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ORIGINAL ARTICLE

Immune inactivation by neuropilin-1 predicts clinical outcome and therapeutic benefit in muscle-invasive bladder cancer

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Abstract

Background Immune checkpoint blockade (ICB) and adjuvant chemotherapy (ACT) have shown clinical benefit in muscle-invasive bladder cancer (MIBC) with only a few predictive biomarkers identified so far. Neuropilin-1 (NRP1) has been identified as a key immune checkpoint and a novel immunotherapeutic target but the clinical significance of NRP1 remains unclear in MIBC.

Methods Three independent cohorts were involved in our study: IMvigor210 Cohort ($n=348$), The Cancer Genome Atlas Cohort (TCGA, $n=391$), and Zhongshan Hospital Cohort (ZSHS, $n=130$). Parallel detection and validation of risk stratification based on NRP1 expression were executed in patients treated with anti-PD-L1 agent and adjuvant chemotherapy (ACT).

Results NRP1 expression conferred poor survival and predicted response to both PD-L1 blockade and cisplatin-based ACT in MIBC. Further exploration revealed high-level NRP1 was extremely associated with infiltration of exhausted $CD8^+$ T cells, immature NK cells and M2 polarized tumor-associated macrophages in MIBC patients. Moreover, elevated NRP1 expression was also correlated with low mutation burden and reduced mutation in cell cycle pathway.

Conclusions Our study firstly identified and validated the clinical implications of NRP1 expression for prognosis and systematic therapeutic responses (PD-L1 blockade and ACT) in MIBC. NRP1 expression was associated with an immunosuppressive microenvironment with dysfunctional effector immune cells. Prospective investigations of its roles in the therapeutic landscape of MIBC warrant more consideration.

Keywords Neuropilin-1 · Muscle-invasive bladder cancer · Tumor microenvironment · Immunotherapy · Adjuvant chemotherapy

Abbreviations

ACT	Adjuvant chemotherapy
DEG	Differentially expressed genes
GA	Gene alteration
GSEA	Gene set enrichment analysis
ICB	Immune checkpoint blockade
IHC	Immunohistochemistry
MIBC	Muscle-invasive bladder cancer
NRP1	Neuropilin-1
OS	Overall survival
RC	Radical cystectomy
RFS	Recurrence-free survival
TMA	Tissue microarray
TMB	Tumor mutation burden
TME	Tumor microenvironment
SCNA	Somatic copy number alteration
ZSHS	Zhongshan Hospital

Précis: NRP1 expression is associated with inferior response to PD-L1 blockade but better response to adjuvant chemotherapy. Immunosuppression by NRP1 expression identified poor prognosis subtype MIBC featured with dysfunctional effector cells infiltration.

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Introduction

Bladder cancer is the most commonly diagnosed urinary malignancy, with over 573,000 new patients worldwide in 2020 [1]. Muscle-invasive bladder cancer (MIBC), in comparison with non-muscle-invasive bladder cancer (NMIBC), is a more malignant type characterized by high aggressiveness and heterogeneity of tumor microenvironment (TME) [2]. MIBC accounts for nearly 30% of bladder cancer cases, of which near a half will eventually progress to metastatic disease [3, 4]. Generally, radical cystectomy (RC) combined with adjuvant or neoadjuvant chemotherapy is the only feasible curative treatment for MIBC [4]. However, the efficacy of cisplatin-based adjuvant chemotherapy (ACT) is still limited due to the suboptimal response rate and unavoidable adverse effects [5]. Cancer immunotherapies, especially immune checkpoint blockade (ICB), have provided new strategies for advanced MIBC treatment as shown in the IMvigor210 study [6] and IMvigor010 trail [7], while most patients fail to derive durable clinical benefit from these agents [8]. To this end, identification of ACT and ICB response biomarkers represents a crucial unmet requirement to refine patient risk stratification, mitigate inappropriate use of regimens, and develop new agents for the non-responders.

Neuropilin-1 (NRP1), originally discovered as a neuronal and endothelial cell receptor for embryonic, axon guidance and angiogenesis [9–11], expresses on several immune cell types where it plays an integral role in regulating immune response [12]. Notably, it has been identified as a marker for regulatory T cells (Treg cells) in their suppression of anti-tumor immunity. NRP1 is elevated in Treg cells in tumors and identifies "stable" Treg cells [13, 14]. Although transcriptional upregulation of NRP1 on $CD8^+$ T cells is highly concordant with multiple immune checkpoints, such as PD1, CTLA4, and LAG3, its function is distinct in limiting memory $CD8^+$ T cell differentiation [15]. Targeting NRP1 exhibits the potential to restore durable anti-tumor immunity in lung cancer [16]. Additionally, NRP1 promotes tumor development through tumor growth, angiogenesis, and cancer metastasis [17–19]. However, the clinical significance of NRP1 in MIBC remains unclear because no previous study has detected the association between NRP1 expression and therapeutic responsiveness in MIBC.

