Original Article

Tumor hypoxia in immune infiltration and prognosis of bladder cancer

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Background: Bladder cancer (BC) is the sixth most common cancer and the ninth leading cause of cancer death among men in the world. Previous studies have shown that tumor hypoxia plays an important role in the occurrence and development of BC, but the role of tumor hypoxia in the prognosis and immune infiltration of BC remains unclear. Our aim was to perform a bioinformatics analysis combined with a clinical analysis to explore the roles of hypoxia in BC.

Methods: We acquired datasets (GSE13507, GSE5287, and GSE1827) containing mRNA expression information from BC cohorts from the Gene Expression Omnibus (GEO) and measured the Hypoxia score using the Gene Set Variation Analysis (GSVA). Then we used X-tile method and log-rank test and Pearson's correlation test to analyze the relation among the Hypoxia score and the clinicopathological and immunological characteristics of BC and used stepwise Cox regression analysis to establish a Prognostic model.

Results: Hypoxia was found to be closely associated with tumor grade, pathological type, invasion, and prognosis of BC in our study. Moreover, we determined that hypoxia was closely related to the infiltration abundance of multiple immune cells through a correlation analysis, and the tumor immune cell infiltration was further found to be significantly associated with the tumor grade and tumor type of BC. Furthermore, we constructed several models based on the Hypoxia score and tumor immune infiltration with C-indexes ranging from 0.703 and 0.888, which showed good performance in predicting the prognosis of BC.

Conclusions: Our study showed that hypoxia plays an important role in the progression, prognosis, and tumor immune infiltration of BC. Our models based on hypoxia and tumor immune infiltration play a guiding role in the prognosis and treatment of BC patients.

Keywords: Hypoxia score; tumor immune infiltration; bladder cancer (BC); prognosis; Gene Expression Omnibus (GEO)

Submitted Dec 26, 2023. Accepted for publication Jun 02, 2024. Published online Jul 26, 2024.
doi: 10.21037/tcr-23-2375
View this article at: https://dx.doi.org/10.21037/tcr-23-2375

Introduction

Bladder cancer (BC) is the sixth most common cancer and the ninth leading cause of cancer death among men worldwide (1). According to the clinical manifestation and prognosis, BC can be divided into mucosal invasive BC (MIBC) and non-MIBC (NMIBC). NMIBC accounts for about 75% of BC patients, and has a good prognosis (2). Approximately 26–55% of NMIBC patients will relapse within five years, and of these, 2.4–19% will develop into MIBC (3). Earlier studies have shown that patient age, tumor stage, tumor size, lymph node metastasis,

and tumor pathological grade are closely linked to the prognosis of BC (4,5). In the past decade, with the rapid development of high-throughput technologies such as gene chips and next-generation sequencing, the study of tumorigenesis and progression has made great progress. At present, many studies have identified a large number of prognostic biomarkers for BC (6,7). However, the mechanism of tumor occurrence and development is very complex, and it is associated with widespread genetic abnormalities, and not to only one or several biomarkers. Thus, an increasing number of studies have been carried out to identify the molecular mechanisms involved in tumorigenesis and development through gene sets, which include various genes with similar biological characteristics (8).

The Molecular Signatures Database (MSigDB) is one of the most widely used repositories of gene sets (9). The HALLMARK gene set is a part of the MsigDB that conveys a specific biological state or process (10). Previous studies have shown that the prognosis and therapeutic response of some tumors are closely related to many HALLMARK gene sets (11,12). Among the different tumor modulators, hypoxia is the key process involved in tumor microenvironment evolution. Hypoxia may be caused by the excessive proliferation of tumor cells or the dysregulation and leakage of elements by the tumor microvascular environment (13). Many tumor properties are associated with tumor hypoxia, including an impaired immune response, metabolic reprogramming, proliferation of tumor stem cells, stimulation of tumor angiogenesis, promotion of tumor invasion and metastasis, and the increase in genomic instability, promotion of apoptosis, and cell proliferation (14). Furthermore, Shou *et al.*'s study suggested that hypoxia plays an important role in evaluating the prognosis of melanoma (15), while Milosevic with his team found that hypoxia is associated with local recurrence and early biochemical recurrence in prostate cancer after radiotherapy (16). In the study by Lin *et al.*, a high Hypoxia score indicated poor prognosis in glioma patients, and hypoxia could be used as an independent prognostic factor (17). In addition, many studies have shown that tumor hypoxia is associated with the increased risk of malignant tumor, tumor progression, and metastasis, resistance to radiotherapy and chemotherapy, and adverse clinical outcomes (18,19). Currently, few studies have examined the effects of hypoxia on the immune response and prognosis of BC. Thus, our aim was to perform a bioinformatics analysis combined with a clinical analysis to explore the roles of hypoxia in BC to provide a new direction for a better understanding BC. We present this article in accordance with the TRIPOD reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-2375/rc).

Highlight box

Key findings

- In our study, we found that hypoxia plays an important role in the immunity, progression and prognosis of bladder cancer (BC).

What is known and what is new?

- Previous studies have shown that tumor hypoxia plays an important role in the occurrence and development of BC, but the role of tumor hypoxia in the prognosis and immune infiltration of BC remains unclear.
- In this manuscript, we studied and discussed the relationship between hypoxia and immunity and prognosis of BC.

What is the implication, and what should change now?

- According to our findings, it can play a role of reference and guidance in the clinical treatment of BC.

