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Immunoproteasome subunits are novel signatures for predicting efficacy of immunotherapy in muscle invasive bladder cancer

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Abstract

Background How to select muscle-invasive bladder cancer (MIBC) patients who are sensitive to immunotherapy is an unmet medical need. This study aimed to explore the role of immunoproteasome subunits as a novel signature for predicting efficacy of immunotherapy in MIBC.

Methods The expression profile of immunoproteasome subunits of MIBC and normal tissues was evaluated from data of The Cancer Genome Atlas (TCGA) and of the Chongqing University Cancer Hospital (CQUCH) cohort. Survival analysis and response to immunotherapy was further explored and compared between immunoproteasome subunits^{high} and immunoproteasome subunits^{low} MIBC patients in the TCGA, the CQUCH and the IMvigor210 cohort. The association of the expression of immunoproteasome subunits with immune checkpoint molecules and the tumor immune microenvironment was explored by immunohistochemistry staining and bioinformatic analysis in MIBC of these three cohorts.

Results The expression of the immunoproteasome subunits PSMB8, PSMB9 and PSMB10 was significantly upregulated in MIBC. MIBC patients with high expression of immunoproteasome subunits, especially high expression of PSMB9, showed a trend of prolonged overall and progression free survival, which was further significantly improved in response to immunotherapy. Bioinformatics and immunohistochemistry staining revealed a positive correlation of the expression of immunoproteasome subunits with the expression of immune checkpoint molecules, with T cell activation and with T cell-mediated cytotoxicity.

Conclusions Immunoproteasome subunits, in particular PSMB9, are immune microenvironment-related molecules of MIBC and are promising signatures for survival prediction in response to immunotherapy of MIBC.

Keywords Muscle invasive bladder cancer, Immunoproteasome, Molecular subtyping, Immune checkpoint, Immunotherapy

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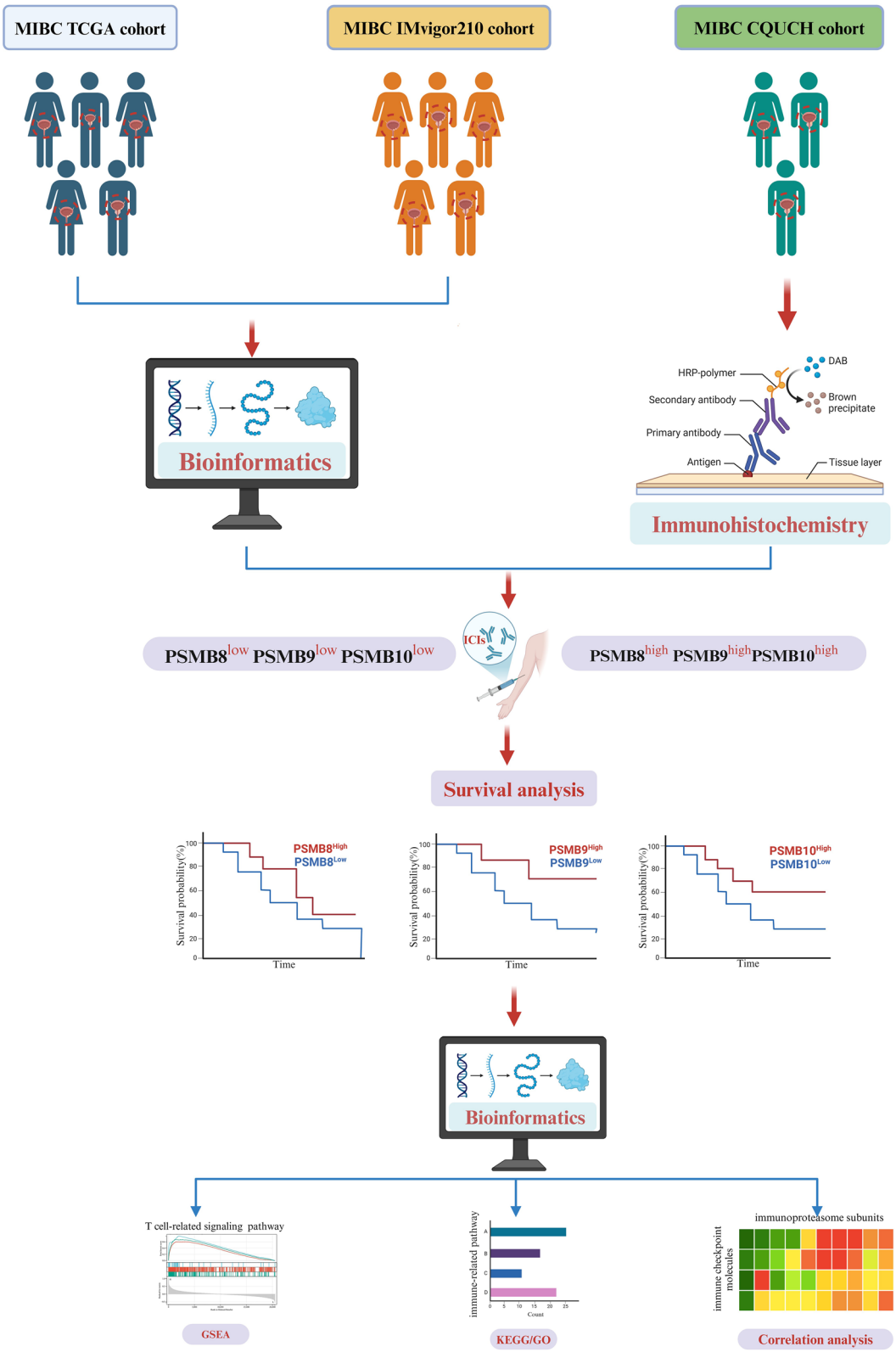
Graphical Abstract



Introduction

Muscle invasive bladder cancer (MIBC) accounts for 30% of first diagnosed bladder cancer, and is more aggressive with a shorter survival in contrast to non-muscle invasive bladder cancer (NMIBC) [1]. The gold standard treatment for MIBC is cisplatin-based neo-adjuvant chemotherapy and radical cystectomy [2]. Despite that this strategy improves prognosis of MIBC, approximately 40% of patients with MIBC are insensitive to neo-adjuvant chemotherapy, resulting in disease progression, operation delay and shortened survival [3]. Therefore, four well-known subtyping systems ((1.) Lund University [4], (2.) University of North Carolina [5], (3.) MD Anderson Cancer Center [6], and (4.) TCGA subtypes [7]) using molecular biomarkers were developed to classify MIBC into three to five molecular subtypes. In the last ten years, this classification system made an important contribution in identifying patients who may benefit from chemotherapy.

For chemotherapy insensitive or intolerant MIBC patients, immunotherapy is an attractive alternative to improve prognosis. In the recent 10 years, immune checkpoint inhibitors (ICIs), such as Pembrolizumab (Keytruda®) and Nivolumab (Opdivo®), have been shown to improve the prognosis of some chemotherapy insensitive or intolerant MIBC patients in neo-adjuvant, adjuvant or salvage phase immunotherapy [8–12]. Nevertheless, not all MIBC patients are sensitive to ICIs. Therefore, how to select immunotherapy sensitive MIBC patients has remained an unmet medical need. Although programmed death receptor ligand-1 (PD-L1) expression is routinely tested in advance to decide the use of ICIs in the clinics, PD-L1 is still a controversial biomarker for predicting a positive response to ICIs in MIBC due to its divergent expression associated with improved survival in response to ICIs [13, 14]. Therefore, the main obstacle for selecting immunotherapy sensitive MIBC patients is the lack of discriminative molecular biomarkers.

Immunoproteasomes bearing the catalytic β-type subunits PSMB8 (LMP7), PSMB9 (LMP2) and PSMB10 (MECL-1) are constitutively expressed in immune cells and replace their standard 20S proteasome counterparts in inflamed tissues that are exposed to interferon (IFN)-γ or tumor necrosis factor (TNF) [15–17]. Apart from processing proteins for MHC-I antigen presentation [18], the immunoproteasome subunits functionally regulate the production of pro-inflammatory cytokines, the differentiation of T cells and the infiltration of effector T cells into the tumor microenvironment. In the last decade, the immunoproteasome was found to have a wide range of expression among different cancers, including renal cell carcinoma, prostate cancer, colorectal cancer, non-small cell lung carcinoma and triple-negative breast carcinoma [19–23]. In addition, a number of studies found that the expression levels of immunoproteasome subunits in different tumors predict prognosis in different types of cancers [24]. Last year, a study from Elhawary et al. evaluated single nucleotide polymorphisms (SNPs) in PSMB9 and PSMB8 and determined an association between these SNPs and the susceptibility to urothelial bladder cancer [25]. These data highlight the potential role of immunoproteasome subunits as promising molecular biomarkers to discriminate immunotherapy responsive or non-responsive patients.