Herein, to decipher the therapeutic landscape of MIBC based on the NRP1 expression, we parallelly analyzed the prognostic and therapeutic merit of NRP1 expression by the genomic and histological data from The Cancer Genome Atlas (TCGA), Zhongshan Hospital (ZSHS) and IMvigor210 study, and correlated NRP1 expression with immune contexture and key pathway alterations in MIBC. Our findings firstly shed light on the predictive significance of NRP1 expression for ACT and ICB therapy in MIBC, which may guide the precise treatment decision and point anti-NRP1 as a novel potential immunotherapeutic approach for MIBC.

Materials and methods

Study population

Three independent cohorts: IMvigor210 Cohort (348 patients treated with PD-L1 inhibitor atezolizumab in IMvigor210 clinical trial) [20], The Cancer Genome Atlas (TCGA) Cohort and Zhongshan Hospital (ZSHS) Cohort were included in this study. IMvigor210 study was a single-arm phase II clinical trial to investigate atezolizumab in patients with metastatic urothelial carcinoma (NCT02108652, NCT02951767), while RECIST (Response Evaluation Criteria in Solid Tumors) v1.1 was used to assess therapeutic response [21]. Responders were defined as complete response (CR) and partial response (PR) as per RECIST, while non-responders were defined as patients with stable disease (SD) and progressive disease (PD). Relevant clinical data of IMvigor210 Cohort were downloaded from http://research-pub.gene.com/IMvigor210CoreBiologies/.

The clinical and genomic information of 412 cases with bladder cancer from TCGA program was acquired via TCGA-Assembler 2.0.5 in May 2018. TCGA Cohort was established for further analysis according to the following inclusion criteria: (i) data integrality of mRNA expression ($n=408$) and overall survival ([OS], $n=405$); (ii) without neoadjuvant chemotherapy ($n=395$); (iii) pathologically diagnosed as MIBC ($n=391$).

With the approval of the Clinical Research Ethics Committee of ZSHS, 215 patients who survived RC from 2002 to 2014 were followed up regularly till July 2016. 130 cases were ultimately enrolled in ZSHS on the basis of consistent criteria: (i) data integrality of follow-up ($n=215$); (ii) pathologically recognized as MIBC ($n=142$); (iii) without other systematic treatment except for ACT and RC ($n=142$); (iv) without dot loss on Formalin-fixed paraffin-embedded (FFPE) tumor microarray (TMA) ($n=130$).

Clinical and pathological features of IMvigor210, TCGA, and ZSHS Cohorts were presented in Supplementary Table 1 and Supplementary Table 2.

RNA-seq data and processing

RNA-seq data of TCGA Cohort and IMvigor210 Cohort were retrieved along with the acquisition of clinical information and were normalized with the formula $\log_2(\text{FPKM}+1)$. The infiltration of 22 immune cells in the TME was

calculated by CIBERSORT algorithm [22], and the absolute score of each case was determined as the sum of 22 cells abundance. Moreover, immune and stromal scores were evaluated by ESTIMATE method [23]. The molecular subtype of each patient was identified through *BLCAsubtyping* package from https://github.com/cit-bioinfo/BLCAsubtyping [24]. The gene signatures were downloaded from https://gsea-msigdb.org, and related information was detailed in Supplementary Table 3.

Genomic analysis and variant assessment

Tumor mutation burden (TMB) was identified as the number of exonic, nonsynonymous single nucleotide variants (SNV), and indel mutations per megabase of genome examined (mutation/Mb) [25]. However, normalized TMB data for each patient in TCGA Cohort was calculated with results of whole-exome sequencing (WES) and *maftools* package, while TMB data of IMvigor210 Cohort was yielded with targeted large-panel sequencing [20]. For this study, we focused our mutational and copy-number variation (CNV) analyses on genes involved in selected pathways. CNV data of TCGA Cohort was downloaded from http://www.cbioportal.org. In IMvigor210 Cohort, CNV status and mutation data were downloaded together with clinical information, which was partly available due to the sequencing method as described in the clinical trial [20]. Gene alterations (GA) were defined as either nonsense, missense, frameshift, splice-site variants affecting consensus nucleotides or deleterious homozygous deletions and amplifications.

Assay methods

Our previous study had introduced the TMA construction (Shanghai Outdo Biotech Co, Ltd) [26]. Additionally, single and double immunohistochemistry (IHC) staining for NRP1 and other molecules were displayed in Supplementary Table 4 and were completed based on the previously detailed protocols [27]. NRP1 expression and immune cells infiltration were evaluated by two pathologists (Dr. Chen and Dr. Zhang) unaware of clinical information independently with NanoZoomer-XR (Hamamatsu) and Image Pro plus 6.0 digitally. To quantify the expression level of NRP1 protein, an IHC score of the cytoplasmic and membranous staining was calculated by multiplication of the intensity (stratified as negative (0), low (1), moderate (2), and high (3)) and percentage of positive cells (0–100%), which generated an IHC score ranging from 0 to 300. IHC score for each individual was evaluated as the average of three representative fields (200 × magnification) for each dot. The cut-off values were determined by the median value in each study cohort. In TCGA Cohort and IMvigor210 Cohort, the cut-off points were 3.888 and 5.518, respectively (normalized NRP1 mRNA expression), while the cut-off point in ZSHS Cohort was 164 (IHC score)/200 × magnification field.