Methods

BC data acquisition and processing

Datasets (GSE13507, GSE5287, and GSE1827) containing mRNA expression information from BC cohorts were acquired from the Gene Expression Omnibus (GEO). The standardization and normalization of gene expression data in the three datasets were consistent with our previous study (20). The prognostic information relative to the GSE13507, GSE5287, and GSE1827 datasets were obtained from the PRECOG database (https://precog.stanford.edu). The detailed clinicopathological features of GSE13507 were reported in the study by Kim *et al.* (21). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Score of hypoxia and immune cell infiltration in BC

We measured the Hypoxia score using the Gene Set Variation Analysis (GSVA) (22,23). The single sample gene set enrichment analysis (ssGSEA) was used to determine a score of immune cell infiltration (24,25).

Tumor hypoxia and clinical-prognostic characteristics of BC

The relationship between the Hypoxia score, tumor types (NMIBC, MIBC), tumor progression (muscle/non-muscle invasion), and tumor grades was analyzed using the Wilcoxon rank sum test. We divided the 165 patients of the GSE13507 dataset into two groups based on the X-tile method (version 3.6.1, Yale University School of Medicine) (26). and made use of the log-rank test to evaluate the correlation between Hypoxia score and prognosis of BC. A P value <0.05 (two-sided) was used to define statistical significance.

Effect of the Hypoxia score on immune infiltration

Pearson's correlation test was used to measure the association between the Hypoxia score and different tumor-infiltrating immune cells. The effects of the Hypoxia score on immune infiltration were further evaluated in the GSE5287 and GSE1827 datasets. GSE5287 datasets contained 30 patients and GSE1827 datasets contained 80 patients. A P value <0.05 (two-sided) was considered statistically significant.

Construction and verification of prediction models

We used a stepwise Cox regression analysis (27,28) to establish prognosis models for recurrence-free survival (RFS), overall survival (OS), cancer-specific survival (CSS), and progression-free survival (PFS). Variables were removed from the model if their removal resulted in a lower Akaike information criterion (AIC). Then, a nomogram was constructed for model visualization. Further, we adopted Harrell's concordance index (C-index) and performed receiver operating characteristic (ROC) curve analysis to validate the performance of our models.

Statistical analysis

All statistical analyses were conducted using R 4.1.0 (R Foundation for Statistical Computing, Beijing Foreign Studies University, Beijing, China).

Results

Relationship between Hypoxia score and clinicoprognostic characteristics

Using the GSE13507 dataset, we found that the Hypoxia score was significantly higher in patients with high tumor grade (*Figure 1A*) (P<0.001), which was associated with non-muscle invasive progression (*Figure 1B*) (P<0.01), but no clear statistical relationship was observed in muscle invasive progression (*Figure 1C*) (P>0.05). Furthermore, the Hypoxia score was lower in the NMIBC than in MIBC samples (*Figure 1D*) (P<0.001), which was also confirmed in the GSE1827 dataset (*Figure 1E*) (P<0.001).

Effect of the Hypoxia score on prognosis

To evaluate the effects of the Hypoxia score on the prognosis and progression, 165 BC patients were divided into high and low Hypoxia score groups. Survival analysis showed that high Hypoxia score was significantly correlated with poor OS (*Figure 2A*), CSS (*Figure 2B*), RFS (*Figure 2C*), and PFS (*Figure 2D*) (P<0.05). In addition, we evaluated the PFS of NMIBC and MIBC patients, and the results showed that high Hypoxia score was significantly correlated with poor PFS in NIMBC (*Figure 2E*) (P<0.05), but was not associated with MIBC (*Figure 2F*) (P>0.05).

Relationship between the Hypoxia score and immune infiltration

Since immune infiltration is closely related to tumor progression and prognosis, we examined the correlation between the Hypoxia score and tumor-infiltrating immune cells. We found that hypoxia was statistically positively correlated with the tumor infiltration of most immune cells (*Figure 3*). Similar results were observed in the GSE5287 (Figure S1) and GSE1827 (Figure S2) datasets, which further supported the reliability of our results. The same correlation trend for the eleven immune cells subsets was detected in the three datasets (*Figure 4*, Figures S3,S4), and included monocytes, effector memory $CD8^+$ T cells, natural killer cells, activated dendritic cells, regulatory T cells, myeloid-

