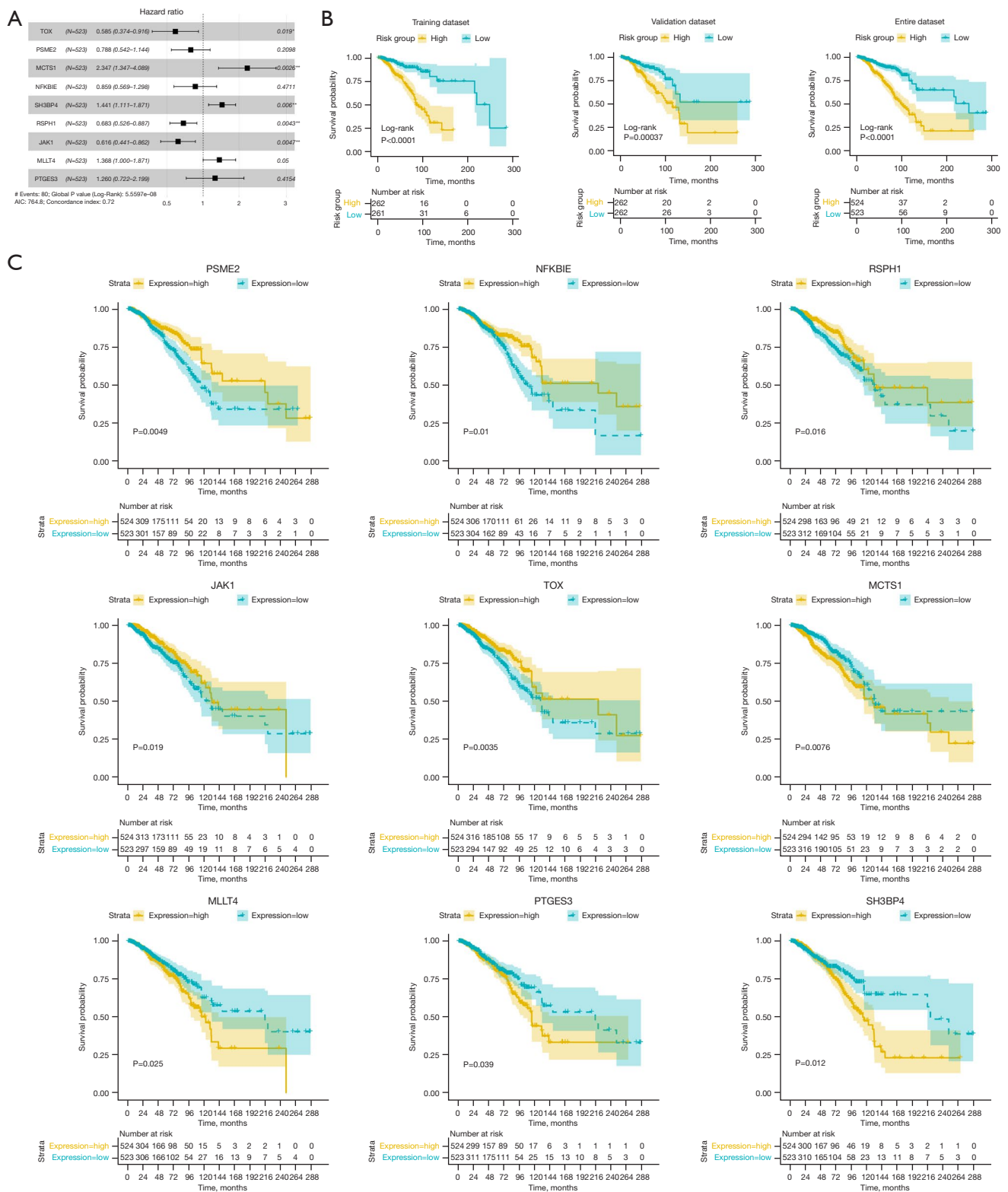
Figure 2 Construction of the breast cancer prognostic risk signature. (A) Independent prognostic immune genes included in the risk model. *, P<0.05; **, P<0.01; (B) Kaplan-Meier analyses of patients in the high- and low-risk groups in the training (left), validation (center), and entire (right) datasets. (C) Kaplan-Meier analyses of the prognostic immune genes included in the risk model. AIC, Akaike information criterion.