In this study, we investigated the clinical value of immunoproteasome subunits in predicting the response to immunotherapy of MIBC patients by clinical and bioinformatic analysis. The expression of immunoproteasome subunits indicated an immune-activated tumor microenvironment. Furthermore, a high expression of immunoproteasome subunits correlated with an improved clinical outcome of MIBC patients receiving immune checkpoint inhibitor treatment. We propose immunoproteasome subunits as promising new molecular biomarkers to improve the current MIBC molecular subtyping system, which helps to identify MIBC patients who may benefit from immunotherapy.

Materials and methods

Study cohort

This study enrolled three independent cohorts, including Chongqing University Cancer Hospital (CQUCH) cohort, The Cancer Genome Atlas (TCGA) cohort, and IMvigor210 cohort. Sixty-seven pathologically verified MIBC patients staging II-IV as AJCC Staging System from Chongqing University Cancer Hospital were enrolled in the CQUCH cohort and followed-up from November 2017 to June 2024 (Supplementary Table 1). Among the enrolled 67 patients, thirty patients received neoadjuvant, adjuvant or salvaged chemotherapy alone, eighteen patients received neoadjuvant, adjuvant or salvaged immunotherapy with immune checkpoint inhibitors alone, and nineteen patients received above chemotherapy sequentially combined with immunotherapy (Supplementary Tables 2–5). Patients received follow-up check-up every three months in the first year, every 6 months for the next two years, and once per year afterward. This study was approved by the Clinical Research Ethics Committee of Chongqing University Cancer Hospital (No. CZLS2022261-A). Written informed consent was obtained from each patient.

RNA sequencing and clinical data of 411 patients with bladder cancer originally registered in the TCGA database were downloaded from http://www.cbioportal.org/. Thirty-seven patients were excluded for pathological NMIBC and undefined staging. Another fifty-nine

patients without clear survival or sequencing data were further excluded. Three hundred and fifteen MIBC patients were finally enrolled in this study as the TCGA cohort in which one patient lacked of overall survival data (Supplementary Table 5).

RNA sequencing and clinical data of 348 patients treated with the PD-L1 inhibitor atezolizumab in the IMvigor210 clinical trial were downloaded from http://research-pub.gene.com/IMvigor210CoreBiologies. Fifty patients were excluded for the lack of clinical evaluation. Two hundred and ninety-eight patients were then enrolled in our study as IMvigor210 cohort (Supplementary Table 5).

Endpoints

The primary outcomes of survival analysis in this study were progression-free survival (PFS) and overall survival (OS). PFS was defined as the time from the beginning of the first treatment with chemotherapy or immunotherapy to the end of the last treatment with chemotherapy or immunotherapy caused by intolerance or measurable disease progression on imaging according to the RECIST guidelines or death from any cause. OS was defined as the time from the beginning of the first treatment with chemotherapy or immunotherapy to the death from any cause.

Immunohistochemistry staining

Immunohistochemistry staining was performed on MIBC tissues and normal bladder tissues from patients in the CQUCH cohort using the avidin–biotin peroxidase complex method. Briefly, after deparaffinization and rehydration through graded solutions of ethanol/water, antigen sites in sections were retrieved by boiling in 0.1 M citrate buffer (pH 6.0) for 10 min. Slides were then treated with 3% hydrogen peroxide to inactivate endogenous peroxidases. Following incubation in phosphate-buffered saline containing 10% species-appropriate normal serum to block non-specific binding at room temperature for 1 h, sections were incubated in a humidified chamber with primary antibodies against PSMB8 (1:100; proteintech, Wuhan, China), PSMB9 (1:100; proteintech), PSMB10 (1:100; proteintech) and PD-L1 (1:100; proteintech) using isotype-matched IgGs as negative controls at 4 °C overnight. The bound antibodies were detected using biotinylated secondary antibodies and incubated with HRP-streptavidin (Vector, USA) at 37 °C for 20 min. After washing, specific expression was visualized using a yellow diaminobenzidine reagent kit (Vector, USA) according to the manufacturer's instructions. Specimens were counterstained with haematoxylin. For quantitative comparison of PSMB8, PSMB9 and PSMB10, each positively stained area was first scored as 1, 2, or 3 per high-power (×200) field according to staining intensity by an independent pathologist blinded to the experimental design. The ratios of positively stained areas to total traced areas were then determined and expressed as percentage on high-power (×200) images using color segmentation in Image-Pro Plus. The final immunohistochemistry (IHC) staining score of each slide was a product of positive staining ratios and staining score per area. The IHC score of PD-L1 staining was the sum of tumor cell proportion score (TPS), immune cell proportion score (IPS) and immune cell positive score (ICP). TPS ≥ 25% or ICP > 1% and IPS ≥ 25% or ICP = 1% and IPS = 100% served as positive expression.

Correlation analysis

Gene data of the TCGA and the IMvigor210 cohort in this study was sourced from databases https://www.cancer.gov/ccg/research/genome-sequencing/tcga and http://research-pub.gene.com/IMvigor210CoreBiologies/. All data underwent rigorous preprocessing steps to ensure data quality and the accuracy of this study.

The immune cells infiltrated in MIBC were sourced from LM22 (https://www.nature.com/articles/nmeth.3337). The expression level of IFN-γ and TNF as well as infiltrated immune cells in MIBC of the TCGA and the Imvigor210 cohort was calculated by using the cibersort.R tool (https://rdrr.io/github/singha53/amritr/src/R/supportFunc_cibersort.R) running in RStudio (version 4.4.1). The correlation between the expression of immunoproteasome subunits and IFN-γ, TNF, infiltrated immune cells as well as thirty-four immune checkpoint genes in MIBC of the TCGA and the Imvigor210 cohort was then analyzed by Spearman correlation coefficient running in RStudio (version 4.4.1). The correlation between the expression of immunoproteasome subunits and the expression of PD-L1 in MIBC of the CQUCH cohort shown as IHC score was analyzed by Pearson correlation coefficient.

Functional and pathway enrichment analysis

Gene ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways analysis were performed to evaluate the association of immunoproteasome subunits with immune-related functions and signaling genes in MIBC of the TCGA cohort by using the "cluster profiler" R package (version 4.4.1).

Gene-set enrichment analysis (GSEA)

GSEA software (version3.0) was used to explore the underlying immune functional pathways, which correlated with the expression of the immunoproteasome subunits PSMB8, PSMB9 and PSMB10 in MIBC of the TCGA cohort. Briefly, the predefined gene set 'c5.go.bp.