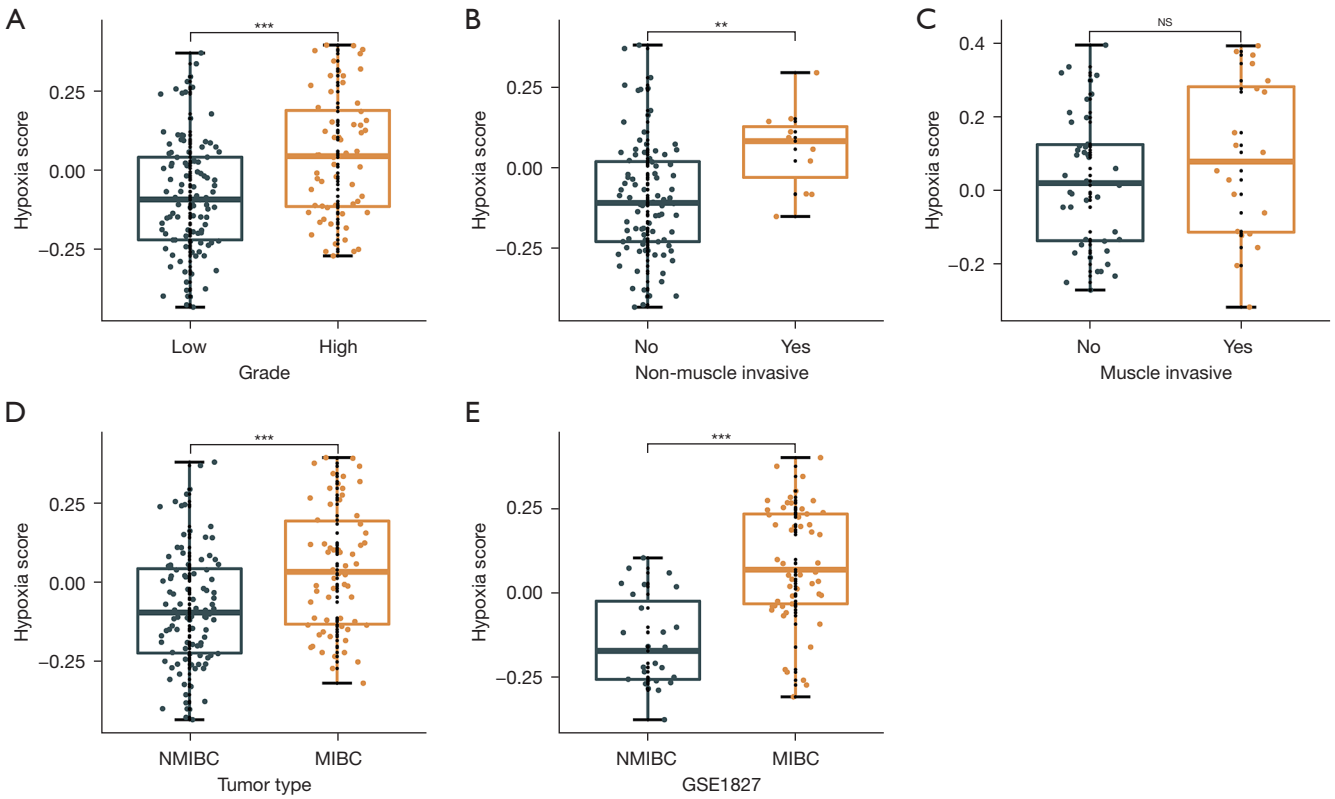

Figure 1 Relationship between hypoxia and clinical-prognostic characteristics in BC. (A) The Hypoxia score in low- and high-tumor grades; (B) the Hypoxia score in patients with and without non-muscle invasive progression; (C) the Hypoxia score in patients with and without muscle invasive progression; (D) the Hypoxia score in different tumor types; (E) validation of the Hypoxia score in GSE1827. **, P<0.01; ***, P<0.001; NS, no statistically significant difference (P>0.05). NMIBC, non-muscle invasive bladder cancer; MIBC, muscle invasive bladder cancer; BC, bladder cancer.

derived suppressor cells (MDSCs), immature dendritic cells, gamma delta T cells, central memory $CD4^+$ T cells, plasmacytoid dendritic cells, and activated $CD4^+$ T cells.

Relationship between tumor immune cell infiltration and clinicopathological features

Based on the above results, we further analyzed the relationship between the eleven immune infiltrating cells and the clinicopathological features of BC. The results showed that seven immune cells were significantly correlated with tumor grade and muscular tissue invasion, including activated $CD4^+$ T cell, gamma delta T cells, natural killer cells, activated dendritic cells, MDSC, regulatory T cells, and plasmacytoid dendritic cells (*Figure 5A*) (P<0.05). Through Wilcoxon rank sum test analysis, a higher intratumoral infiltration of the seven immune cell subsets was detected in the high-grade samples (*Figure 5B*) (P<0.05) and MIBC samples (*Figure 5C*) (P<0.05). Similarly, we used the GSE1827 dataset as an external cohort to verify our results, and we confirmed that the same seven immune cell subsets also scored higher in the MIBC samples (Figure S5) (P<0.05).

Construction and verification of the prognostic models

According to the results of stepwise Cox regression analysis based on AIC values, we chose age, grade, and scores for effector memory $CD8^+$ T cells, natural killer cells, and activated dendritic cells to develop a nomogram for RFS (*Figure 6A*). The area under the curve (AUC) values of ROC curves at 3, 5, and 8 years were 0.813, 0.722, and 0.684, respectively, and the C-index value was 0.703 (*Figure 6B*). The age, tumor type, Hypoxia score, and gamma delta T cell score were chosen for the construction of the nomogram for OS (*Figure 6C*). The AUC values at 3 years