Statistical analysis

The Chi-squared test was applied to detect the relation of NRP1 expression with clinicopathological parameters, molecular subtypes, and gene alterations. The predictive value of NRP1 expression for the survival benefit from ACT and PD-L1 blockade was evaluated by the log-rank test. Mann–Whitney test was conducted to inquire the diversity of immune infiltration between NRP1 high and low expression subgroups. GO (Gene Ontology) analysis, KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis, and differential expression genes analysis were accomplished by limma, edgeR, and clusterProfiler packages (http://www.r-project.org/). A two-sided *P* value less than 0.05 was considered as significant variation, all the statistical methods mentioned above were completed through IBM SPSS Statistics 25.0 (SPSS Inc., Chicago, IL) and R software 4.0.4.

Results

NRP1 expression indicated therapeutic resistance to PD-L1 inhibitor

Study cohorts and exclusion criteria were displayed in Supplementary Fig. 1A. Herein, to explore the clinical significance of the novel immune checkpoint NRP1, we observed significantly enrichment of NRP1 expression in the immunotherapy non-responders in IMvigor210 Cohort ($P = 0.026$, Fig. 1a), suggesting that NRP1 may be associated with immunotherapy resistance. Further survival analysis also indicated that high expression of NRP1 predicted poor prognosis after immunotherapy ($P = 0.044$, Fig. 1b). Currently, it has been revealed that immune and molecular indicators may be used to precisely distinguish immunotherapy-sensitive populations, including IHC biomarkers like immune phenotype and PD-L1 expression on immune cells (IC) and molecular subtypes [20, 24], which motivated us to investigate the predictive significance of NRP1 beyond above biomarkers. We explored the association of NRP1 mRNA level with TCGA2017 subtype in TCGA and IMvigor210 Cohort [28], showing that patients in NRP1 high group were mostly classified into "basal-squamous," "luminal-infiltrated," and "neuronal" subtype (Supplementary Fig. 2). Intriguingly, NRP1 expression predicted adverse survival in PD-L1 negative or immune excluded or basal-squamous patients (Fig. 1c). The combined stratification of NRP1 and current markers could indeed distinguish the therapeutic response to immunotherapy more elaborately.