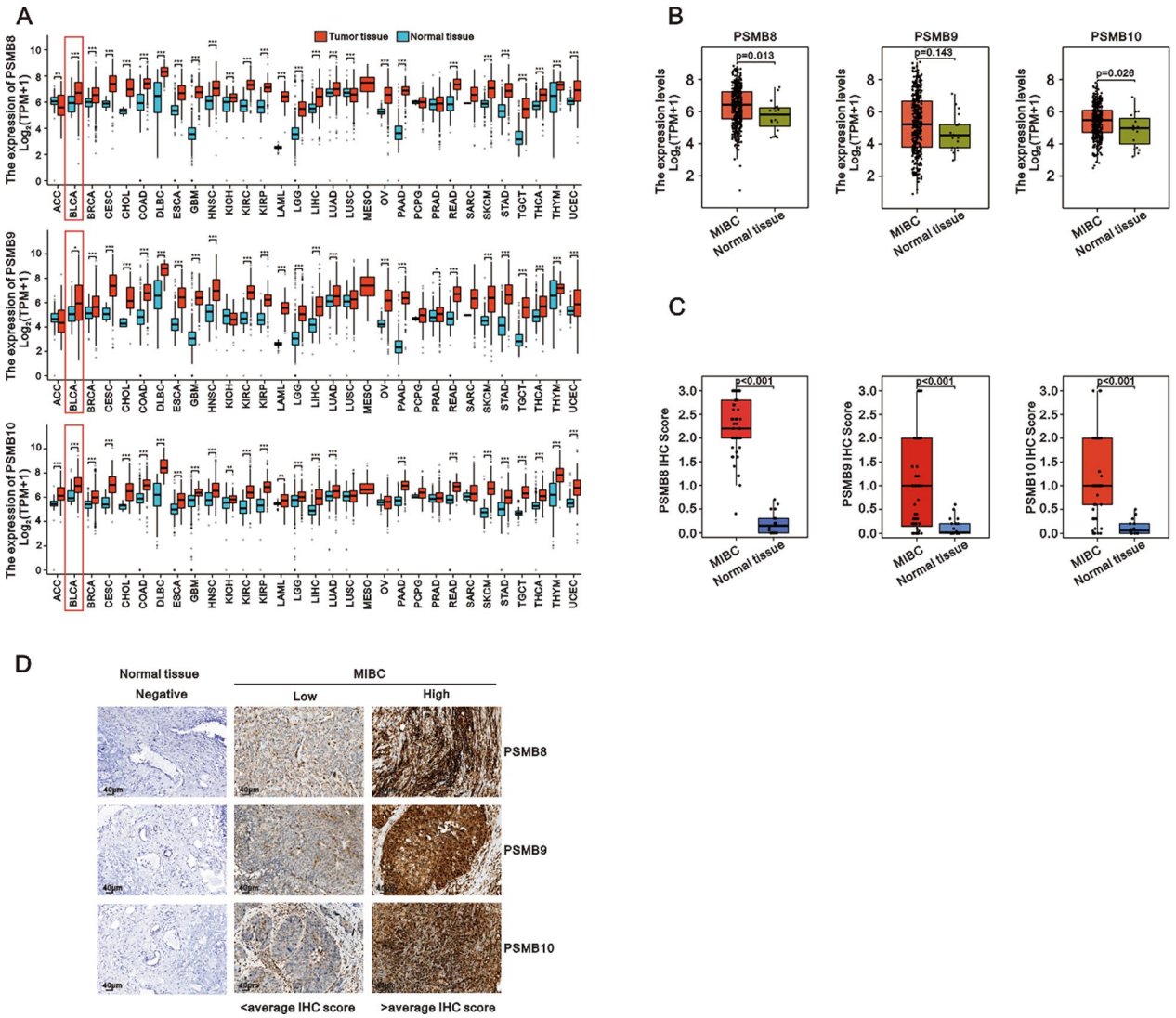


Fig. 1 Immunoproteasome expression profile in muscle-invasive bladder cancer (MIBC). **A** Expression of immunoproteasome subunits PSMB8, PSMB9 and PSMB10 in pan-tumor tissues and corresponding normal tissues derived from the TCGA dataset. Red boxes outline upregulation of PSMB8, PSMB9 and PSMB10 in bladder cancer tissues in contrast to normal tissues. Data are expressed as individual spots per subject with mean of the logarithm of transcripts per million. *p < 0.05, **p < 0.01, ***p < 0.001. **B** Expression of PSMB8, PSMB9 and PSMB10 in MIBC (n = 369) and normal tissues (n = 19) from the TCGA cohort. Data are expressed as individual spots per subject with mean ± SEM of the logarithm of transcripts per million. P-values are indicated in each graph. **C** Immunohistochemistry staining scores of PSMB8, PSMB9 and PSMB10 in MIBC (n = 67) and normal tissues (n = 18) derived from the CQUCH cohort. Data are expressed as individual spots per subject with mean ± SEM of each group. P-values are indicated in each graph. **D** Representative positive and negative immunohistochemistry stainings of PSMB8, PSMB9 and PSMB10 in MIBC and normal tissues derived from the CQUCH cohort. Scale bar: 40 μm. Positive stainings are divided into low and high expression by an average immunohistochemistry staining score

v7.4.symbols.gmt' from the Molecular Signatures Database were employed. A normalized enrichment score (NES) was calculated as the primary GSEA statistic. The threshold values of statistical significance were set as |NES| > 1, normalized P-values (NOM P-values) < 0.05, and false discovery rate (FDR) < 0.25. The results of the GSEA analysis were then visualized by Sanger box tools (http://www.sangerbox.com/tool).

Statistical analysis

Data are presented as median or mean ± SEM. The Student-t test was used to evaluate the statistical significance

of immunoproteasome subunit expression levels between normal tissues and cancer tissues, and PD-L1 expression levels between immunoproteasome subunit^{high} and immunoproteasome subunit^{low} in MIBC. The statistical significance of patients' clinic-pathological parameter was evaluated using the Chi-square test. Kaplan–Meier estimates were used to characterize the event-time distribution of the endpoints. Stratified Cox proportional hazard models were used to evaluate hazard ratios and to test the significance of the above described time-to-event endpoints. Values of $P < 0.05$ were considered statistically significant.

Results

Expression of immunoproteasome subunits are elevated in different types of tumor tissues including MIBC

We first explored the expression of the immunoproteasome subunits PSMB8, PSMB9 and PSMB10 in multitype of normal tissues and cancers based on the TCGA database. We found that in contrast to corresponding normal tissues, the expression of immunoproteasome subunits is significantly elevated in most cancer types, such as breast cancer, diffuse large B cell lymphoma, glioblastoma multiforme, lung adenocarcinoma, ovarian cancer, and also in bladder cancer (Fig. 1A). We further confirmed the expression of the immunoproteasome subunits PSMB8, PSMB9 and PSMB10 in MIBC (staging T2-T4) based on the TCGA database. Bioinformatic analysis revealed that PSMB8 and PSMB10 were significantly upregulated, and PSMB9 was moderately elevated in MIBC compared with normal tissues (Fig. 1B). To further verify the results of the bioinformatic analysis, which is based on a public database, we collected 67 MIBC tissues and 18 normal adjacent tissues from CQUCH to perform immunohistochemistry stainings for PSMB8, PSMB9 and PSMB10. Relative expression levels of PSMB8, PSMB9 and PSMB10 in each slide were evaluated by immunohistochemistry staining scores. Immunohistochemistry staining scores of PSMB8, PSMB9 and PSMB10 in MIBC tissues were higher than in normal bladder tissues which reached scores similar to the negative staining (Fig. 1C). In slides of MIBC tissues, the final staining intensity lower than the average immunohistochemistry staining score was defined as "low expression" of PSMB8, PSMB9 and PSMB10. Otherwise, it was defined as "high expression" of PSMB8, PSMB9 and PSMB10 (Fig. 1D).

Elevated expression of immunoproteasome subunits in MIBC indicates better overall prognosis of patients

To further elucidate the role of the expression of immunoproteasome subunits in predicting prognosis of MIBC patients, we categorized the MIBC patients of the CQUCH and the TCGA cohort into two population according to the expression level of each immunoproteasome subunit by average immunohistochemistry staining scores (Fig. 1D). We found that there were no differences in PFS (median, 33.0 vs. 26.0 months; $P=0.577$) and OS (median, 33.0 vs. 34.0 months; $P=0.920$) between the PSMB8^{high} and the PSMB8^{low} MIBC population in the CQUCH cohort. The PFS (median, 31.0 vs. 26.0 months; $P=0.402$) and OS (median, 34.0 vs. 32.0 months; $P=0.393$) were also not different between the PSMB10^{high} and the PSMB10^{low} MIBC population. However, the PSMB9^{high} MIBC population was linked to a significantly increased PFS value (median, 34.0 vs. 25.0 months; HR, 0.52; 95% CI, 0.28–0.97; $P=0.041$) and an obvious trend of a prolonged OS (median, 38.0 vs. 31.0 months; HR, 0.55; 95% CI, 0.28–1.08; $P=0.085$) compared with the PSMB9^{low} MIBC population in the CQUCH cohort (Fig. 2A). The trend of improved PFS values (median, 30.2 vs. 22.7 months; HR, 0.77; 95% CI, 0.55–1.06; $P=0.112$) and OS values (median, not reached vs. 46.8 months; HR, 0.71; 95% CI, 0.48–1.04; $P=0.079$) in the PSMB9^{high} MIBC population was validated in the TCGA cohort (Fig. 2B). In addition, both PFS and OS were also significantly prolonged in the PSMB8^{high} and the PSMB10^{high} MIBC population compared with the PSMB8^{low} and the PSMB10^{low} MIBC population in the TCGA cohort (Fig. 2B). These results suggest that high expression of the immunoproteasome subunits PSMB8, PSMB9 or PSMB10 predict better survival prognosis of MIBC patients.

MIBC patients with elevated expression of immunoproteasome subunits benefit more from immunotherapy

The IMvigor210 cohort, in which patients received immune checkpoint inhibitor treatment, was used to further explore whether immunoproteasome subunits could serve as candidate biomarkers to predict the response to immunotherapy. We found that the OS was significantly prolonged in the PSMB8^{high}, PSMB9^{high} and PSMB10^{high} MIBC population in contrast to the PSMB8^{low} (median, 15.6 vs. 8.7 months; HR, 0.60; 95% CI 0.45–0.80; $P=0.0004$), PSMB9^{low} (median, 13.3 vs. 9.2 months; HR, 0.69; 95% CI 0.52–0.92; $P=0.037$) and PSMB10^{low} (median, 14.8 vs. 8.8 months; HR, 0.67; 95% CI 0.50–0.89; $P=0.007$) MIBC population (Fig. 3A).