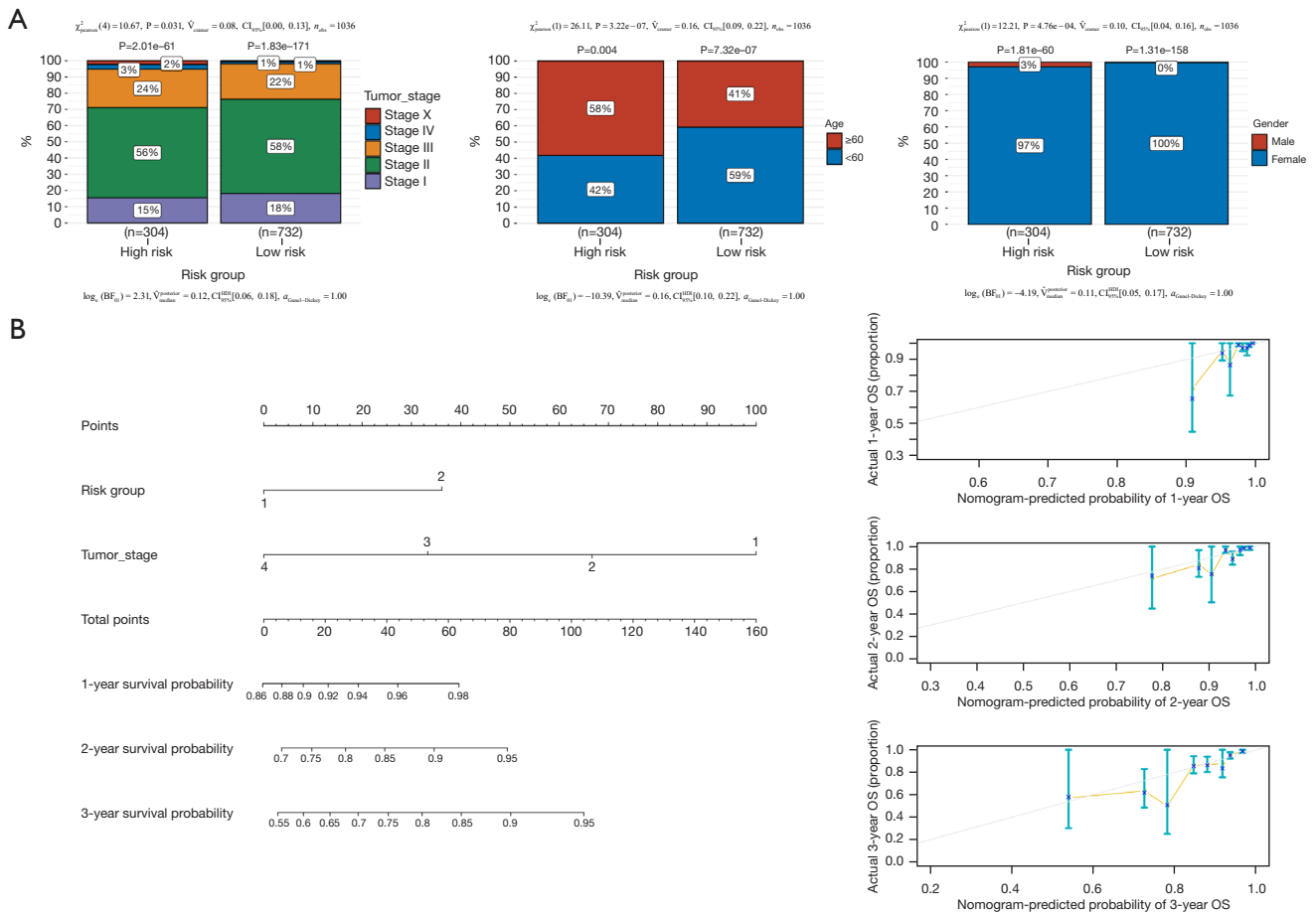
Figure 3 Identification of independent prognostic clinical factors in high- and low-risk groups. (A) Proportional distribution of clinical factors of patients with breast cancer. (B) Nomograms for survival prediction of patients with breast cancer based on independent prognostic clinical factors. BF, bayes factor; OS, overall survival.