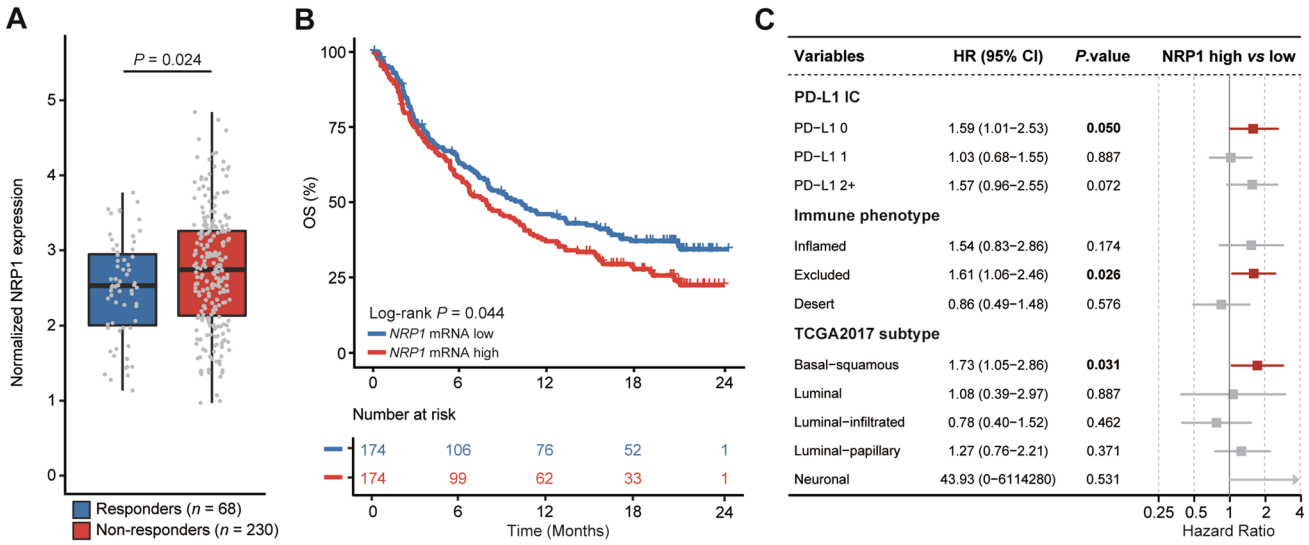

Fig. 1 NRP1 expression predicted inferior response to PD-L1 blockade. **a**–**b** The association of NRP1 expression with atezolizumab responses (**a**, IMvigor210 Cohort, $n=298$) and overall survival (b, IMvigor210 Cohort, $n=348$). **c** Hazard ratio of death with NRP1 high versus low in the context of PD-L1 expression on immune cells, immune phenotype and TCGA2017 subtypes was evaluated by univariate cox analysis in atezolizumab-applied patients in IMvigor210 Cohort ($n=348$). The two-sided P values were calculated by the Mann–Whitney U test. Log-rank test was conducted for Kaplan–Meier curves. OS—overall survival; CI—confidence interval; HR—hazard ratio; IC—immune cells. Specimens were scored as IHC IC0, IC1, IC2 + if $<1\%$, $\geq 1\%$ but $<5\%$, $\geq 5\%$ of immune cells were PD-L1 positive, respectively

NRP1 expression yielded poor prognosis but favorable response to adjuvant chemotherapy

To further explore the clinical significance of NRP1 expression, we investigated whether NRP1 was predictive of chemotherapy response in MIBC. Depending on whether receiving ACT or not, TCGA Cohort and ZSHS Cohort were further subdivided into patients with ACT (71 and 62, respectively) and patients without ACT (175 and 68, respectively). To elucidate the clinical significance of NRP1 expression in MIBC, Kaplan–Meier curves and log-rank test were applied to assess OS and RFS between NRP1 high/low subgroups in the TCGA and ZSHS Cohorts. In both cohorts, NRP1 high subgroup indicated inferior prognosis in patients without ACT (TCGA: OS: $P=0.025$, RFS: $P=0.025$; ZSHS: OS: $P<0.001$, RFS: $P<0.001$; Fig. 2a and Supplementary Fig. 3A). Intriguingly, patients with high NRP1 mRNA expression could potentially possessed superior survival benefit from ACT (OS: $P=0.260$; RFS: $P=0.027$). Consistently, histologic data in ZSHS Cohort showed the positive relationship between NRP1 expression and ACT responses (OS: $P=0.036$; RFS: $P=0.005$; Fig. 2b and Supplementary Fig. 3B). Representative images of IHC staining for NRP1 expression were exhibited in Supplementary Fig. 1B.

Characterization of the immune enriched but suppressive microenvironment with elevated NRP1 expression

The specific distinction of the tumor microenvironment has a profound impact on response to chemotherapy and immunotherapy [29, 30]. Hence, we attempted to depict the immune infiltration between NRP1 high and low groups to expound the fundamentals to the predictive value of NRP1. As illustrated in Fig. 3a and b, tumors with high NRP1 expression were characterized by high immune infiltration and abundant stroma scores according to the ESTIMATE algorithm. In particular, patients with high NRP1 expression exhibited abundant immune cells infiltrates with reduced activated dendritic cells. Meanwhile, the expression of immune checkpoints and suppressive molecules was remarkably up-regulated in NRP1 high group, which was consistent with previous study [15]. To validate the findings described above, IHC staining was adopted and yielded the same results (Fig. 3c and Supplementary Fig. 4). The pro-tumor cells (M2 macrophages, mast cells, neutrophils, and Th2 cells), immunosuppressive cytokines (IL-10 and TGF-β) and checkpoint expression were enriched in NRP1 high subgroup (Fig. 3d and e). We noticed that the primary tumoricidal immune cells