To investigate whether the positive correlation of the expression of immunoproteasome subunits and the response to immunotherapy also occurred in MIBC patients from our CQUCH cohort, thirty-seven eligible patients receiving immunotherapy were categorized into an immunoproteasome subunit^{high} and an immunoproteasome subunit^{low} MIBC population. Over the

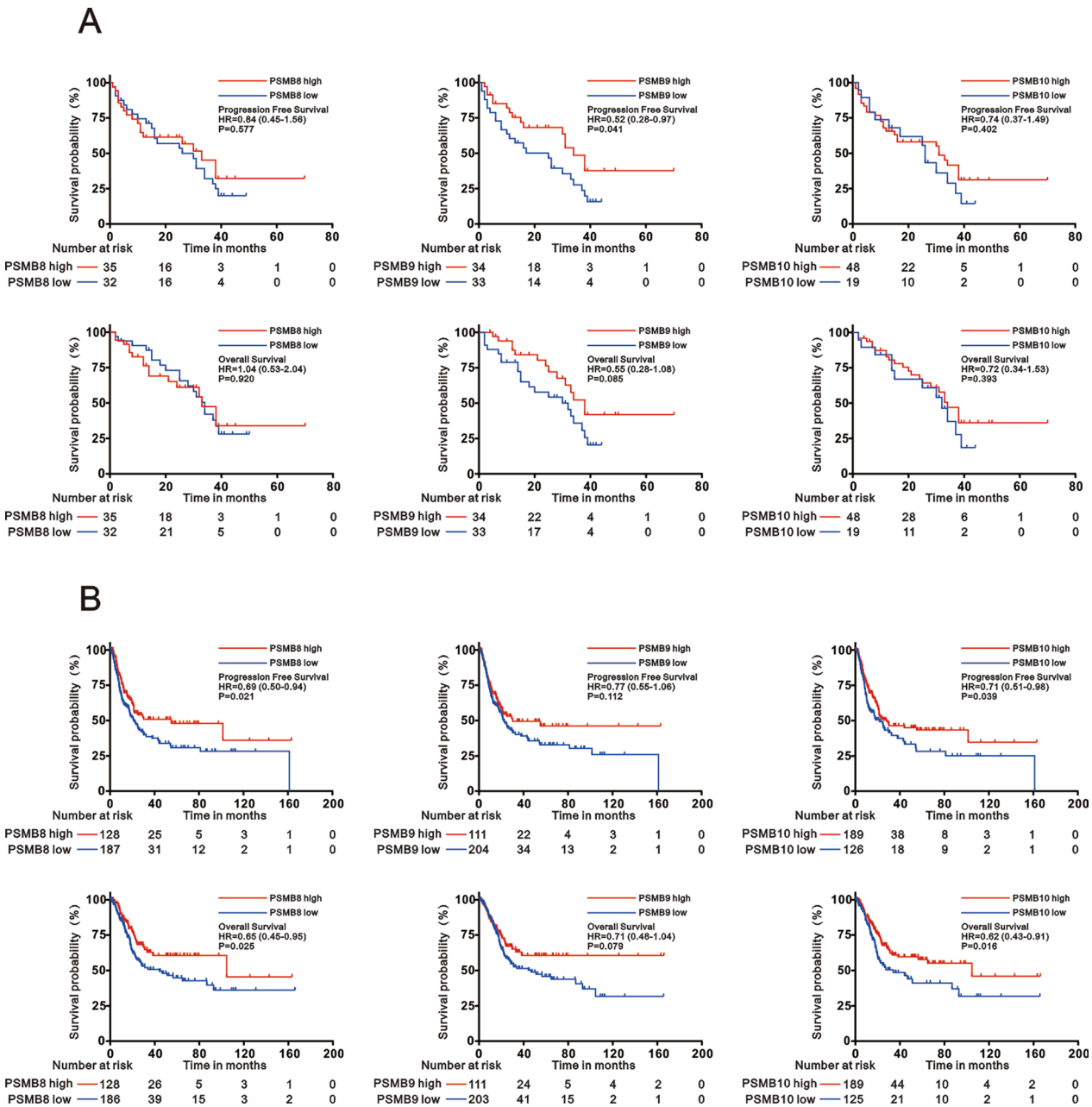


Fig. 2 Association of the expression of immunoproteasome subunits with survival prognosis in MIBC patients. **A** Kaplan–Meier curve of progression-free survival (upper panel) and overall survival (lower panel) for MIBC patients in the CQUCH cohort with high and low expression of PSMB8, PSMB9 and PSMB10. **B** Kaplan–Meier curve of progression-free survival (upper panel) and overall survival (lower panel) for MIBC patients in the TCGA cohort with high and low expression of PSMB8, PSMB9 and PSMB10

course of 80 months, both PFS and OS values showed that PSMB9^{high} and PSMB10^{high} MIBC populations significantly benefited from immune checkpoint inhibitor treatment compared with the PSMB9^{low} (mPFS, not reached vs. 16.0 months; $P=0.009$; mOS, not reached vs. 20.0 months; $P=0.013$) and the PSMB10^{low} (mPFS, not reached vs. 13.0 months; $P=0.041$; mOS, not reached vs. 15.0 months; $P=0.043$) MIBC population (Fig. 3B). Although PFS and OS values of the PSMB8^{high} MIBC population indicated an improved survival in response

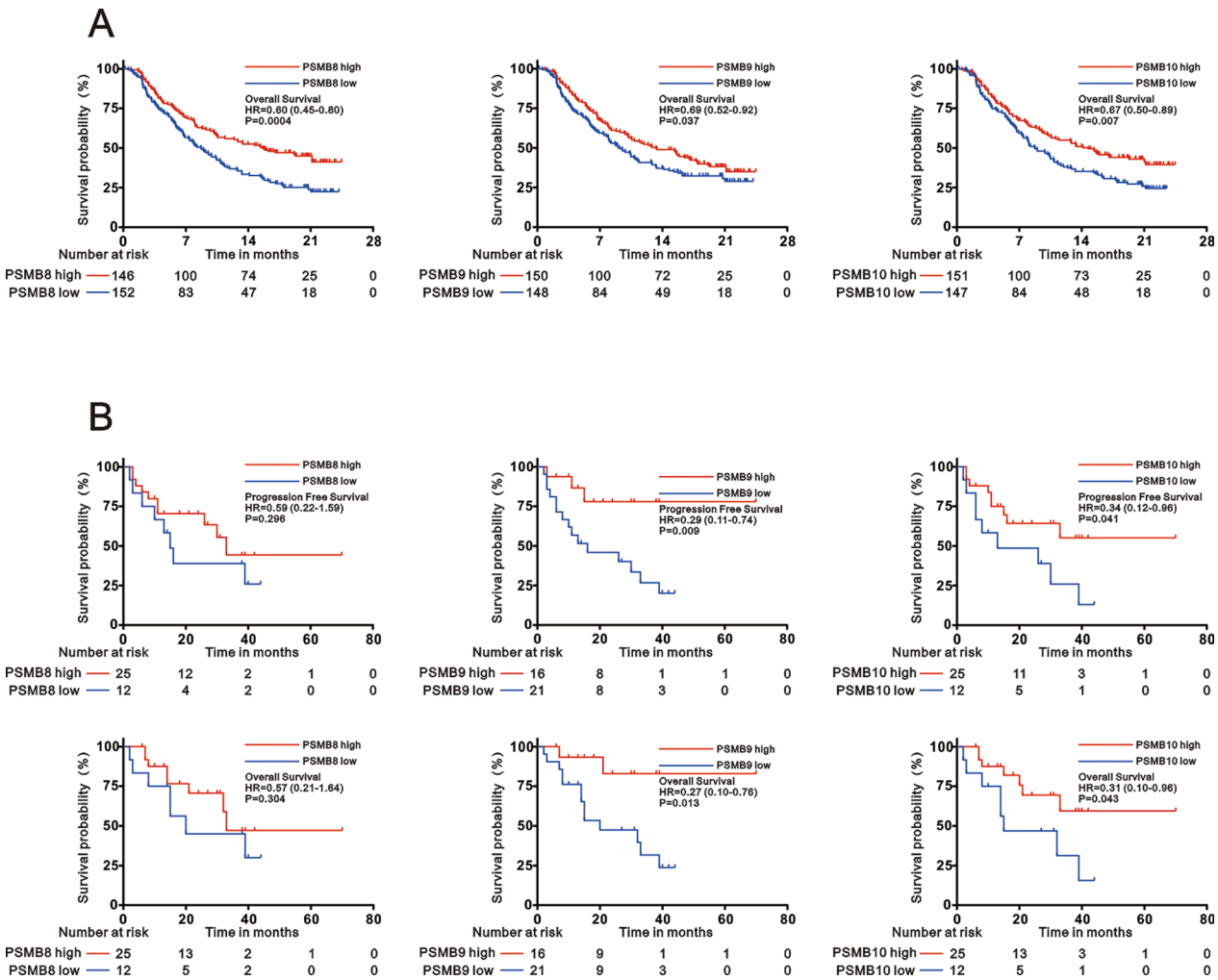


Fig. 3 Association of the expression of immunoproteasome subunits with survival prognosis in MIBC patients receiving immunotherapy. **A** Kaplan–Meier curve of overall survival for MIBC patients receiving immune checkpoint inhibitor treatment in the IMvigor210 cohort with high and low expression of PSMB8, PSMB9 and PSMB10. **B** Kaplan–Meier curve of progression-free survival (upper panel) and overall survival (lower panel) for MIBC patients receiving immune checkpoint inhibitor treatment in the CQUCH cohort with high and low expression of PSMB8, PSMB9 and PSMB10

to immunotherapy compared with the PSMB8^{low} MIBC population, data did not reach statistical significance (mPFS, 33.0 vs. 15.0 months; $P=0.296$; mOS, 33.0 vs. 20.0 months; $P=0.304$) (Fig. 3B).