Associations between risk score and clinical characteristics

To investigate the clinical value of the risk score in breast cancer, the correlation between the risk score and clinical characteristics of patients with breast cancer was analyzed. The results revealed observable differences in age, sex, and stage between the high- and low-risk groups (chi-square test, P<0.05; *Figure 3A*). Additionally, univariate and multivariate Cox regression analyses suggested that tumor stage and risk score could be independent prognostic factors in patients with breast cancer (*Table 2*). The nomogram plot for independent prognostic factors is shown in *Figure 3B*.

Immune infiltration and checkpoints analyses of the risk signature

Differences in immune abundance between the high- and low-risk groups were determined. As illustrated in *Figure 4*, the infiltration expression of memory B cells, T cells, activated NK cells, and macrophages between the two groups was significantly different. In addition, checkpoint analysis demonstrated that, compared with that in the low-risk group, the expression of immune checkpoint genes, including *LAG3*, *PDCD1*, *HAVCR2*, and *CTLA4*, was downregulated in the high-risk group (*Figure 5*, Student's *t*-test).

Table 2 Univariate and multivariate Cox regression analyses of the clinical characteristics of patients with breast cancer

Clinical characteristics	Univariate Cox regression		Multivariate Cox regression	
	HR (95% CI for HR)	P value	HR (95% CI for HR)	P value
Tumor stage	1.84 (1.24–2.72)	0.00238	2.03 (1.52–2.72)	1.53E-06
Sex	1.69 (1.18–2.43)	0.00452	0.484 (0.0669–3.51)	0.473
T stage	0.79 (0.653–0.957)	0.0159	0.914 (0.686–1.22)	0.542
Risk group	0.719 (0.53–0.975)	0.0339	0.393 (0.275–0.561)	3.02E-07
N stage	0.759 (0.5–1.15)	0.194	–	–
M stage	1.22 (0.852–1.75)	0.279	–	–
Age	1.17 (0.77–1.77)	0.467	–	–

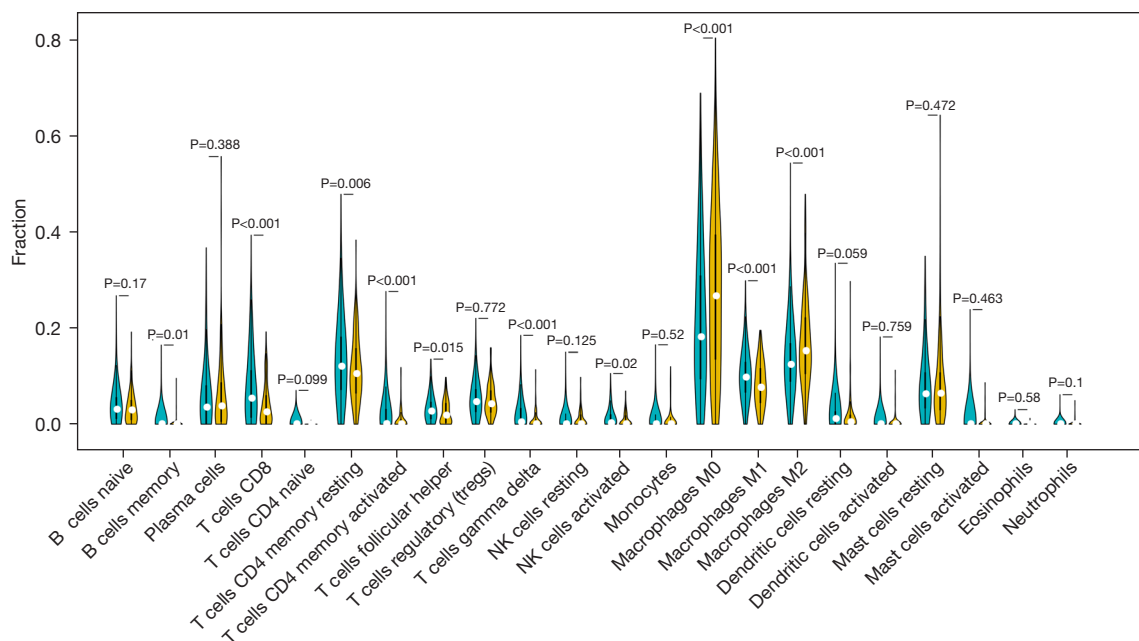T, tumor; N, node; M, metastasis; HR, hazard ratio; CI, confidence interval.