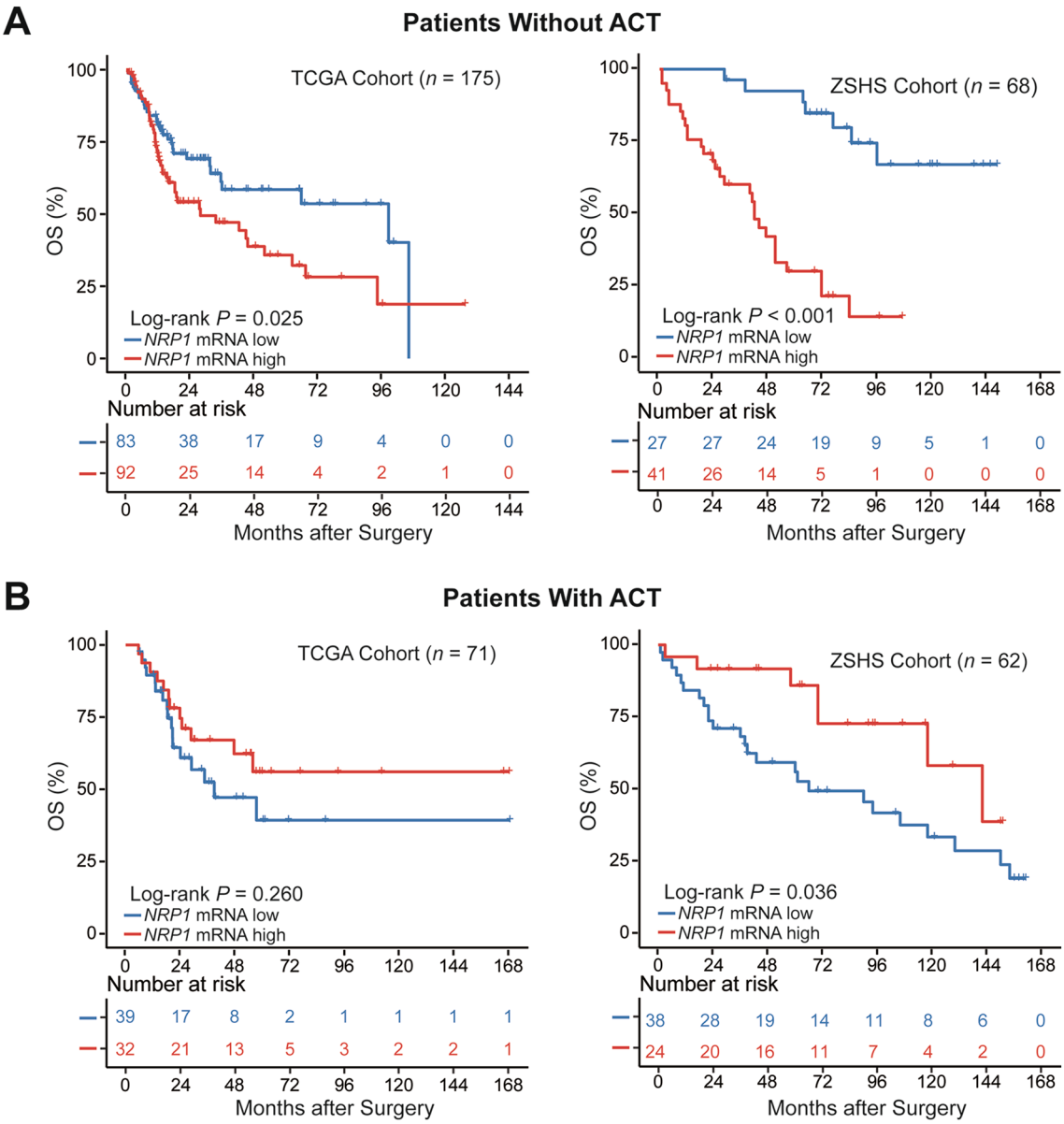

Fig. 2 NRP1 expression yielded poor prognosis but favorable response to adjuvant chemotherapy. **a**–**b** The relationship of NRP1 expression with clinical outcomes (**a**, TCGA Cohort, $n=175$; ZSHS Cohort, $n=68$) and chemotherapy benefit (**b**, TCGA Cohort, $n=71$; ZSHS Cohort, $n=62$) in MIBC. The overall survival (OS) was compared between NRP1 high and NRP1 low patients. Log-rank test was conducted for Kaplan–Meier curves. ACT, adjuvant chemotherapy

(CD8$^+$ T cells and NK cells) were also elevated in NRP1 high group, which was contrary to our assumption. To this end, GSEA (gene set enrichment analysis) was executed to identify the functional status of the CD8$^+$ T cells and NK cells. The results revealed that NRP1-related dysfunctional CD8$^+$ T cells had an exhausted phenotype and NK cells were in the limbo of immaturity (Fig. 3f and Supplementary Fig. 5). In conclusion, high NRP1 expression was strongly correlated with an immunosuppressive contexture with dysfunctional effector cells in MIBC patients.

To characterize the underlying biological process affected by NRP1 expression, we inquired differentially expressed genes between the high and low expression of NRP1 groups within TCGA Cohort and IMvigor210 Cohort in parallel. Genes satisfied both Log2 (fold change) > 1 and $P<0.05$ were regarded as up-regulated. As displayed in Supplementary Fig. 6A, the expression of multiple chemokines and related receptors elevated together with NRP1. Harnessing the up-regulated genes to perform GO analysis and KEGG analysis, pathways relative to chemokines were significantly