To further validate the expression of immunoproteasome subunits in predicting immunotherapy efficacy to MIBC, we analyzed the efficacy of immunotherapy in the immunoproteasome subunit^{high} MIBC population and in the immunoproteasome subunit^{low} MIBC population. First, in the immunoproteasome subunit^{high} population, PSMB9^{high} MIBC patients receiving immunotherapy revealed a significantly improved PFS (median, not reached vs. 31.0 months; HR, 0.32; 95% CI 0.12–0.87; $P=0.025$) and OS (median, not reached vs. 31.0 months; HR, 0.31; 95% CI 0.11–0.90; $P=0.031$) compared with patients not receiving immunotherapy. The significantly prolonged PFS (median, not reached vs. 30.0 months; HR, 0.42; 95% CI 0.19–0.92; $P=0.029$) and a trend of improved OS (median, not reached vs. 31.0 months; HR, 0.48; 95% CI 0.21–1.11; $P=0.084$) was also observed in the PSMB10^{high} MIBC population receiving immunotherapy. Even though not statistically significant, both PFS (median, 33.0 vs. 11.0 months; HR, 0.46; 95% CI 0.14–1.11; $P=0.060$) and OS (median, 33.0 vs. 18.0 months; HR, 0.34; 95% CI 0.11–1.04; $P=0.059$) values in the PSMB8^{high} MIBC immunotherapy group were increased compared with patients not receiving immunotherapy (Fig. 4A). Second, immunotherapy did

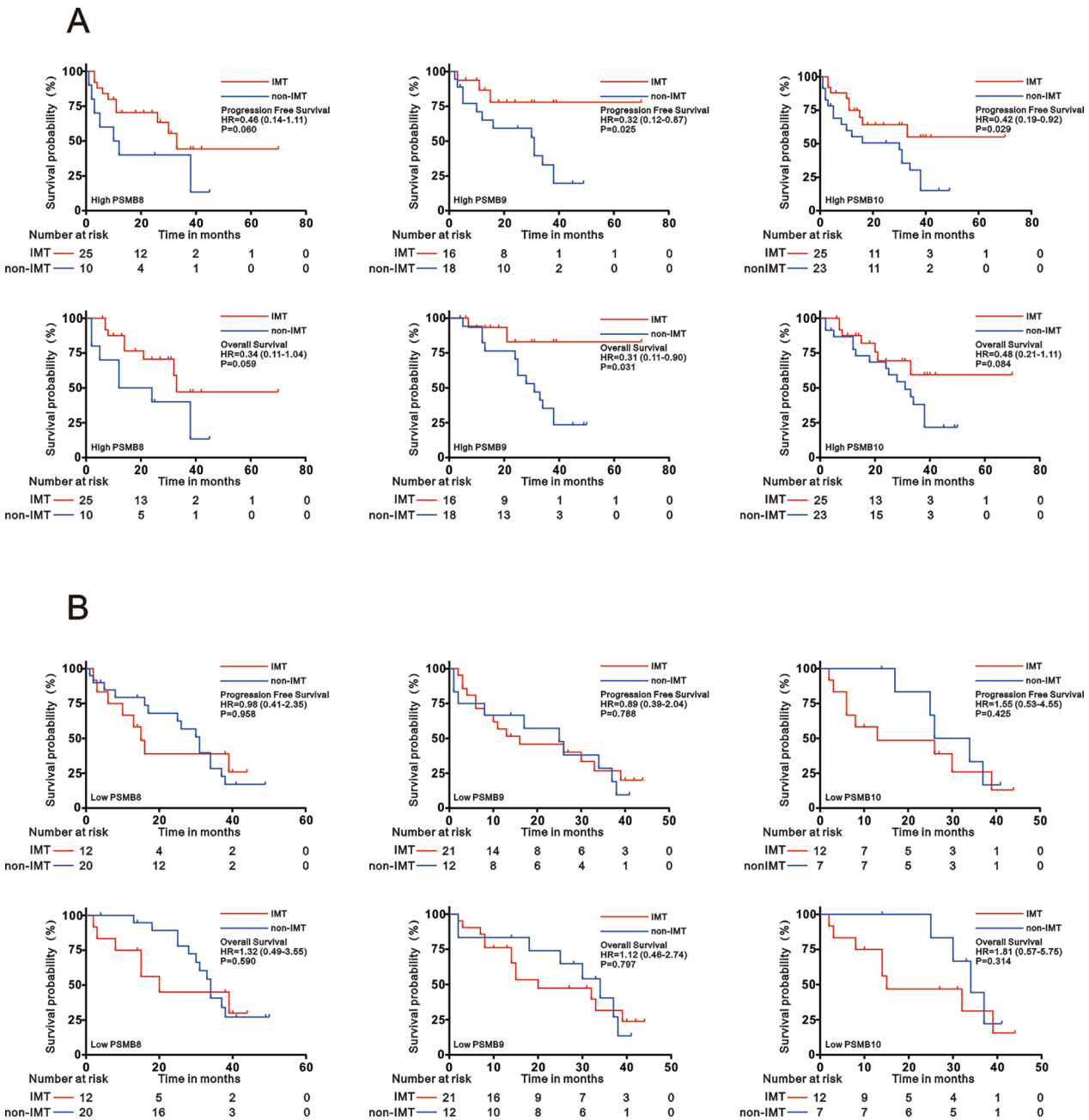


Fig. 4 Effect of immunotherapy (IMT) on survival prognosis in MIBC patients with different expression levels of immunoproteasome subunits. **A** Kaplan–Meier curve of progression-free survival (upper panel) and overall survival (lower panel) for MIBC patients receiving IMT or not receiving (non-IMT) immune checkpoint inhibitor treatment in the CQUCH cohort with high expression of PSMB8, PSMB9 and PSMB10. **B** Kaplan–Meier curve of progression-free survival (upper panel) and overall survival (lower panel) for MIBC patients receiving IMT or not receiving (non-IMT) immune checkpoint inhibitor treatment in the CQUCH cohort with low expression of PSMB8, PSMB9 and PSMB10

not improve PFS and OS in the immunoproteasome subunit^{low} MIBC population, neither for PSMB8, nor PSMB9 nor PSMB10 (Fig. 4B). Hence, high expression of the immunoproteasome subunits PSMB8, PSMB9 or PSMB10 in MIBC correlates with the efficacy of immune checkpoint inhibitor treatment.

Immune checkpoint molecules correlate with the expression of immunoproteasome subunits but they are not predictive for survival in response to immunotherapy in MIBC

Efficacy of ICIs strongly depends on the expression of immune checkpoint molecules. To investigate why

immunoproteasome expression could predict the immunotherapy response, we first performed expression correlation analysis between immunoproteasome subunits and immune checkpoint molecules in MIBC. The heatmaps of the correlation analysis showed that immune checkpoint molecules were significantly associated with PSMB8, PSMB9 and PSMB10 expression in MIBC of the TCGA and the IMvigor210 cohort (Fig. 5). PSMB8, PSMB9 and PSMB10 expression significantly and positively correlated with BTLA, PDCD1 (PD-1), CD274 (PD-L1), PDCD1LG2 (PD-L2), CTLA4, LAG3 and TIGIT both in the TCGA (Fig. 5A) and the IMvigor210 cohort (Fig. 5B). Furthermore, PD-L1 immunohistochemistry stainings of fifteen MIBC tissues derived from our CQUCH cohort revealed that the expression level of PD-L1 (shown as IHC score) was significantly upregulated in MIBC tissues derived from PSMB9^{high} patients in contrast to PSMB9^{low} expressing MIBC tissues. The PD-L1 IHC score was non-significantly increased in PSMB8^{high} and PSMB10^{high} compared with PSMB8^{low} and PSMB10^{low} expressing MIBC tissues (Fig. 5C). The expression correlation analysis between PD-L1 and immunoproteasome subunits in MIBC of the CQUCH cohort further showed that the expression of PSMB9 was significantly and positively correlated with PD-L1 expression, while PSMB8 and PSMB10 were not (Fig. 5D). Since the expression of immunoproteasome subunits was found to predict a positive response to immunotherapy in MIBC on the one hand, and also positively correlates with the expression of immune checkpoint molecules on the other hand, we further analyzed whether PD-L1 expression is also predictive for ICI treatment in MIBC. In our CQUCH cohort, there was no significant difference in OS (median, 18.0 vs. 14.0 months; HR, 0.20; 95% CI 0.03–1.20; $P=0.079$), and also no difference in PFS (median, 11.0 vs. 10.5 months; HR, 0.67; 95% CI 0.15–2.54; $P=0.534$) between PD-L1^{high} and PD-L1^{low} patients receiving ICI treatment (Fig. 5E). This was confirmed (OS: median, 13.3 vs. 9.2 months; HR, 0.76; 95% CI 0.57–1.02; $P=0.068$) in the larger IMvigor210 cohort including 298 participants with immunotherapy (Fig. 5F). Hence, the expression of immunoproteasome subunits, especially PSMB9, correlates with immune checkpoint molecules, but the latter is not predictive for survival in response to immunotherapy in MIBC.