Figure 4 Immune cell infiltration into the TIME according to the risk signature. The infiltration levels of memory B cells, T cells, activated NK cells, and macrophages between the high- and low-risk groups were significantly different. Blue fractions, low-risk group cell infiltration levels; Golden fractions, high-risk group cell infiltration levels. NK, natural killer; TIME, tumor immune microenvironment.

KEGG pathway analysis

A total of 13 differentially enriched KEGG pathways between the high- and low-risk groups were identified using GSVA (*Figure 6*). These pathways involved response-related functions and some immune-related diseases and were downregulated in the high-risk groups.

Discussion

The treatment of breast cancer has been a substantial clinical challenge owing to poor overall survival (OS) and the heterogeneous clinical characteristics of this type of cancer. Currently, relevant immunotherapies have shown sustained antineoplastic activity and controlled adverse
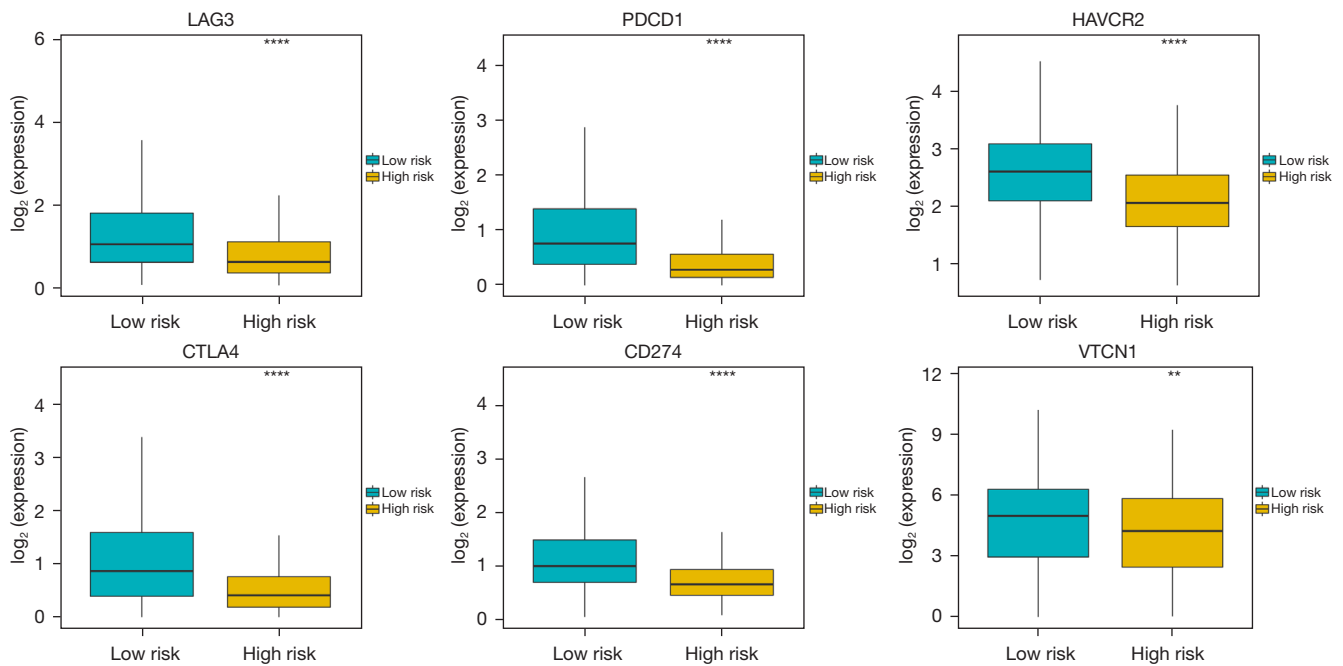
Figure 5 Checkpoint analyses of the breast cancer risk signature. The expression of immune checkpoint genes in the high-risk group were downregulated in comparison to that of the corresponding genes in the low-risk group. **, P<0.01; ****, P<0.0001.
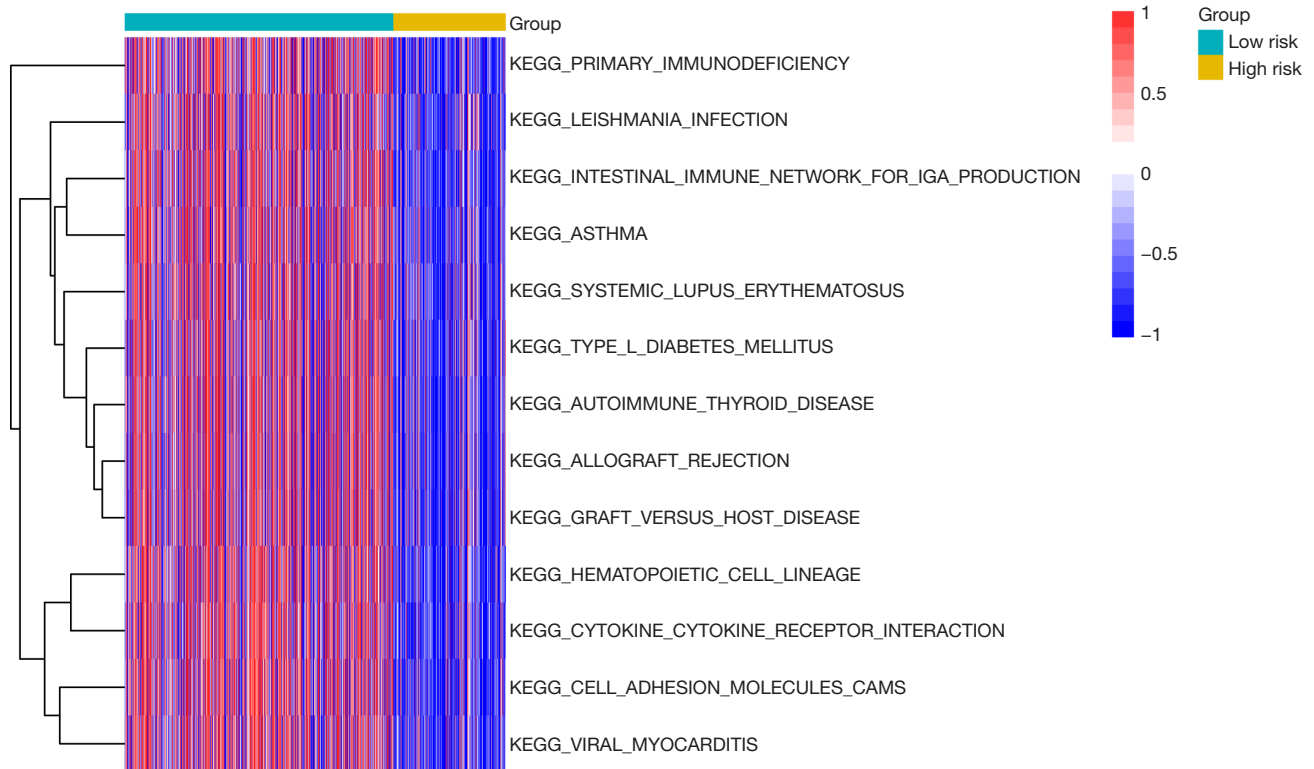