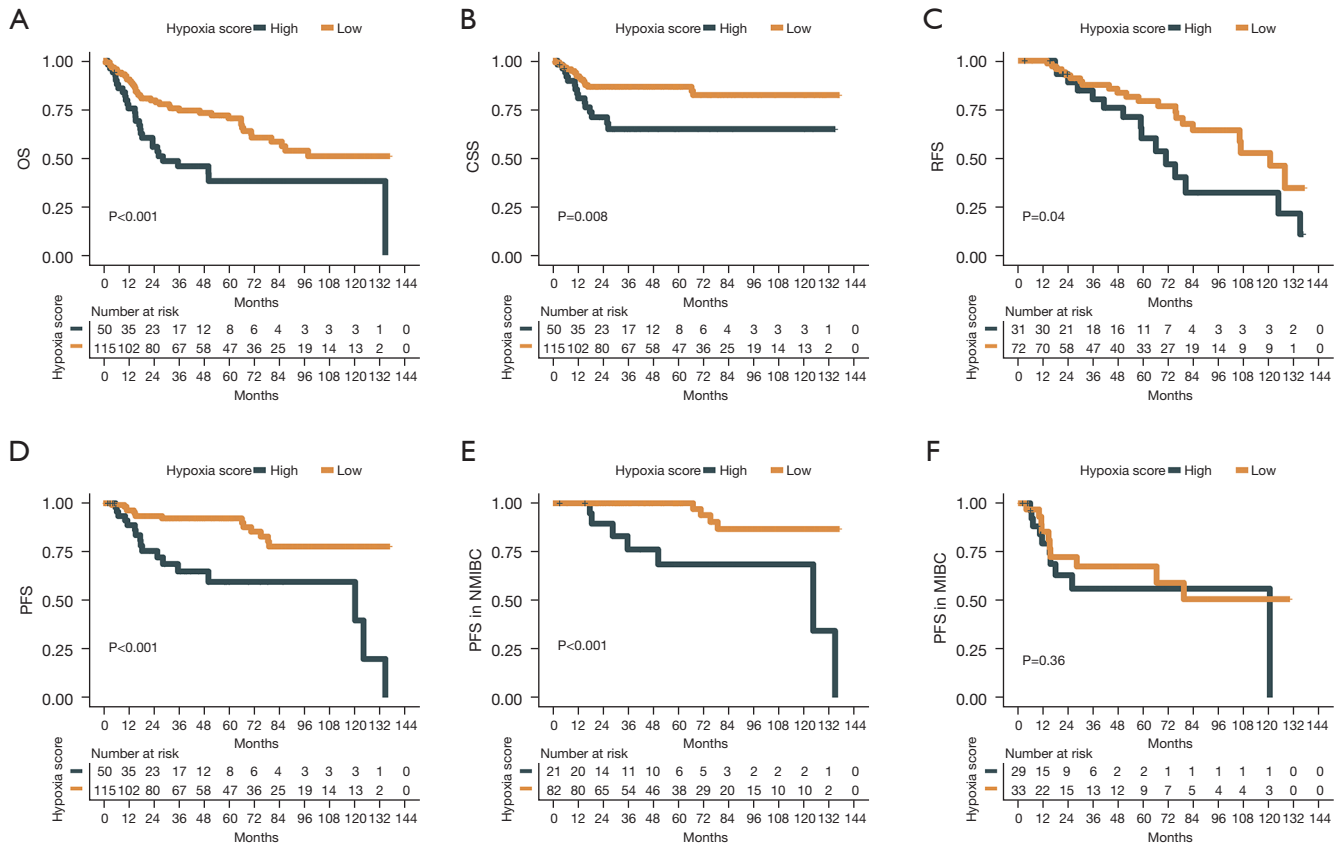

Figure 2 Survival analysis of the Hypoxia score in bladder cancer. Survival curves of the Hypoxia score for OS (A), CSS (B), RFS (C), PFS (D), PFS in NMIBC (E), and PFS in MIBC (F). OS, overall survival; CSS, cancer-specific survival; RFS, recurrence-free survival; PFS, progression-free survival; NMIBC, non-muscle invasive bladder cancer; MIBC, muscle invasive bladder cancer.

(0.844), 5 years (0.849), and 8 years (0.843) were all greater than 0.840, and the C-index value was 0.782 (*Figure 6D*). A nomogram for CSS with a robust C-index (0.888) was built based on age, tumor type, natural killer cell score, and gamma delta T cell score. The AUC values at 3 years (0.940), 5 years (0.956), and 8 years (0.929) were all over 0.920 (*Figure 6E,6F*). A nomogram for PFS was constructed based on age, tumor type, Hypoxia score, and effector memory $CD8^+$ T cell score (*Figure 6G*). The AUC values of 3, 5, and 8 years were all greater than 0.860, and the C-index value was 0.856 (*Figure 6H*). These results indicated that the prediction models based on the Hypoxia score or/and tumor-infiltrating immune cells can preferentially predict CSS and PFS in BC.

Discussion

With a deeper understanding of tumor biology in recent years, increasing evidence appears supporting the close association between occurrence and progression of tumors and widespread genetic abnormalities. Therefore, a comprehensive analysis based on gene sets is becoming increasingly important. In this study, we found that a tumor hypoxia-related gene set was closely related to the clinicopathological features of BC, including tumor grade, invasion, and progression, and a high Hypoxia score indicated worse outcomes. Numerous previous studies have also confirmed that hypoxia is involved in the growth, angiogenesis, metastasis, chemosensitivity, and prognosis of BC. Xia *et al.* (29) found that sulforaphane inhibits the proliferation of NMIBC through suppression of hypoxia-inducible factor (HIF)-1α-mediated glycolysis under hypoxic conditions. Wei *et al.* (30) found that hypoxia promotes the growth of BC and is associated with a poor prognosis. Reiher *et al.* (31) found that hypoxia could regulate angiogenesis of BC, primarily through its effects