Expression of immunoproteasome subunits is associated with inflammatory factors which are potent in predicting a response to immunotherapy in MIBC

Immunoproteasome subunits are expressed in immune cells and in inflamed tissues that are exposed to IFN-γ or TNF [15–17]. We then analyzed a potential correlation between the expression of immunoproteasome subunits and IFN-γ and TNF in MIBC to explore a potential reason for the expression of immunoproteasome subunits as predictive markers for immunotherapy efficacy in MIBC in the TCGA and the IMvigor210 cohort. The TCGA cohort showed a significant and positive association between the expression of immunoproteasome subunits and IFN-γ (Fig. 6A). Similarly, in the IMvigor210 cohort the expression of the immunoproteasome subunits PSMB8, PSMB9 and PSMB10 significantly and positively correlated with both IFN-γ and TNF expression (Fig. 6B). Further survival analysis revealed a significantly improved OS for IFN-γ^{high} MIBC patients compared with IFN-γ^{low} patients in the IMvigor210 cohort (median, 15.4 vs. 8.2 months; HR, 0.62; 95% CI 0.46–0.83; $P=0.001$) (Fig. 6C). In addition, TNF^{high} MIBC patients showed a trend in OS improvement in contrast to TNF^{low} patients in the IMvigor210 cohort (median, 11.7 vs. 9.6 months; HR, 0.84; 95% CI 0.63–1.13; $P=0.252$) (Fig. 6D). Therefore, the expression of immunoproteasome subunits is positively associated with the immunoproteasome inducible factors IFN-γ and TNF. Hence, both immunoproteasome subunits and IFN-γ or TNF are potent markers to predict the response to immunotherapy in MIBC patients.

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Fig. 5 Correlation between the expression of immunoproteasome subunits and immune checkpoint molecules in MIBC. Correlation heatmap of the association of immune checkpoint molecules with the expression of immunoproteasome subunits PSMB8, PSMB9 and PSMB10 in MIBC of the TCGA cohort (**A**) and the IMvigor210 cohort (**B**). Color bar on the right side of each heatmap shows the correlation coefficient. Positive correlation is shown in red, negative and blue. **C** Comparison of PD-L1 expression between immunoproteasome subunits PSMB8^{high}, PSMB9^{high}, PSMB10^{high} and PSMB8^{low}, PSMB9^{low}, PSMB10^{low} in MIBC of the CQUCH cohort. Data are depicted as mean ± SEM of individual data points. **D** Scatter diagram for expression correlation between PD-L1 and the immunoproteasome subunits PSMB8, PSMB9 and PSMB10 in MIBC of the CQUCH cohort. Data are expressed as individual dot per subject. **E** Kaplan–Meier curve of progression-free survival (left panel) and overall survival (right panel) for MIBC patients receiving immune checkpoint inhibitor treatment in the CQUCH cohort with high and low expression of PD-L1. **F** Kaplan–Meier curve of overall survival for MIBC patients receiving immune checkpoint inhibitor treatment in the IMvigor210 cohort with high and low expression of PD-L1

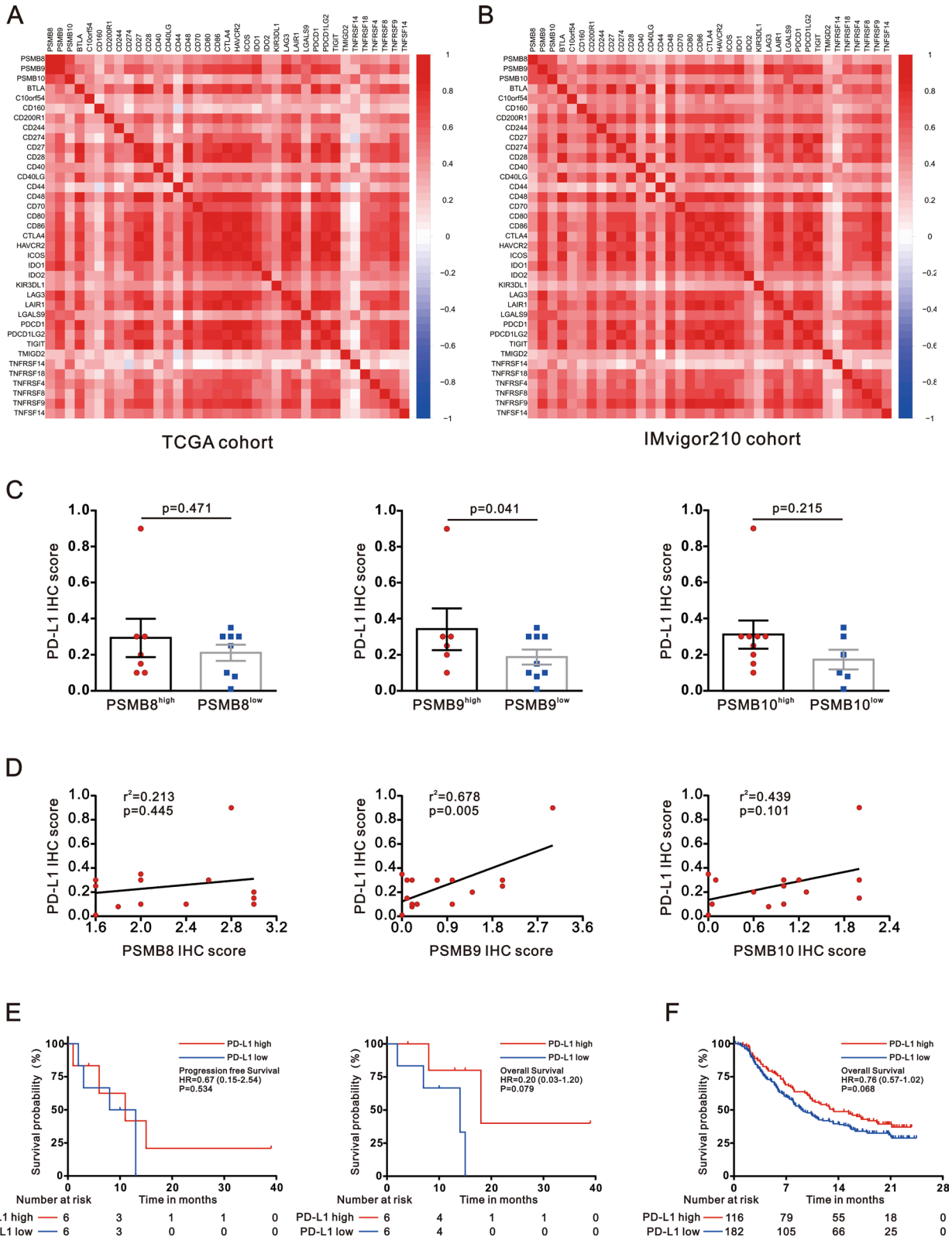

Fig. 5 (See legend on previous page.)