Figure 6 Differentially enriched KEGG pathways between the high- and low-risk groups. KEGG, Kyoto Encyclopedia of Genes and Genomes.

reactions, indicating that the TIME in breast cancer requires further exploration (28). m6A methylation, the most common form of mRNA modification, has been shown to contribute to malignant progression and influence clinical prognosis in many tumors (29-32), including breast cancer. Nevertheless, the role of m6A regulator-related immunogenes in breast cancer has not been fully elucidated. Hence, in this study, we explored the function of m6A regulator-related immune genes in the malignant progression, prognosis, and immune microenvironment of breast cancer.

The risk signature of breast cancer m6A regulator-related immunogenes, including *TOX*, *PSME2*, *MCTS1*, *NFKBIE*, *SH3BP4*, *RSPH1*, *JAK1*, *MLLT4*, and *PTGES3*, was constructed. *TOX* is a transcription factor involved in the regulation of T cell failure in chronic infections and cancers (33). Moreover, *TOX* is negatively correlated with the infiltration of several immune cell types in TIME (34). Higher levels of proteasome activator subunit 2 (*PSME2*) have been observed in breast carcinomas than in metastatic sites (35). In addition, multiple copies in T-cell lymphoma-1 (*MCTS1*) is an oncogene that is highly expressed in several cancer tissues; *MCTS1* promotes cell proliferation and migration and inhibits apoptosis of lung cancer cells by regulating the E2F1 and c-Myc signaling pathway (36). NF-κB inhibitor epsilon (*NFKBIE*) abnormality is a common genetic event in B-cell malignancies, and *NFKBIE* deletion may be a new adverse prognostic marker in primary mediastinal B-cell lymphoma (37). Moreover, the tumor suppressive effects of *SH3BP4* have been revealed; that is, *SH3BP4* can act as a negative feedback regulator of Wnt signaling by regulating the subcellular localization of β-catenin (38). In the breast, the level of Janus kinase 1 (*JAK1*) mRNA is correlated with breast cancer prognosis and the level of immune infiltration, suggesting that *JAK1* can be used as a prognostic biomarker (39). Moreover, in our model, patients with high expression of the genes encoding *PSME2*, *NFKBIE*, *RSPH1*, *JAK1*, and *TOX* had better outcomes than those with low expression. Conversely, patients with high *PTGES3*, *MLLT4*, *SH3BP4*, and *MCTS1* expression had worse prognoses than those with low expression. All these findings suggest that the risk signature is not only an important supplement for breast cancer analysis but also a novel approach for the prognostic analysis of breast cancer.

Accumulating evidence has focused on TIME and the fact that breast cancer is a highly immunogenic tumor (40). In the present study, the m6A-related immunogene risk signature not only contributed to prognosis prediction, but also indicated how the TIME of breast cancer is affected. Briefly, the infiltration levels of memory B cells, T cells, activated NK cells, and macrophages between the high- and low-risk groups were different. In the immune landscape analysis of inflammatory breast cancer, memory B cells and T cells showed significant enrichments (41). M1 and M2 macrophages derive from monocytes and have opposite immune functions. M1 macrophages can promote inflammatory responses and inhibit tumor growth, whereas M2 macrophages play immune-inhibitory and tumor-promoting roles (42,43). Taken together, the present study provides a comprehensive evaluation of the m6A-related immunogene risk signature, which will help understand the characteristics of TIME cell infiltration and promote personalized new therapies by determining responses to immunotherapy.

Immunotherapy, represented by ICB, has shown impressive clinical efficacy in a small subset of patients with long-lasting responses; however, the clinical benefits for most patients remain unsatisfactory (14). Hence, the prediction of ICB responses according to the immune properties of the TIME is key to increasing the success of existing ICBs and developing new immunotherapeutic strategies (44). In this study, the immune checkpoint genes *LAG3*, *PDCD1*, *HAVCR2*, and *CTLA4* were downregulated in the high-risk group compared to those in the low-risk group. The data indicate that the m6A-related immunogene risk signature is observably associated with the tumor immune response, and the established m6A-related immunogene risk signature will help predict the response to immunotherapy in breast cancer.

In addition, the m6A-related immunogene risk signature pathways were significantly different between the high- and low-risk groups. These different pathways were mainly associated with immune responses such as the intestinal immune network for IgA production and primary immunodeficiency as well as with some immune-related diseases such as autoimmune thyroid disease. These results also demonstrate the important predictive role of m6A-related immunogenes in the risk signature identified in this study.

The present study shows that the m6A regulator-related risk prognostic signature could predict the prognosis of breast cancer and offer hints on the TIME. However, some limitations still exist. Although the signature and key genes identified in this study were validated in the datasets, experimental validation is still needed for further clinical applications.

Conclusions

This study comprehensively identified and systematically profiled m6A-related immunogenes in breast cancer. Moreover, an m6A-related immunogene prognostic model, including *TOX*, *PSME2*, *MCTS1*, *NFKBIE*, *SH3BP4*, *RSPH1*, *JAK1*, *MLLT4* and *PTGES3*, was constructed. Taken together, this study not only provides clinical information for prognostic analyses of breast cancer but also provides novel insights into immunotherapeutic strategies.

Acknowledgments

Footnote

Reporting Checklist: The authors have completed the TRIPOD reporting checklist. Available at https://tcr.amegroups.com/article/view/10.21037/tcr-22-1335/rc

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-22-1335/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work 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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