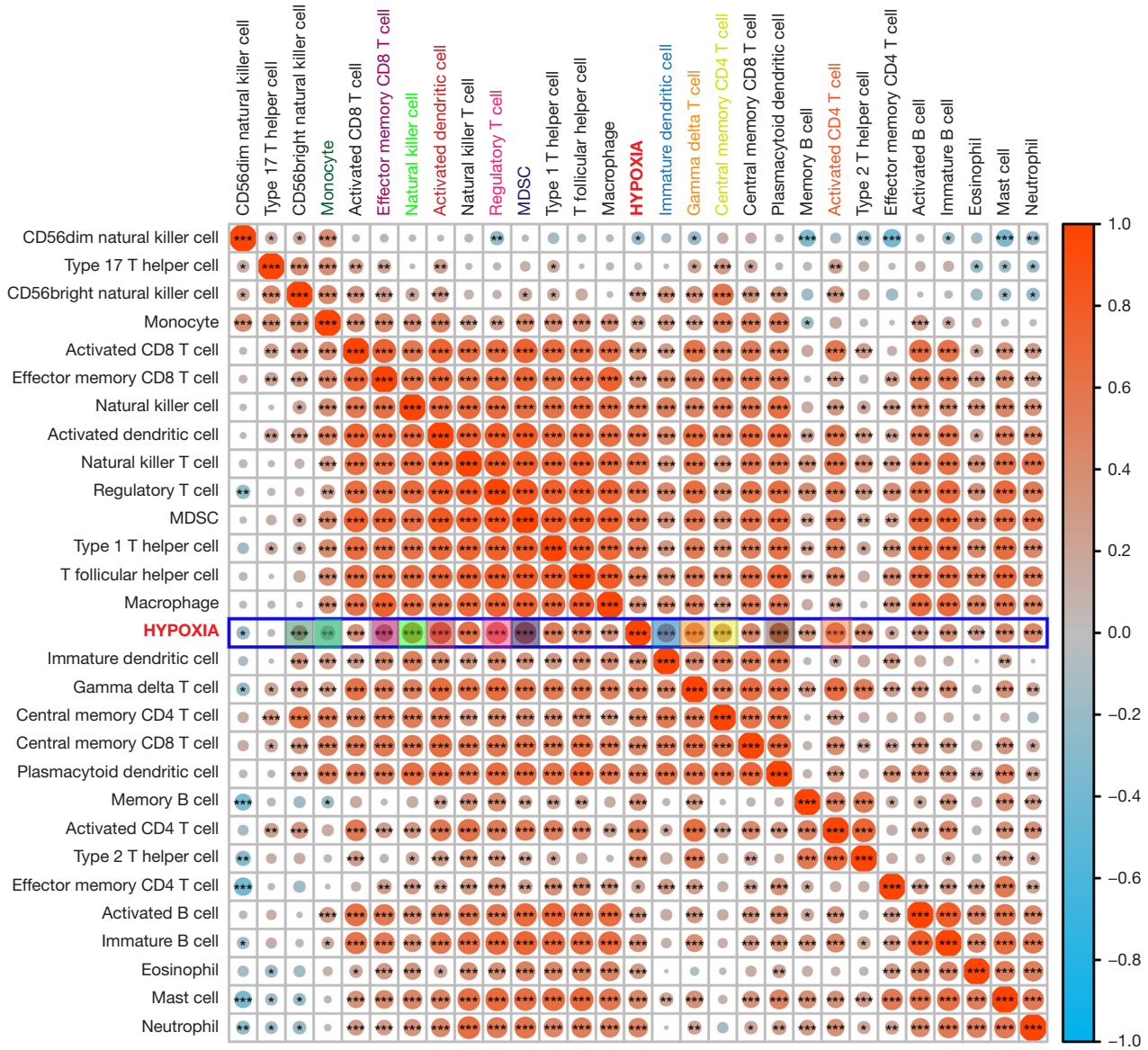

Figure 3 Correlation between the Hypoxia score and tumor immune infiltration in bladder cancer. *, P<0.05; **, P<0.01; ***, P<0.001. MDSC, myeloid-derived suppressor cell.

on VEGF. Studies by Yang *et al.* and Lv *et al.* revealed that hypoxia could induce migration and invasion of BC cells (32,33). In addition, intratumoral hypoxia has been shown to reinforce the resistance of cisplatin and gemcitabine treatment through multiple pathways (34-36) and shows a strong and independent prognostic value for BC patients (37,38). These previous studies further support the reliability of our results.

Currently, the role of hypoxia on immune infiltration in BC remains unclear. Early studies showed that immune infiltration could influence the development of BC (39,40). and hypoxia has also been proposed to play a key role in tumor immune cell infiltration. Hypoxic conditions are mainly implicated in changing the expression of molecular markers and inducing immune cell transport to generate an immunosuppressive phenotype through HIF-1-dependent processes (41). In pancreatic cancer, hypoxia could increase the number of regulatory T cells and prevent the activation