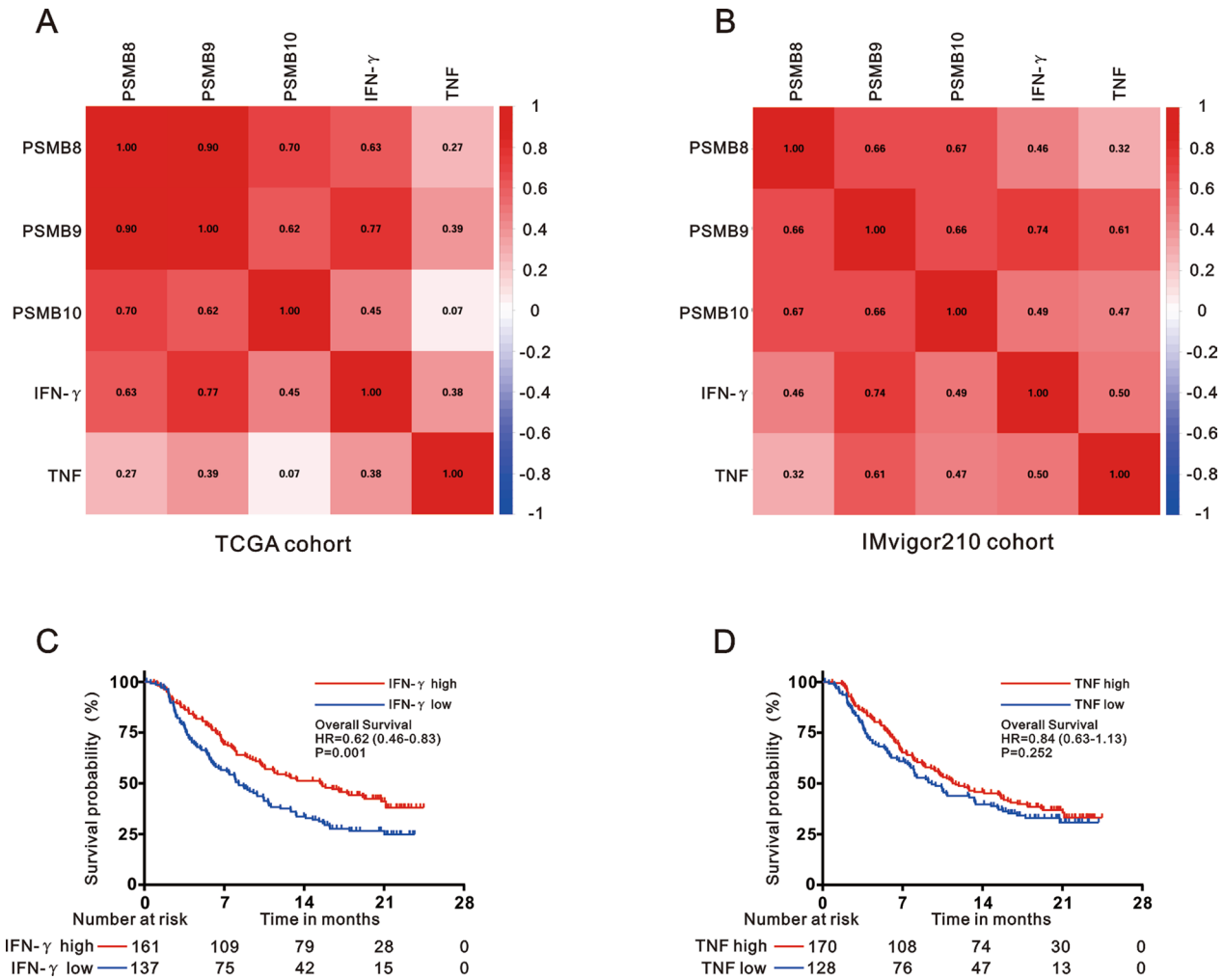

Fig. 6 Association of immunoproteasome subunits with inflammatory factors in MIBC. Expression correlation heatmap of the association of IFN-γ and TNF with the immunoproteasome subunits PSMB8, PSMB9 and PSMB10 in MIBC of the TCGA cohort (**A**) and the IMvigor210 cohort (**B**). Correlation coefficient between each two molecules is written in each square. Color bar on the right side of each heatmap shows the correlation coefficient. Positive correlation is shown in red, negative and blue. **C**, **D** Kaplan–Meier curve of overall survival of MIBC patients receiving immune checkpoint inhibitor treatment in the IMvigor210 cohort with high and low expression of IFN-γ or TNF

The expression of immunoproteasome subunits is associated with an inflammatory tumor microenvironment

Since the efficacy of ICI treatment is dependent on the infiltration of immune cells into the tumor microenvironment [26], we evaluated the association of immunoproteasome subunits with tumor infiltrating immune cells in MIBC. The TCGA cohort showed that the expression of immunoproteasome subunits positively correlated with effector immune cells, including $CD8^+$ T cells, memory activated $CD4^+$ T cells and M1 macrophages, and negatively associated with inhibitory or resting immune cells, including regulatory T cells and naïve $CD4^+$ T cells (Fig. 7A). In addition to $CD8^+$ T cells and M1 macrophages, the IMvigor210 cohort also showed that immunoproteasome subunits positively associated with activated NK cells, and negatively associated with resting NK cells (Fig. 7B).

Next, we explored the related biological functions of immunoproteasome subunits in MIBC of the TCGA cohort by gene ontology (GO) analysis. Expression of the immunoproteasome subunits PSMB8, PSMB9 and PSMB10 was strongly associated with immune response processes such as immune effector processes, positive regulation of immune responses and T cell activation (Fig. 8A). KEGG analysis (Kyoto Encyclopedia of Genes and Genomes) (Fig. 8B) and GSEA analysis (Gene Set Enrichment Analysis) (Fig. 8C) further revealed that

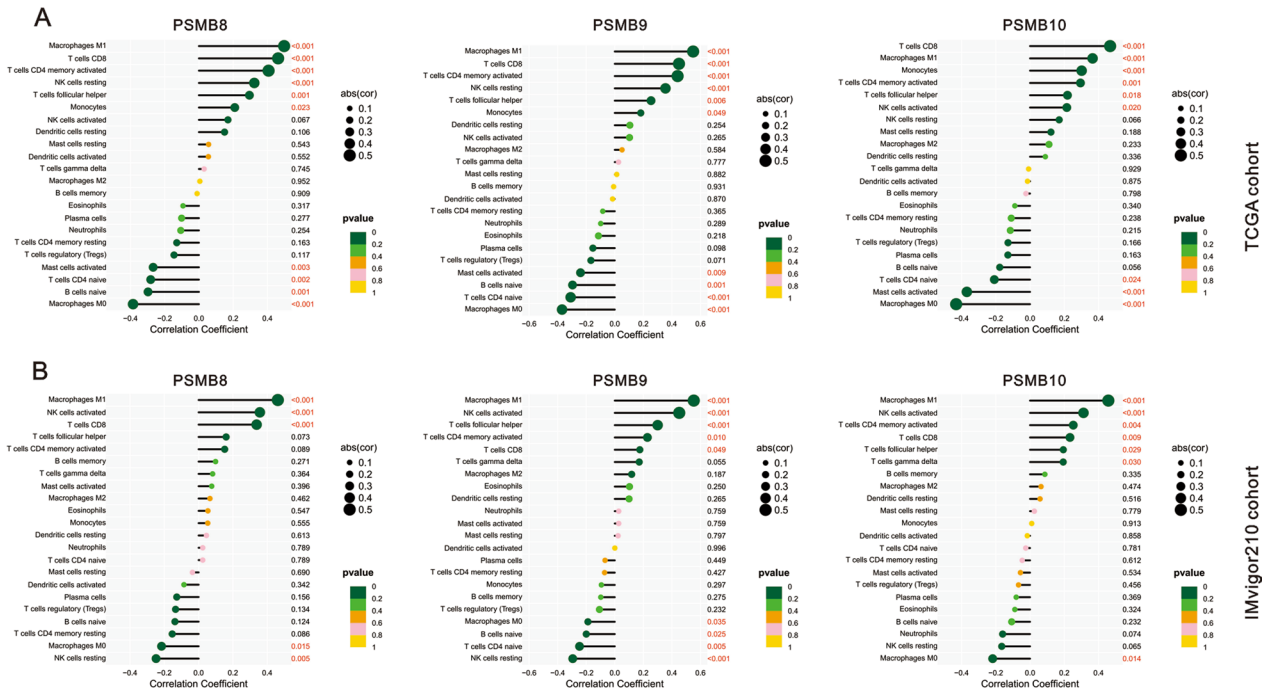

Fig. 7 Association of immunoproteasome subunits with tumor infiltrating immune cells in MIBC. Correlation lollipop charts of tumor infiltrating immune cells which were associated with the expression of the immunoproteasome subunits PSMB8, PSMB9 and PSMB10 in MIBC of the TCGA cohort (**A**) and the IMvigor210 cohort (**B**). Lollipop size shows the correlation coefficient between each infiltrating immune cell type and each immunoproteasome subunit. P values were labeled on the right side of each chart. Values of $P < 0.05$ were considered statistically significant and marked in red

these related immune response processes positively correlated with NK cell mediated cytotoxicity, T cell differentiation, PD-L1 expression, PD-1 checkpoint pathways in cancer, T cell mediated cytotoxicity, T cell activation, and T cell receptor signaling pathways. These results suggest that the expression of immunoproteasome subunits strongly correlated with pro-inflammatory immune pathways contributing to an anti-tumor microenvironment.

Discussion

Chemotherapy and immunotherapy have become the recommended treatment to MIBC at advanced or metastatic stage or in the perioperative period [2]. However, platinum-based chemotherapy has not met satisfactory response and survival rates, and immunotherapy failed in 50% of MIBC patients [13]. Therefore, how to select sensitive patients for chemotherapy or immunotherapy is a central issue in the field of urologic oncology. Fortunately, four molecular subtyping systems have been established to identify chemotherapy sensitive MIBC patients in the last decade [4–7]. However, a similar subtyping system to select for immunotherapy sensitive MIBC patients has remained an unmet medical need due to the lack of discriminative molecular biomarkers.