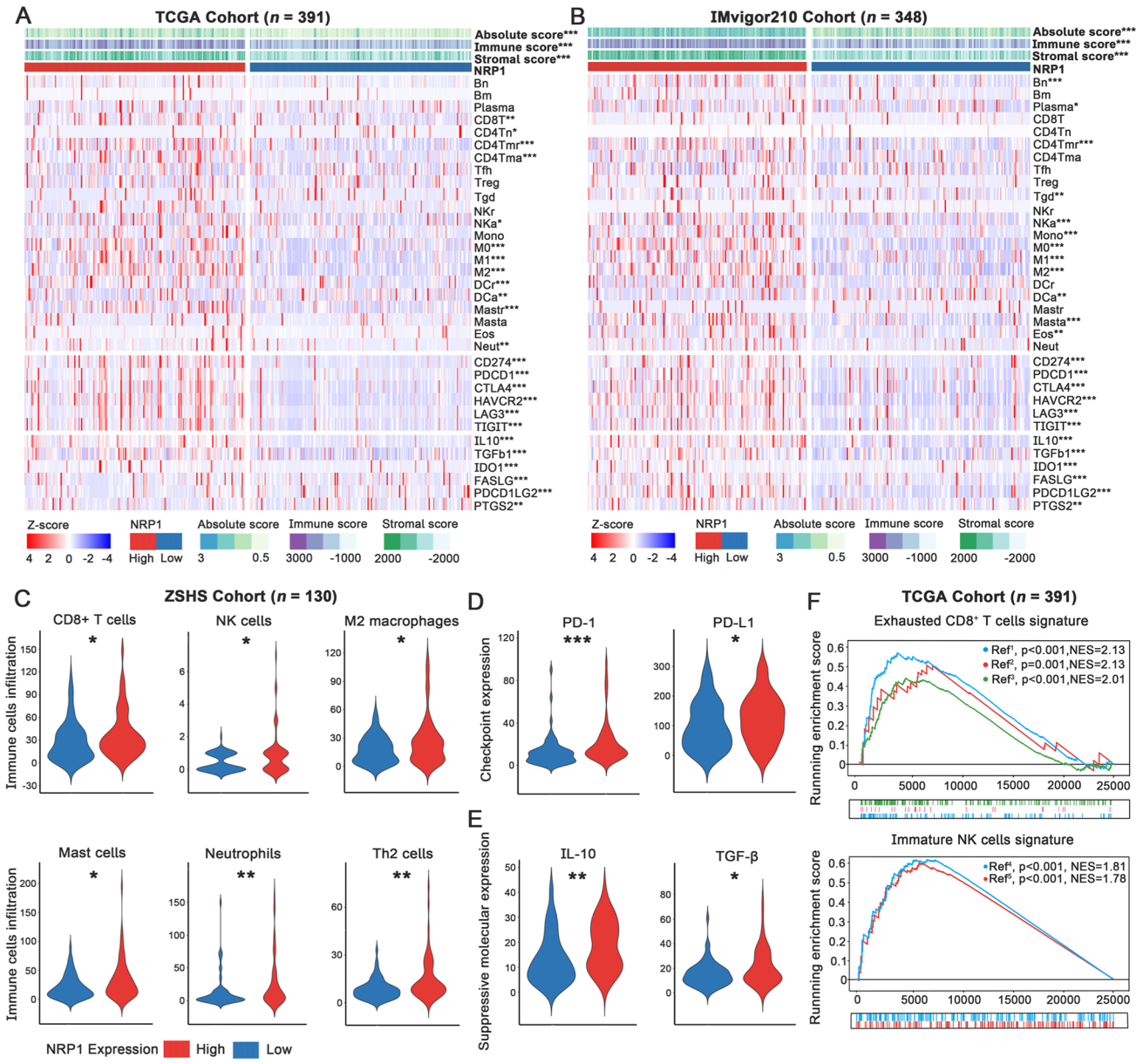

Fig. 3 NRP1 expression shaped an immune enriched but suppressive microenvironment. **a**–**b** Heatmap showing ESTIMATE scores, 22 immune cell subsets (CIBERSORT), immune checkpoints and suppressive molecular expression in NRP1 high and NRP1 low groups in TCGA Cohort (**a**, $n=391$) and IMvigor210 Cohort (**b**, $n=348$). **c**–**e** Immune cells infiltration (**c**), checkpoints expression (**d**) and suppressive molecular expression (**e**) were evaluated through IHC in ZSHS Cohort ($n=130$). $CD8^+$ T cells, M2 macrophages, mast cells, neutrophils, Th2 cells, PD-1, IL-10 and TGF-β were identified as positive cells. NK cells were evaluated with proportion (%) of positive cells in each patient while PD-L1 expression was assessed with IHC score. **f** The functional phenotypes of $CD8^+$ T cells and NK cells were identified by GSEA analysis in TCGA Cohort. The two-sided *P* values were calculated by the Mann–Whitney *U* test. $*P<0.05$, $**P<0.01$, $***P<0.001$. IHC, immunohistochemistry; Bn, naive B cells; Bm, memory B cells; Plasma, plasma cells; CD8T, $CD8^+$ T cells; CD4Tn, naive $CD4^+$ T cells; CD4Tmr, resting memory $CD4^+$ T cells; CD4Tma, activated memory $CD4^+$ T cells; Tfh, follicular helper T cells; Tregs, regulatory T cells; Tgd, γδT cells; NKr, resting nature killer cells; NKa, activated nature killer cells; Mono, monocytes; M0, M0 macrophages; M1, M1 macrophages; M2, M2 macrophages; DCr, resting dendritic cells; DCa, activated dendritic cells; Mastr, resting mast cells; Masta, activated mast cells; Eos, eosinophils; Neut, neutrophils; NES, normalized enrichment score

enriched in NRP1 high expression group (Supplementary Fig. 6B and 6C), which implied NRP1 mediated immune cells recruitment to establish suppressive TME and recapitulated the finding of previous research [16, 18, 31–33]. Besides, NRP1 expression was reported to play critical role in tumor angiogenesis, which was a key resistance mechanism to immunotherapy in MIBC [18, 34]. Therefore, we detected and validated the strong correlation between NRP1

mRNA expression and angiogenesis signature (Supplementary Fig. 7).