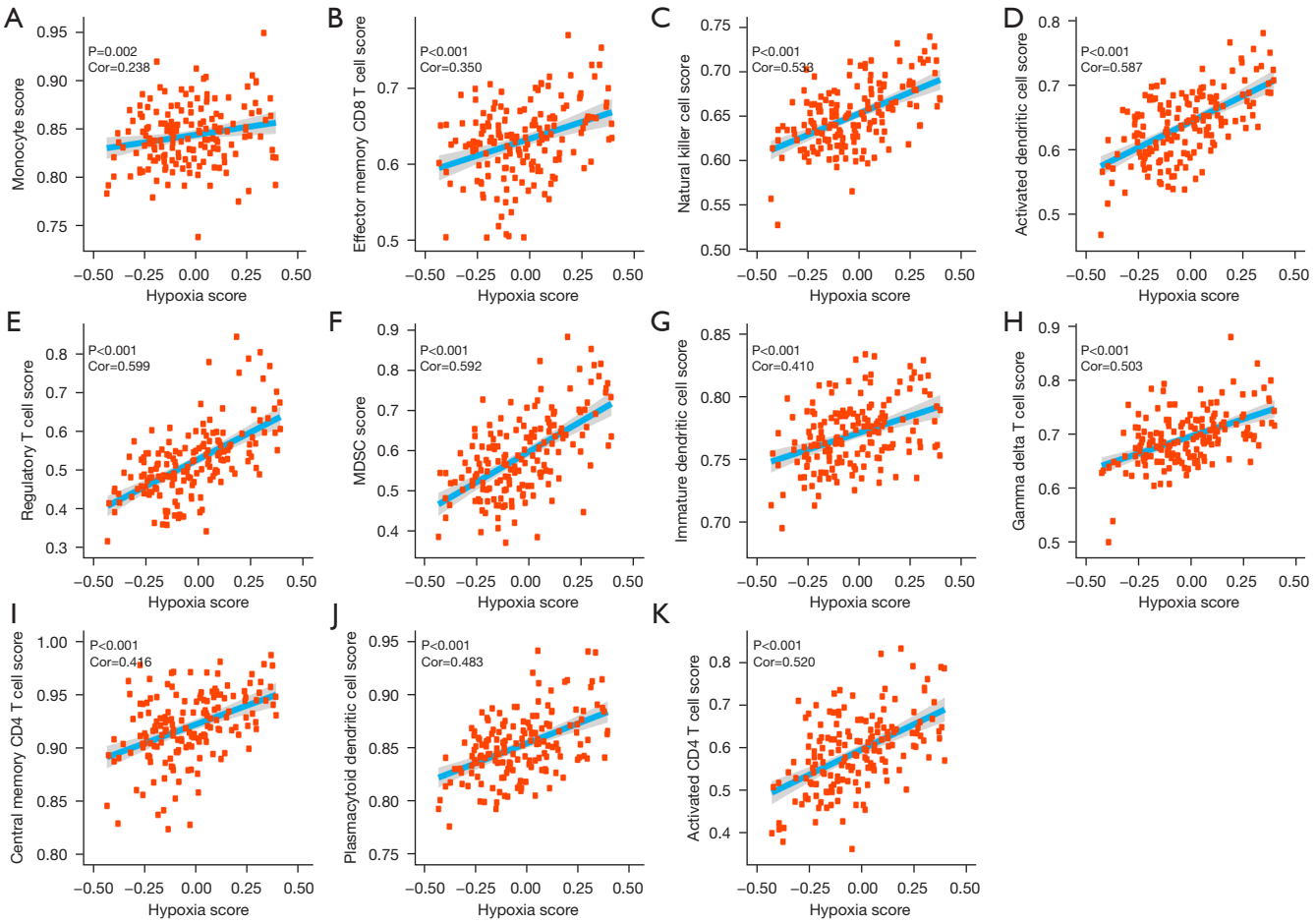

Figure 4 Relationship analysis of the Hypoxia score and scores for monocytes (A), effector memory CD8$^+$ T cells (B), natural killer cells (C), activated dendritic cells (D), regulatory T cells (E), MDSC (F), immature dendritic cells (G), gamma delta T cells (H), central memory CD4$^+$ T cells (I), plasmacytoid dendritic cells (J), and activated CD4$^+$ T cells (K). MDSC, myeloid-derived suppressor cell; Cor, Pearson's correlation coefficient.

of effector T cells through the production of cytokines and the increases in expression of effector CTLA-4 (42). The reduction of hypoxic conditions could restore T cell infiltration and increase the sensitivity of prostate cancer to immunotherapy (43). In this study, we analyzed the relationship between the Hypoxia score and different tumor infiltrating immune cells. The results showed that the infiltration abundance of monocytes, effector memory CD8$^+$ T cells, natural killer cells, activated dendritic cells, regulatory T cells, MDSCs, immature dendritic cells, gamma delta T cells, central memory CD4$^+$ T cells, plasmacytoid dendritic cells, and activated CD4 T cells were significantly positively correlated with the Hypoxia score. Similar results were also observed through an independent external verification. Therefore, these findings suggested that hypoxia may be involved in regulating the infiltration of multiple immune cells in BC. Of course, the molecular mechanism of hypoxia in regulating immune infiltrating cells remains to be further evaluated.