In advance of immunotherapy, PD-L1 expression in tumors of patients is commonly tested because it is the molecular basis of a successful ICI therapy [27]. ICIs block the binding of immune checkpoint molecules, thereby maintaining the cytotoxic activity of T cells, which can continue to infiltrate the tumor microenvironment and attack tumor cells [26]. Immune "cold tumors" lack the infiltration of anti-tumor immune cells, such as $CD8^+$ T cells, into the tumor microenvironment. Even though PD-L1 is expressed in these cold tumors, ICIs fail to achieve anti-tumor activity. On the contrary, ICI therapy induces a significant anti-tumor effect in "hot tumors" demonstrated by infiltration of cytotoxic $CD8^+$ T cells into the tumor microenvironment [28]. Furthermore, the low positive rate (10–33%) of PD-L1 expression in MIBC [29–31], also observed in our CQUCH cohort (26.7%), also limits the predictive accuracy of PD-L1 as a marker for a response to ICIs. Taken together, PD-L1 as a predictive and reliable biomarker is still controversial due to its divergent association with OS and PFS in patients with late stage MIBC who receive ICIs [13, 14]. In agreement, we also show that PD-L1 expression is not suitable to predict ICI efficacy, although only fifteen patients were tested for PD-L1 expression in this study. Hence, molecular biomarkers associating with cytotoxic immune cells

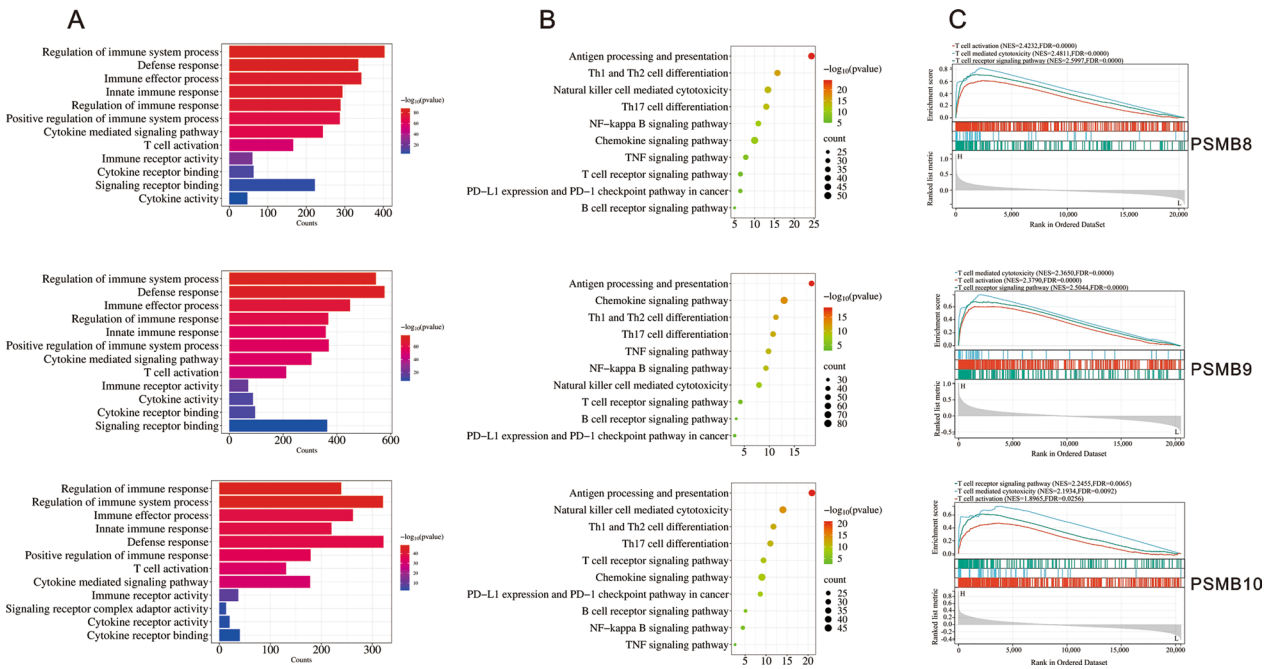

Fig. 8 Association of immunoproteasome subunits with different immune-related functions and signaling in MIBC. **A** GO analysis of the association of the immunoproteasome subunits PSMB8, PSMB9 and PSMB10 with immune-related functions in MIBC of the TCGA cohort. Color bar shows the p-value on the right side of each chart. The number of related genes were shown as the length of the column of each chart. **B** KEGG analysis of the association of the immunoproteasome subunits PSMB8, PSMB9 and PSMB10 with immune-related signaling in MIBC of the TCGA cohort. Color bar shows the p-value on the right side of each chart. The number of related genes were shown by dot size. **C** GSEA analysis of the association of the immunoproteasome subunits PSMB8, PSMB9 and PSMB10 with different immune cell functions in MIBC of the TCGA cohort

and an active immune response in the tumor microenvironment are urgently needed in MIBC.

Immunoproteasome subunits are expressed in immune cells [15, 17], indicating that they are potential biomarkers for predicting tumor immune cell infiltration and an active immune response in the tumor microenvironment. In this study, we found that the expression of immunoproteasome subunits, especially PSMB9, positively correlated with the infiltration of effector immune cells ($CD8^+$ T cells, M1 macrophages and NK cells) and an active immune response (NK cell-and T cell-mediated cytotoxicity). In contrast, expression of immunoproteasome subunits was negatively associated with immune suppressive regulatory T cells, suggesting that immunoproteasome subunits are biomarkers for immune "hot tumors" in MIBC. Furthermore, immunoproteasomes are also induced in an inflammatory environment due to the expression of IFN-γ [15–17]. T cells and NK cells are the main cell types secreting IFN-γ. Hence, the positive correlation of immunoproteasome subunits (especially PSMB9) with T cells, NK cells, and IFN-γ observed in this study confirms the association of immunoproteasomes with an inflammatory tumor microenvironment. Moreover, tumor infiltrating immune cells, which mainly express immunoproteasomes [32, 33], additionally contribute to immunoproteasome subunit expression in the tumor, indicating an active ongoing immune response associated with immunoproteasome subunits. In addition, infiltrating tumor specific T cells are a pre-requisite for a successful immunotherapy [26]. Therefore, the immunoproteasome expression in T cells might be a reason for the positive correlation of immunoproteasome subunit expression in MIBC and a successful immunotherapy.

Investigation of the predictive mechanism of immunoproteasome subunits in ICIs therapeutic efficacy was limited since data on infiltration of immune cells, expression of immune response markers and inflammatory cytokines were not available from our CQUCH cohort. Furthermore, the limited number of participants in our CQUCH cohort may cover the predictive role of PSMB8 and PSMB10. The differences in the predictive role of PSMB8, PSMB9 and PSMB10 for the therapeutic efficacy of ICIs among different cohorts need to be further investigated. Moreover, the low detection rate of PD-L1 in our CQUCH cohort may mask its role in predicting a positive response to ICI treatment. Tests combining PD-L1 and immunoproteasome subunits may further improve survival prediction of patients with MIBC in response to immunotherapy. The recent advances in antibody–drug

conjugates for urothelial carcinoma treatment [34], suggest using biomarkers directly linking therapeutic targets to therapeutic efficacy.

In conclusion, this study suggests that immunoproteasome subunits, in particular PSMB9, are promising biomarkers to predict the response to immunotherapy in MIBC, probably due to their unique expression in the anti-tumor inflammatory microenvironment.

Abbreviations

MIBC	Muscle invasive bladder cancer
CQUCH	Chongqing University Cancer Hospital
GO	Gene ontology
GSEA	Gene-set enrichment analysis
ICP	Immune cell positive score
IFN	Interferon
IHC	Immunohistochemistry
IPS	Immune cell proportion score
KEGG	Kyoto Encyclopedia of Genes and Genomes
PFS	Progression free survival
OS	Overall survival
TCGA	The Cancer Genome Atlas
TNF	Tumor necrosis factor
TPS	Tumor cell proportion score

Supplementary Information

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Supplementary material 1.

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None.

Author contributions
X.J.W., H.Y., T.Z. and H.C. performed bioinformatics analysis and drafted the manuscript. Y.L. and N. L. contributed to clinical data collection and analysis. XY.C. and QM. J. performed pathological staining and analysis. M.B. contributed to manuscript refinement and conceptual instruction. F.Y. and J.L. were in charge of conceptual and experimental design and data interpretation. All the authors have read and approved the final manuscript.

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Data availability
Data generated or analyzed during this study are included in this published article and its supplementary information files are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
The study was approved by the Clinical Research Ethics Committee of Chongqing University Cancer Hospital (No. CZLS2022261-A) and performed by the Declaration of Helsinki for human subject protection. No personal information of patients was involved.

Consent for publication
All the patients were provided with written informed consent before enrolment.

Competing interests
The authors declare no potential conflicts of interest.

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