Genomic landscape and driver pathway alterations based on NRP1 expression

Driver gene mutations and functional alteration in specific pathways have been revealed as latent determinants for therapy response. Hence, we selected pathways which might affect ACT and ICB response to comprehend the underlying relationship between pathway alterations and NRP1 expression. In both cohorts, the mutation frequencies of genes involved in cell cycle pathway were significantly decreased in NRP1 high subgroup (Fig. 4a and b). Growing evidence has revealed the tumor mutation burden (TMB) is key determinant of response to ICB, we subsequently explored the

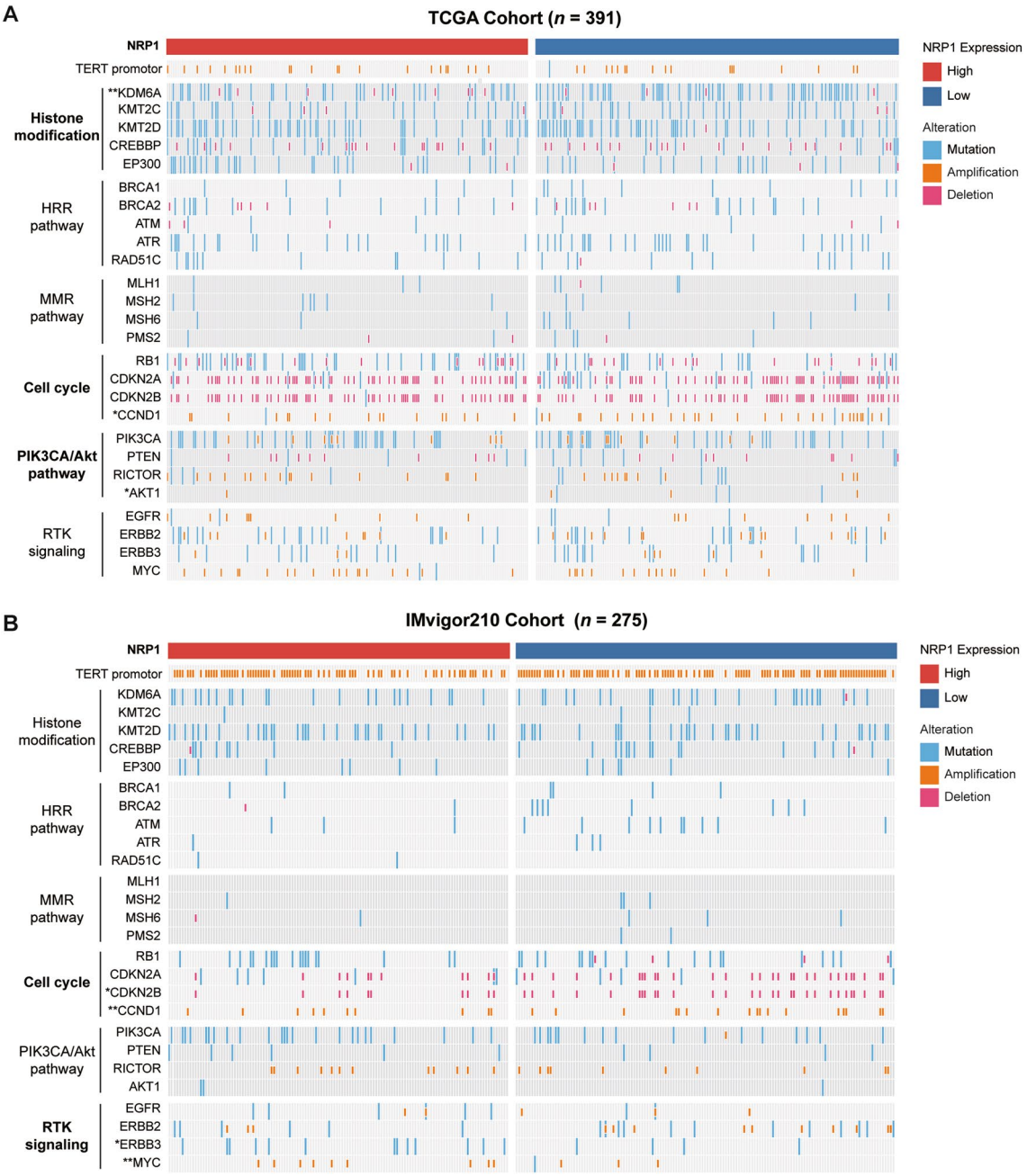

Fig. 4 Genetic alteration landscape based on NRP1 expression. **a**, **b** Distribution of driver pathway alterations in NRP1 expression high and low groups in TCGA Cohort (**a**, $n = 391$) and IMvigor210 Cohort (**b**, $n = 275$). All reported alterations were deleterious or likely deleterious. The two-sided P values were analyzed by chi-squared test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. HRR, homologous recombination repair; MMR, mismatch repair; RTK, receptor tyrosine kinase