In previous studies, some immune infiltrating cells have been proposed as candidate markers for the diagnosis and prognosis of tumors (44,45). Wu *et al.* (46) indicated that, in the process of the immune response in BC, immune cell clusters with different immune infiltrates and mutational properties may affect tumor development and the sensitivity to treatment as well as prognosis. In this study, we further analyzed the relationship between immune infiltration and clinicopathological characteristics of BC. The results showed

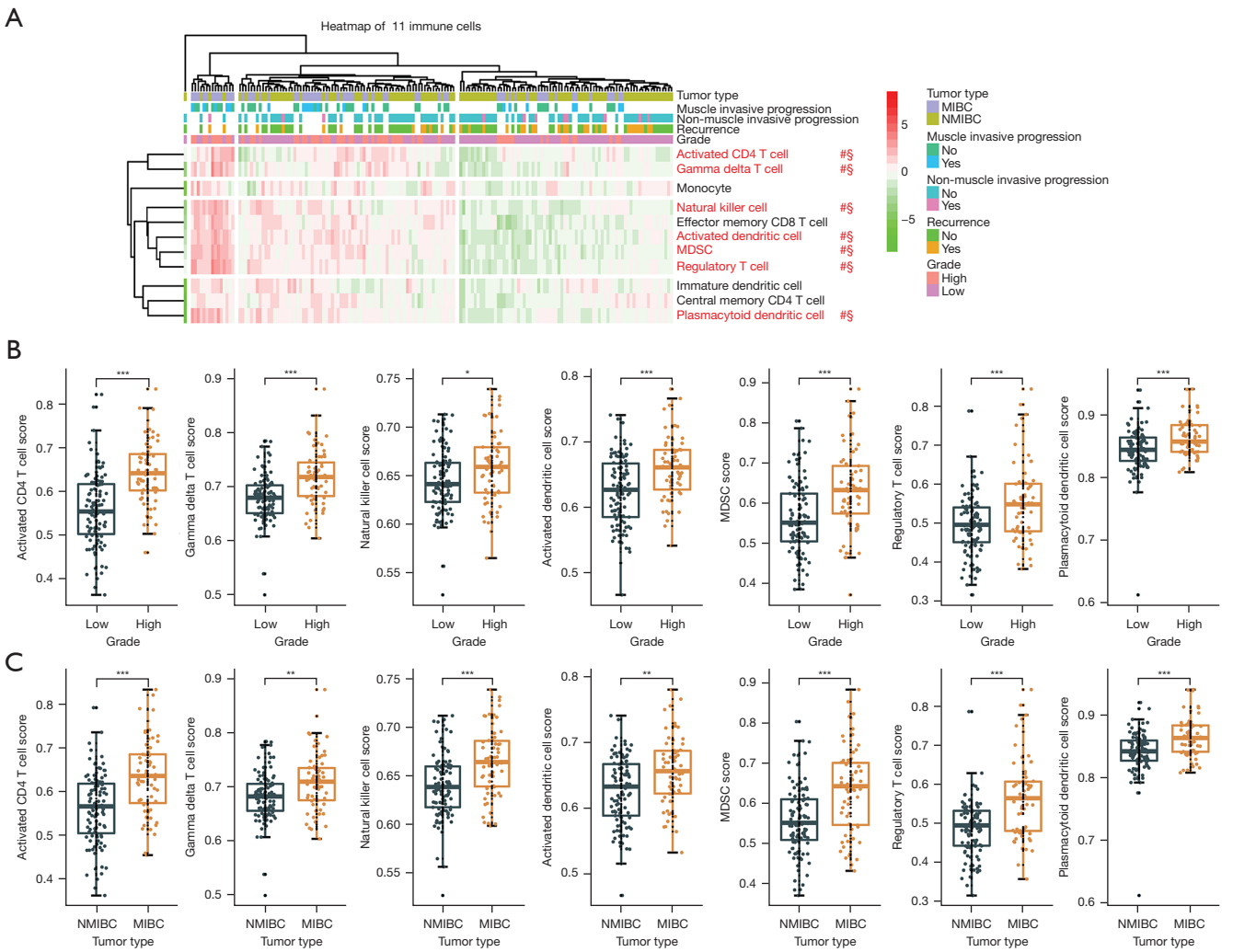

Figure 5 Relationship between tumor infiltrating immune cells and clinicopathological characteristics in bladder cancer. (A) Heatmap showing the differences in 11 tumor infiltrating immune cells in tumor samples with different clinical characteristics; (B) differential analysis of seven tumor infiltrating immune cells in low- and high-grade tumors; (C) differential analysis of seven tumor infiltrating immune cells between NMIBC and MIBC types. §, P<0.05 in NMIBC and MIBC. #, P<0.05 in low and high tumor grade. *, P<0.05; **, P<0.01; ***, P<0.001. MDSC, myeloid-derived suppressor cell; MIBC, muscle invasive bladder cancer; NMIBC, non-muscle invasive bladder cancer.

that activated CD4$^+$ T cells, gamma delta T cells, natural killer cells, activated dendritic cells, MDSC, regulatory T cell, and plasmacytoid dendritic cell were significantly correlated with the tumor grade and muscular invasion. These findings may be helpful to further reveal the immune response mechanism in the development of BC.

To apply the results of this study to the clinic, prognostic models for RFS, OS, CSS, and PFS of BC patients based on the Hypoxia score and the degree of tumor immune infiltration were constructed. Our models showed good performance in predicting prognosis, especially for CSS and PFS, which may provide guidance to clinicians during treatment decision-making and prognosis evaluation. The performance of our models requires further validation in an independent external dataset.

Conclusions

In conclusion, our study showed that hypoxia was closely associated with tumor grade, pathological type, invasion, and prognosis of BC, and played a key role in the infiltration of multiple immune cells. In addition, tumor

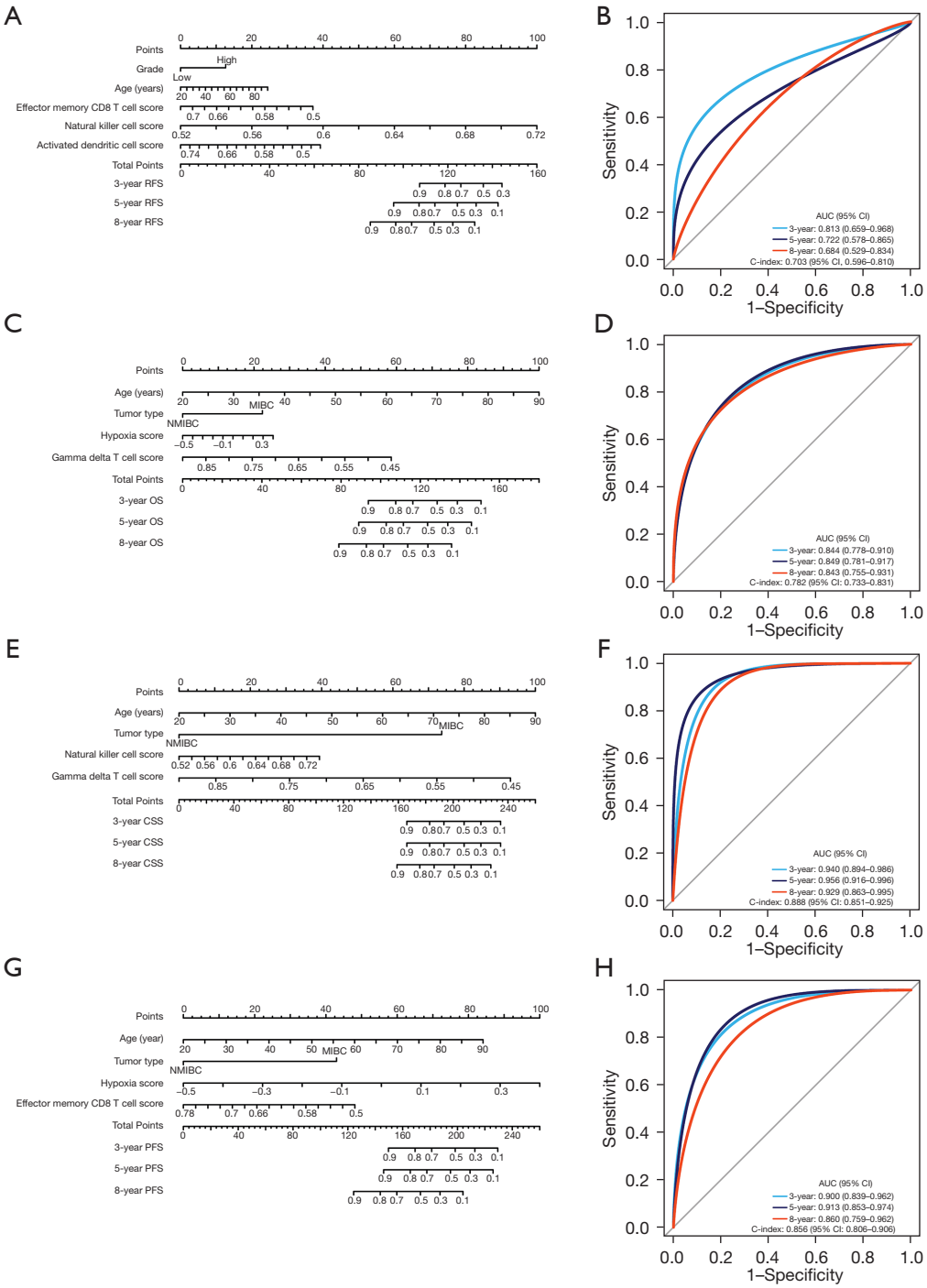

Figure 6 Construction and validation of prognostic models in bladder cancer. Nomograms for predicting 3-, 5-, and 8-year RFS (A), OS (C), CSS (E), and PFS (G). Verification of the prognostic models via the ROC curve and C-index for RFS (B), OS (D), CSS (F), and PFS (H). AUC, area under the curve; NMIBC, non-muscle invasive bladder cancer; MIBC, muscle invasive bladder cancer; RFS, recurrence-free survival; OS, overall survival; CSS, cancer-specific survival; PFS, progression-free survival; ROC, receiver operating characteristic.

immune infiltration was significantly correlated with tumor grade and tumor types of BC. These findings may help us better understand the pathogenesis and development of BC. Furthermore, the predictive models based on hypoxia and tumor immune infiltration showed very good performance in predicting the prognosis of BC, which may contribute to guiding prognosis estimation and treatment of BC patients.

Acknowledgments

We thank the GEO working groups for generating public data.
Funding: This work was supported by the Natural Science Foundation of Guizhou Province of China (grant No. QKHCG [2019] 4433 and grant No. QKHJC-ZK [2021] YB468).

Footnote

Reporting Checklist: The authors have completed the TRIPOD reporting checklist. Available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-2375/rc

Peer Review File: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-2375/prf

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-2375/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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Cite this article as: Xu F, Yao X, Zhou N, Hu Z, Guo C, Zhou H, Yan X. Tumor hypoxia in immune infiltration and prognosis of bladder cancer. Transl Cancer Res 2024;13(7):3273-3284. doi: 10.21037/tcr-23-2375