distribution of TMB between NRP1 high/low subgroups. Remarkably, patients characterized by high mutation burden were more distributed in the group with NRP1 low expression in IMvigor210 Cohort (Supplementary Fig. 8). Taken together, our results indicated that NRP1 expression correlated with reduction in TMB and alterations in cell cycle pathway.

Discussion

Chemotherapy has been the primary choice for cancer patients for the past few decades [35], while ICB therapy provides a novel promising paradigm in cancer treatment. Nonetheless, both the treatments have been verified to be effective but limited in MIBC [8]. To this end, biomarkers that reflect the intrinsic biology of the tumor and can be used to predict response-specific therapies are of particular interest. Numerous previous studies had revealed novel immune checkpoint NRP1 played a significant role in the immune microenvironment [15, 16], and correlated with adverse clinical outcomes [36]. Additionally, a recent study revealed NRP1 expression promoted proliferation, angiogenesis, invasion, and immune evasion of bladder cancer [33]. In our study, we found that NRP1 level could predict active response to ACT but refractory to atezolizumab, which suggested that NRP1 might guide the precise therapy for MIBC patients.

TME has been proved to be closely related to the responsiveness to chemotherapy and immunotherapy [29, 30]. The immune contexture related to high NRP1 expression was investigated and validated as an enriched but immunosuppressive milieu, which indicated inferior clinical outcomes in non-ACT applied patients. On the other hand, mutation and overexpression in cell cycle pathway genes have been previously associated with chemotherapeutic resistance [37], consistent with the alteration landscape characterized by NRP1 low expression. For immunotherapy, the inhibitory TME may be caused by NRP1 signaling independent of the PD-L1 pathway, thereby mediating PD-L1 therapeutic resistance, which was also demonstrated in previous studies [15, 16], suggesting the potential of NRP1-targeted therapy alone or in combination with other agents in MIBC. To date, only one clinical trial is evaluating the targeting potential in combination with Nivolumab (ASP1948) [36].

Several molecular subtypes have been defined based on the transcriptome profiling method in MIBC, which resembles the acknowledged subtypes of breast cancer. Among them, basal-squamous patients had an inferior prognosis despite abundant cytotoxic immune cells [24]. However, basal-squamous patients were more eligible for immunotherapy than all other subtypes, which might ascribe to the distinct capability of DNA damage repair across the molecular subtypes [38, 39]. From the results, we identified a group of basal squamous patients with high NRP1 expression resistant to immunotherapy, suggesting potential utility in combining immune features and molecular subtypes to distinguish patients sensitive to specific treatments.

There were still some limitations in our study. Despite a negative correlation between NRP1 expression and TMB was observed in both cohorts, however, the calculation and normalization methods were varied between TCGA and IMvigor210 cohorts due to distinct sequencing techniques [40, 41], which could introduce a bias in our analysis. Given the retrospective and exploratory design of our study, the results still require further validation. Besides, patients in IMvigor210 Cohort were diagnosed with metastasis urothelial carcinoma (mUC). Currently, atezolizumab is approved for previously treated mUC, untreated cisplatin-ineligible mUC, and mUC that has progressed after platinum-based chemotherapy [7]. Therefore, the immune microenvironment and molecular characteristics between the TCGA Cohort and IMvigor210 Cohort were slightly different.

In conclusion, we identified and validated the prognostic value of intratumoral NRP1 expression. Our results provided pre-clinical evidence that intratumoral NRP1 expression was not only a feasible surrogate of precise immunotherapeutic selection but was also an independent predictor for ACT, which may guide risk stratification and treatment decisions of MIBC patients.

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Authors' contributions YY, HZ, KJ, and RY for acquisition of data, analysis and interpretation of data, statistical analysis and drafting of the manuscript; ZL, HZ, CL, XS, SY, YC, LL, and LX for technical and material support; JX, YZ, and ZW for study concept and design, analysis and interpretation of data, drafting of the manuscript, obtained funding, and study supervision. All authors read and approved the final manuscript.

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Data and materials availability Data and materials generated that are relevant to the results are included in this article. Other data are available from the corresponding author Prof. Xu upon reasonable request.

Declarations

Conflict of interest The authors declare that they have no competing interests.

Ethics statement This study was approved by the Clinical Research Ethics Committee of Zhongshan Hospital, Fudan University (No. B2015-030). Written informed consent was obtained from each patient.

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